Solution-active Structural Alternations in Liganded Hemoglobins C (β6 Glu → Lys) and S (β6 Glu → Val)*

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Based upon existing crystallographic evidence, HbS, HbC, and HbA have essentially the same molecular structure. However, important areas of the molecule are not well defined crystallographically (e.g. the N-terminal nonhelical portion of the α and β chains), and conformational constraints differ in solution and in the crystalline state. Over the years, our laboratory and others have provided evidence of conformational changes in HbS and, more recently, in HbC.

We now present data based upon allosteric perturbation monitored by front-face fluorescence, ultraviolet resonance Raman spectroscopy, circular dichroism, and oxygen equilibrium studies that confirm and significantly expand previous findings suggesting solution-active structural differences in liganded forms of HbS and HbC distal to the site of mutation and involving the 2,3-diphosphoglycerate binding pocket. The liganded forms of these hemoglobins are of significant interest because HbC crystallizes in the erythrocyte in the oxy form, and oxy HbS exhibits increased mechanical precipitability and a high propensity to oxidize. Specific findings are as follows: 1) differences in the intrinsic fluorescence indicate that the Trp microenvironments are more hydrophobic for HbS > HbC > HbA, 2) ultraviolet resonance Raman spectroscopy detects alterations in Tyr hydrogen bonding, in Trp hydrophobicity at the αβ interface (β37), and in the A-helix (α14/β15) of both chains, 3) displacement by inositol hexaphosphate of the Hb-bound 8-hydroxy-1,3,6-pyrenetrisulfonate (the fluorescent 2,3-diphosphoglycerate analog) follows the order HbA > HbS > HbC, and 4) oxygen equilibria measurements indicate a differential allosteric effect by inositol hexaphosphate for HbC ~ HbS > HbA.

Naturally occurring β6 hemoglobin mutants aggregate into defined structures in the erythrocyte. Sickle cell hemoglobin (HbS, β6 Glu → Val) forms polymers in the deoxy state, whereas HbC (β6 Glu → Lys) forms crystals in the oxy liganded state. A complete understanding of the mechanisms giving rise to deoxy HbS polymers and oxy HbC crystals remains to be elucidated. In the last few years, our laboratory has pursued questions related to mechanisms involved in the ligand-specific, induced crystallization of HbC, starting with site-specific probing of the R-state tetrameric structure of HbC (1, 2). Here, we extend the studies to include a comparison of the R-state of HbS. The R-state of HbS is of particular relevance because oxy HbS exhibits unusual properties compared with HbA, such as mechanical precipitability (3–5), greater unfolding at an air-water interface (6, 7), and increased autooxidation (8–10).

According to existing crystallographic evidence, HbS, HbC, and HbA have essentially the same molecular structure. However, this ignores the following: 1) important areas of the molecule are not well defined crystallographically (e.g. the N-terminal nonhelical portion of the α and β chains), and 2) crystals might constrain conformation compared with that in solution.

Spectroscopic and biochemical findings by our laboratory and others revealed distal (away from the mutation site) conformational changes: for HbS, NMR studies revealed alterations in the pK of surface His, including β2 His affecting the DPG1 binding site (11, 12). Optical spectroscopies and –SH group reactivity demonstrate a long-range tertiary effect of the β6 Val substitution around the heme pocket (13–16). Organic phosphates decrease the β93 sulphydryl reactivity more for oxy HbA than for oxy HbS, which is indicative of weaker organic phosphate binding to HbS compared with HbA (16). Polymerization-independent functional differences were shown for HbS compared with HbA (17, 18). Hemoglobin HbC > HbS > HbA bind to the cytosolic side of the erythrocyte membrane (19–21). It is noteworthy that the membrane binding site for hemoglobin is the 11-amino acid N-terminal of the cytosolic side of band 3 that fits into the hemoglobin DPG pocket (22). A close examination of subunit dissociation studies indicates a slight but reproducible difference between the intersubunit bonds for HbA and HbS and the A5 hybrid (23). This is in contrast to functional and ligand binding studies presented by others (e.g. Refs. 24 and 25). Despite all of the reported differences (5–21), it is generally concluded that the similarities between HbA and HbS are emphasized and viewed as more significant. The “small differences” are typically regarded as having no structural or functional relevance (e.g. Refs. 26 and 27).

