Conformational Changes in Oxyhemoglobin C (Gluβ6 → Lys) Detected by Spectroscopic Probing

(Rceived for publication, September 12, 1995)

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Hemoglobin C (Gluβ6 → Lys) shares with hemoglobin S (Gluβ6 → Val) the site of mutation, but with different consequences: deoxygenated forms polymers, whereas oxy-HbC readily forms crystals. The molecular mechanism for this property of oxy-HbC is unknown. Since no detailed oxy-HbC crystal structural information exists, spectroscopic probing is used in this study to investigate possible solution-phase conformational changes in HbC compared with HbA. Intrinsic fluorescence combined with UV resonance Raman data demonstrate a weakening of the Trpβ135-Serβ172 hydrogen bond that most likely leads to a displacement of the A helix away from the E helix.

The β6 hemoglobin variants, in particular, sickle cell hemoglobin (HbS) and HbC, are aggregating hemoglobins and, for decades, have been of interest to structural biologists, pathophysiologists, and clinicians. HbC is the second most commonly encountered abnormal hemoglobin in the United States and, next to HbS and HbE, the third most prevalent hemoglobin variant worldwide (1). HbC (Gluβ6 → Lys) shares with HbS (Gluβ6 → Val) the site of mutation at position β6, but the consequences of the specific substitutions are very different. In red blood cells, deoxygenated HbS forms polymers, whereas HbC forms intracellular crystals in the oxygenated state as demonstrated by absorption spectroscopy as well as by their tendency to melt upon deoxygenation (2). However, to date, no structural basis for the abnormal properties of these mutant hemoglobins has been defined (3).

In this study, we focus upon conformational changes in HbC as a means of addressing the remaining questions: Is the localized β6 mutation alone responsible for the tendency of HbC to crystallize, or does this mutation induce extended tertiary and/or quaternary conformational changes necessary for crystallization? Hence, to investigate possible dynamic distal conformational changes in HbC compared with HbA, several optical spectroscopies were sequentially used in this study to probe conformational differences in liganded HbC.

EXPERIMENTAL PROCEDURES

Hemoglobin Purification—Hemolysates from AC and CC individuals' washed red blood cells were made by freeze-thawing the cellular debris separated by centrifugation. The hemoglobins were separated and purified on a Whatman CM52 column as described previously (4), and the isolation was verified by isoelectric focusing. The separation of both HbC and HbA from the same AC hemolysates ensures a proper control.

Front-face Fluorescence Spectroscopy—The fluorescence emission of the hemoglobins was obtained using a SLM-AMINCO 8000C photon-counting spectrophotometer with a front-face accessory (5–7). The excitation is at 296 nm, which selectively excites for Trp fluorescence, eliminating the contribution of tyrosines to the fluorescence spectrum (8). Hemoglobin solutions were 1 g%, wherein the hemoglobin dissociation equilibrium is shifted largely to tetramers. Experiments were conducted at pH 6.85 in 0.1 M Hepes buffer. Under these conditions, crystallization will not occur within the time period of the measurements.

Ultraviolet Raman Spectroscopy—The UV resonance Raman spectrometer is composed of a laser source, a 1.5-m single spectrograph equipped with 3600 groove/mm holographic grating, and a liquid N2-cooled CCD detector (Princeton Instruments Inc., Princeton, NJ). The CCD detector has a UV metachrome coating on its chip. The modified intracavity frequency-doubled ring dye laser (Coherent 899) utilizing stilbene 420 is pumped by a multi-UV line output of an argon laser (Coherent Innova 400). To maximize the UV output with acceptable bandwidth, a thin etalon was used instead of two etalons (thick and thin). This laser system generated CW UV output from 218 to 240 nm. The maximum output of this system at 226 nm is 6 milliwatts. The average UV power used is ~1.2 milliwatts, proven to be sufficient for acquiring high quality Raman spectra. The sample was contained in a quartz NMR tube for data collection, maintained at 10 °C, and spun in helical fashion at 5 Hz to minimize local heating and potential photodegradation. The scattered photons were collected with a triplet lens (Fused Suprasil, CVI Laser Corp.) and focused onto the entrance slit (200 μm) of the spectrometer (Sopra, Inc.). The wavelength shift was calibrated with the use of the Raman spectra of cyclohexane and 1,4-dioxane. Spectral acquisitions were carried out as a series of 5-min accumulation; each final spectrum was the sum of three to five of these 5-min spectra. Before summing the spectra, cosmic rays were removed from each spectrum using SpectralCal software (Galactic Industries Corp.). In addition, each spectrum was subtracted from the first one taken from a given sample. If differences were observed, the spectrum was discarded.