A recent study using more sensitive spectroscopic instrumentation, front-face fluorometry, and ultraviolet resonance Raman (UVRR) spectroscopy revealed differences in the H-bonding between β15 Trp and β72 Ser of HbC versus HbA under conditions of 0.3 M perchlorate (1), suggesting a displacement of the A-helix away from the E-helix with a weakening of the H-bond between β15 Trp and β72 Ser. Crystal growth kinetics and spectroscopic studies of HbC in the presence of hemoglobin central cavity-binding proteins and effectors point to differences in the DPG pocket (2).

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‡ The abbreviations used are: DPG, 2,3-diphosphoglycerate; UVRR, ultraviolet resonance Raman; HPT, 8-hydroxy-1,3,6-pyrenetrisulfonate; COHb, carbonmonoxyhemoglobin.
The present study focuses on a comparison of R-state forms of HbC, HbS, and HbA. The comparison is made using intrinsic fluorescence, extrinsic fluorescence spectroscopic probing of the central cavity DPG binding site, UVRR spectroscopy, CD spectroscopy, allosteric perturbation, and oxygen equilibrium studies. These results confirm and significantly expand previous findings and demonstrate a differential response between the R-states of HbC and HbS to central cavity perturbations.

EXPERIMENTAL PROCEDURES

Human Hemoglobin Preparation—HbC, HbA, and HbS were purified and separated from hemolysates obtained from AC, SS, or AS individuals by CM-52 cation exchange chromatography and stripped of organic phosphates by Sephadex G-25 (equilibrated with bis-Tris and 0.1 M NaCl) column chromatography. The eluted hemoglobin was concentrated and chromatographed again on a Sephadex G-25 column equilibrated with a 0.05 M Hepes buffer at the desired pH of the experimental conditions. No significant met (oxidized) hemoglobin content was detected as determined by absorption spectrophotometry.

Front-face Fluorescence Spectroscopy—A SLM 8000C photon counting spectrophotometer adapted with a front-face cuvette holder was used to obtain the steady-state fluorescence intrinsic emission spectra of the hemoglobin solutions as well as the emission from the fluorescent DPG analog, 5-hydroxy-1,3,6-pyrenetrisulfonate (HPT) (1). The properties and binding of HPT to hemoglobins have been described previously in detail (2, 28–30). The addition of effectors to the hemoglobin solutions did not significantly alter the pH.

UV Resonance Raman Spectroscopy—The apparatus used to generate and collect the Raman signal was described previously (1). The excitation wavelength was 228 nm, and the UV power at the sample was 1.0 milliwatt. The spectrophotometric slit width was 200 μm. The Raman signal was collected at 5-min intervals for a total of 30 min. All samples were cooled under a stream of nitrogen gas to 6 °C. To ensure that the hemoglobin ligation state remained unchanged during the course of the experiment, the absorption spectra of each sample were taken before and after UV exposure; no absorption band changes were seen during this experiment. For all hemoglobin variants, the heme concentration was 0.27 mM in 0.1 M Hepes buffer, pH 7.35. The internal calibration standard utilized was a Raman band at 1045 cm⁻¹ from 0.56 mM potassium nitrate. Because the Raman cross-section of the 1045 cm⁻¹ band diminishes upon prolonged UV exposure, a sample-to-sample total UV exposure was tightly controlled by keeping sample irradiation times constant and operating the pump laser in light-controlled mode.

Circular Dichroism Spectroscopy—A Jasco J-720 spectropolarimeter was used to compare the CD spectra of purified and stripped HbC, HbS, and HbA (0.1 mM heme) in 0.1 M Hepes buffer, pH 6.85, with and without an excess of inositol hexaphosphate (1 Hb:5 inositol hexaphosphate). The 220 and 280 nm excitation/monitoring system was used to compare the CD spectra of purified and stripped HbC, HbS, and HbA (0.1 mM heme) in 0.1 M Hepes buffer (no chloride), pH 7.35, as shown in Fig. 2. Within the given UVRR frequency window, tryptophan bands are located at 1558 cm⁻¹ (W3), 1458 cm⁻¹ (W5), 1358 cm⁻¹ (W7), and 1012 cm⁻¹ (W16), whereas tyrosine bands are found at 1615/1595 cm⁻¹ (Y8a/b, 1205 cm⁻¹ (Y7a), and 1175 cm⁻¹ (Y9a) (35–37). When the spectra are normalized at the nitrate internal standard as shown (Fig. 2), both tyrosine and tryptophan bands for COHbS exhibit the greatest cross-section, followed by those for COHbC. Bands for COHbA have the smallest cross-section. The W3 band for each hemoglobin species may be fit to two bands, one of which is centered at 1558.6 ± 0.2 cm⁻¹ (data not shown). The position of the lower wavenumber band was centered at 1547.5 cm⁻¹ for COHbA and COHbC. In the COHbW3 band, however, this lower frequency band is shifted to 1545 cm⁻¹. This frequency shift is illustrated by the 1542 cm⁻¹ difference peak for COHbS-COHbA (Fig. 2). The fractional cross-sections for both W3 bands were essentially identical for all three hemoglobins. It should also be noted that the 1547 cm⁻¹ band for COHbC exhibits a small difference just above the level of noise when compared with COHbA. A comparison of the non-normalized UVRR spectra of the CO forms of the three hemoglobins (data not shown) demonstrates that the peak intensities of the W16 band and the NO₃ internal standard bands are inversely ordered, indicating that the W16 band contribution to the NO₃ standard peak does not affect the global spectral intensity ordering (i.e., HbS > HbC > HbA).

RESULTS

Front-face Fluorescence Spectroscopy—It is well established that the Trp microenvironments of proteins may be probed and defined by fluorescence spectroscopy, including hemoglobin tryptophans and other tetramer microdomains. Non-tryptophan residues have been studied by the binding of extrinsic fluorescent probes (for a review of Hb fluorescence, see Ref. 31).

One approach to understanding the mechanisms of oxy HbC crystallization is to determine the solution-structural differences among the R-state β6 mutant hemoglobins and HbA. Differences in the Trp microenvironment(s) of R-state HbS (sickle cell hemoglobin), HbC, and HbA are revealed by comparing the intrinsic fluorescence spectra (Fig. 1), which show a decrease in intensity for the β6 mutants (HbC < HbS < HbA) and a blue-shift of the emission maximum for HbC and HbS. Tryptophan fluorescence emission maximum shifts are a function of hydrophobicity, whereas tryptophan fluorescence intensity changes are not easily interpretable, arising from any of several contributing factors including nonradiative processes (32–34).

2 T. Spiro, private communication.
These results indicate a differential affinity of IHP for HbA, HbS, HbC.

Probing the Heme Environment by CD Spectroscopy—Small differences in the ellipticity of the oxy liganded state of HbS, HbC, HbA in 0.1 M Hepes buffer, pH 6.85, are enhanced with the addition of excess IHP (Fig. 4). The addition of excess IHP results in a small but 2-fold increase in ellipticity exhibited by intensity changes and a shift from 420.5 nm to longer wavelengths (HbA, 424 nm; HbC, 423; and HbS, 422 nm).

Oxygen Equilibrium Measurements—Oxygen affinity differences between the β6 mutants and HbA (0.05 M Hepes buffer, pH 7.35) as a function of IHP titration are demonstrated by oxygen-equilibrium measurements (Fig. 5). The differences approach a plateau at a 1.5:1 IHP:Hb tetramer molar ratio. This is consistent with the known binding of IHP to the hemoglobin tetramer (1:1 at the DPG binding site) and reports of a secondary lower-affinity binding site.

Stripped HbA compared with HbC and HbS under conditions similar to that of the fluorescence studies (0.05 M Hepes buffer,
Spectroscopic Indications of Structural Differences in HbC and HbS

The difference in $P_{50}$ between HbA and each of the $\beta_6$ mutants under these conditions is significant (Table I).

**Discussion**

To date, there is no detailed mechanistic explanation as to why HbC forms crystals in the oxy state and HbS forms polymers in the deoxy state or why oxy HbS exhibits mechanical instability, greater unfolding at an air-water interface, and a high propensity to oxidize. The ligand-specific induction of these distinct processes weakens the argument that the aggregation phenomena arise exclusively from electrostatic or hydrophobic differences at $\beta_6$. Rather, we hypothesize that the ligand-specific characteristics of each of these $\beta_6$ mutant hemoglobins arise from an intramolecular long-range communication of the conformational change at the $\beta_6$ site. The alteration need not be the same for HbS and HbC.

Spectroscopic and functional (oxygen equilibria) studies presented here strongly suggest that global, solution-active conformational alterations, particularly those involving the A-heelix and the DPG binding pocket, are different in HbS and HbC. The intrinsic fluorescence studies show a small but reproducible shift of the emission maxima to shorter wavelengths for these R-state $\beta_6$ mutants. Because it is well established that tryptophan emission wavelength shifts are a function of hydrophobicity (32–34), the findings indicate that the Trp environment(s) of HbC and HbS compared with HbA are altered in the direction of increased hydrophobicity (HbS > HbC > HbA) (Fig. 1). UVRR comparative spectroscopy helps to dissect the specificities of the Trp alterations.

UVRR difference spectra of hemoglobins typically exhibit two categories of change (37, 40, 41, 42). The first can readily be attributed to quaternary structure-based differences in the $\alpha_1\beta_6$ interface. There are two features that have been especially characterized. The first is a substantial derivative shaped feature associated with Y8a at $-1615$ cm$^{-1}$ that has been attributed to the T- to R-state loss of the $\epsilon_{42}$-$\beta_9$ hydrogen bond in the switch region of the interface. The second is a T- to R-state decrease in the intensity of the low frequency shoulder of the W3 band at $-1548$ cm$^{-1}$ that originates from $\beta_3\beta_6$ Trp in the hinge region of the $\alpha_1\beta_6$ interface.

The second category of change observed in UVRR difference spectra is a series of intensity changes in several of the tyrosine and tryptophan bands that occur without any sizable shifts in the peak frequencies (35, 42–44). A decrease in intensity is observed when comparing either deoxy T or liganded R to deoxy R. The decrease is attributed to a generalized loosening of the global structure including a weakening of the hydrogen bond between the A-heelix tryptophans and their respective bonding partners on the E-heelix. A decrease in the intensity of the tyrosine bands is attributed to a weakening of the hydrogen bond between the penultimate tyrosines and the carbonyls of the FG5 valines. An increase in the intensity of these tyrosine and tryptophan bands is similarly ascribed to a strengthening of these hydrogen bonding interactions due to tighter packing between the A- and E-helices and the H- and F-helices. A UVRR study of C termini-modified hemoglobins indicates that there is a coupling between the packing of the H-heelix and the packing of the A- and E-helices (43). An explanation for this effect was given based on the H-heelix acting as a scaffold that supports the A-heelix. A disruption of the H-heelix as occurs upon deletion of the $\beta_{145}$ Tyr and $\beta_{146}$ His weakens the support of the A-heelix of the $\beta$ chain, resulting in a tighter packing of the A-heelix and E-heelix. This tighter packing is reflected in an increased intensity of the main peak of W3 at $1560$ cm$^{-1}$ due to the increase in hydrogen bonding of $\beta_{15}$ Trp (43).

The UVRR differences observed in the present study tend to fall into the second category. At physiological pH 7.35, COHbC and COHbS show a turn toward greater hydrophobicity in the microenvironment for all three of the Trp residues, $\alpha_{14}$, $\beta_{15}$, and $\beta_{37}$, as reflected in a greater W3 band cross-section for both mutants relative to COHbA. In support of this, at pH 6.35, these effects are shown to be further exaggerated (45). The position of the W3 UVRR band is a function of the dihedral angle, $\chi_2$, which is the angle between the indole residue and the backbone $C_n$ (Ref. 46). An equation relating the W3 frequency to the dihedral angle has been proposed (47). Applying this equation, we find that the 1547.5 cm$^{-1}$ W3 band for $\beta_3\beta_6$ Trp of COHbA and COHbC (Fig. 2) corresponds to a $\chi_2$ dihedral angle of $87^\circ$, whereas the 1545 cm$^{-1}$ band of COHbS maps to a dihedral angle of $81^\circ$. The change in intensity at the 1558 cm$^{-1}$ band assigned to differences in the H-bonding between $\beta_{15}$ Trp and $\beta_{72}$ Ser of the A- and E-helices may be predictive of alterations in the DPG pocket. Similar UVRR findings for the T-state flouromet HbS were reported recently (48).

At physiological pH 7.35, the $-1558$ cm$^{-1}$ UVRR signal from HbS, HbC, and HbA (Fig. 2) indicates a tightening of the H-bond in the order of HbS > HbC > HbA. Our earlier studies (1), which were conducted at pH 6.85, indicated an outward displacement of the A-heelix in HbC compared with HbA. UVRR studies comparing the use of nitrate and perchlorate as the internal standard reveal that the different observations in our earlier study (1) result from the presence of 0.3 M perchlorate, which we speculate may function as an allosteric effector. Studies of the effects of chloride at low pH (45) and other allosteric effectors are underway.

In general, with respect to the UVRR results, in going from HbA to HbC to HbS, there is intensity increase in most of the tyrosine and tryptophan bands. Most significantly, the Y8a band at 1615 cm$^{-1}$ does not shift (no derivative signal), and the differences in W3 are largely but not entirely due to the $\alpha_{14}$, $\beta_{15}$ component at 1560 cm$^{-1}$. Thus the differences among the three liganded hemoglobins can be largely attributed to global packing changes among several of the helices. The wavelength shifts in the intrinsic fluorescence suggest that the increase in packing between helices is accompanied by or is induced by an increase in the degree of hydrophobicity within the globin.

Similar conclusions regarding the increase in A-E-heelix packing in flouromet HbS at pH 6.5 were arrived at by Sokolov and Mukerji (48). They also found that the fluorescence change between HbA and HbS was essentially independent of the quaternary state. This finding, in conjunction with the observed R-T and HbA-HbS UVRR differences, led to the conclusion that $\beta_{37}$ Trp is responsible for the R-T fluorescence difference (31), whereas the A-E-heelix packing changes are responsible for the R-T independent HbA-HbS fluorescence differences, as also shown earlier for R-state HbC (1).
The present results are consistent with these results and conclusions.

It should be noted that in our earlier report (1), solution conditions using pH 6.85 for the spectroscopic studies had been chosen because hemoglobin exhibits a maximal fluorescence emission difference upon R → T switching at this pH. This maximal difference in fluorescence was attributed to alterations at the αβi interface, because it appeared to be obliterated when β43 Glu was covalently modified (49–52). Studies pursuing the structural effects imposed by this pH difference are ongoing.

A direct confirmation of a perturbed central cavity of HbC and HbS is seen with the use of the extrinsic fluorescent DPG analog HPT, which directly probes the DPG binding site (28–30). The results with COHb at pH 6.35, previously shown to form an altered R-state (38, 39), indicate that the affinity of IHP for the hemoglobin discussed earlier follow the order HbA > HbS > HbC. These results, combined with the UVRR findings, indicate an alteration of the DPG pocket at physiological pH. A possible source for this alteration is a β6-induced tightening of the β15 Trp-β72 Ser hydrogen bond, with the consequence that the A-helix moves closer to the E-helix for the β6 mutants HbC and HbS.

The effects of IHP upon the P50 of HbC and HbS compared with HbA provide further support that there is an A-helix perturbation in the β6 mutants that translates to an altered DPG pocket and consequently affects oxygen binding properties (Fig. 5). HbC and HbS stripped of organic phosphates show a small but statistically significant difference in their oxygen affinities when compared with HbA (Table 1). These differences in oxygen affinities are consistent with the suggested A-helix alterations and/or subtle differences in the microenvironment of β37 Trp at the αβi interface for the β6 mutants, both of which are indicated by the spectroscopic data. The differences are enhanced by the addition of the DPG analog IHP.

Structural alterations corresponding to the heme environment are correlated with the change in oxygen affinity upon addition of IHP, as reflected by the CD Soret spectral changes (420 nm). The addition of IHP under the conditions used in this study alters the Soret band in ellipticity and wavelength for oxy HbA > oxy HbC > oxy HbS (Fig. 4). Above 300 nm, the optical activity of the heme results from short and long distance interactions of the heme with the protein matrix, specifically attributed by theoretical calculations to a coupled oscillator interaction between the heme transitions and those of the surrounding aromatic side chains (53, 54). This interpretation is consistent with the altered microenvironments of the aromatic amino acids of these β6 mutants, as indicated by the fluorescence and UVRR spectroscopic results. The implication of differences in the heme environment initially arises from the observation that for HbS, the deoxy form gives rise to polymerization, whereas for HbC, the oxy form promotes intraerythrocytic crystallization. Hence, the CD Soret differences in ellipticity may serve as a direct measurement of conformational changes distally communicated to the heme microenvironment. Differences in the oxyhemoglobin CD spectra of HbA, HbS, and HbC were reported earlier by Melki (55) and of COHbS were reported by Fronticelli et al. (13–15).

For decades, functional studies in which HbS and/or HbC were compared with HbA (conducted under very different conditions e.g. bis-Tris buffer) than those used in the present study) gave rise to the concept that functional and structural differences distal to the site of the β6 mutations did not exist on a significant level (24, 25). Early spectroscopic findings and binding studies questioned these conclusions (11–21, 55, 56), but the dogma prevailed, especially in light of the crystal structure showing no differences distal to the site of mutation, albeit at 3 Å resolution (57, 58).

Padlan and Love (57, 58) found that the deoxy HbS crystal structure exhibits a narrowing of the DPG pocket compared with HbA by a comparison of the eight deoxy hemoglobin S subunits with the symmetry-averaged subunits of deoxy HbA. The biggest structural change is a shift in a hinge-like motion of the A-helices of 1β1 and 2β1 subunits, resulting in a 5 Å displacement of the α carbons of the N-terminal valine and a narrowing of the DPG binding pocket. This finding is consistent with the conclusion presented here. However, Eaton and Hofrichter (27) reason that because no change in the A-helices is observed in the 1β1 and 2β1 subunits, the displacement observed in the 1β2 and 2β2 subunits presumably results from the formation of the intermolecular contacts in the crystal (the E- and F-helices of β subunits in neighboring molecules) and is not a direct result of the substitution of valine at β6. On the contrary, this difference may very well be intrinsic to the mutation as indicated by: 1) the findings presented in this investigation, and 2) the recently published 2.05 Å resolution crystal structure of deoxy HbS by Harrington et al. (59), reporting movement of the A-helices and subtle changes in the positions of the residues in the acceptor pocket that demonstrate plasticity in both the donor and acceptor chains of the lateral contact facilitates binding.

In general, the advent of state of the art spectroscopic techniques with site-specific signal assignments permits superior signal resolution and, consequently, highly detailed structural information for many proteins. For hemoglobins, this has resulted in the uncovering of solution-active structural differences at the tetrameric molecular level and, as exemplified here, distal to the site of the β6 mutations when compared with HbA. Our results point to A-helix alterations that may be the likely candidate in the primary mechanism driving oxy HbC to crystallize and causing destabilization of oxy HbS, which, under deoxy conditions, may give rise to the polymerization process. Similar A-helix perturbations have also been reported by others for both T- and R-state forms of fluoro-met HbS (48). Extensive comparative investigations of the deoxy forms of these mutants are planned.

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