Visible Resonance Raman Spectroscopy—All measurements were made at room temperature (23 °C) in cylindrical quartz or sapphire cells 0.2 mm in width. These measurements were repeated on two separate occasions exhibiting identical spectral patterns. For calibration purposes before and after each sample measurement, Raman spectra were generated using organic solvents with known spectral characteristics. For the low frequency spectra, a 1:1 (w/w) mixture of CH3Br3 + CHBr3 was used and for the high frequency region, indene (C9H6) was used.

Resonance Raman spectroscopic measurements were obtained using the following system. The second harmonic (532 nm, 20 Hz) of a neodymium:YAG laser (Continuum NY-81C) passed through a hydrogen cell (200 p.s.i.) was used to generate the blue (435.71 nm) pulse (~1 mJ, 7-nS pulses). A single laser pulse photolyzes the sample and probes the Raman spectrum of the transients within ~7 ns. Scattered light was collected with a Nikon F1.4 50-mm camera lens and focused with an f-matching lens onto a 150-μm entrance slit of an ISA HR640 monochromator. A notch filter was used to reduce the Raleigh scattering, and a depolarizer was used to reduce grating biases. The detector was a
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RESULTS AND DISCUSSION

Front-face fluorescence (5–7) has been used to probe changes in both the environment of tryptophans and heme-Trp distances in hemoglobins (9). The first positive findings were obtained comparing the intrinsic fluorescence intensities of the oxy and deoxy forms of HbA and HbC as shown in Fig. 1. 296 nm excitation was used to eliminate any resonance energy transfer from Tyr → Trp (9). A significant difference (p = 0.0035) in the fluorescence emission intensity of oxyHbC and oxyHbA is observed. This is not the case for the deoxy derivatives. A significant difference (p = 0.00008) in the percent change in the fluorescence intensity of HbC compared with HbA upon deoxygenation is also shown in Fig. 1, but is accounted for by the initial intensity difference between the liganded species.

This difference in the intrinsic fluorescence can arise from any of the Trp residues (Trp<sup>14</sup>, Trp<sup>15</sup>, and Trp<sup>37</sup>) within Hb. It is also possible that this difference may arise from differences in orientation and efficiency of transfer from tryptophan(s) to the heme. Hence, to further localize the origin of the increase in fluorescence, we used conformationally sensitive vibrational spectra of tryptophans and tyrosines using UV resonance Raman spectroscopy (10–12), building on the findings of Spiro and co-workers (10, 13). These authors showed that the distinct low frequency shoulder of the Trp(W3) UV resonance Raman band at ~1550 cm<sup>-1</sup> arises from Trp<sup>37</sup>, whereas the main band at ~1555 cm<sup>-1</sup> is derived from the composite contribution of Trp<sup>14</sup>/15. Fig. 2 shows a comparison of the UV resonance Raman spectra of the liganded (CO) forms of HbA compared with HbC. All differences can be attributed to the Trp(W3)<sup>37</sup> band since no other spectral differences are observed.

Visible resonance Raman spectroscopy is used to probe the functionally important heme environment (14–16). This method probes heme vibrational modes, many of which are highly sensitive to the local tertiary structure. It is used here to resolve any differences, between HbC and HbA, in the heme and local heme environment that could contribute to the observed fluorescence alteration. Comparisons between the equilibrium deoxy forms and the photoproducts of ligand-bound forms reflect both ligand binding and quaternary structure-induced changes in the heme environment. The low frequency region contains the iron-proximal histidine stretching mode, which is highly sensitive to changes in the functionally important proximal heme pocket. Other bands in the low frequency region reflect the environment of the vinyl and propionate groups. The high frequency region contains modes that are sensitive to the iron displacement and the τ electron density of the porphyrin ring (14, 15).

Fig. 3 (a and b) shows that the high and low frequency regions of the resonance Raman spectra of both the equilibrium deoxy form and the 10-ns transient photoproduct of HbA and HbC are identical within our spectral resolution. Identical proximal and distal heme pocket environments would be consistent with the recent report by Shapiro et al. (17) that there are no differences between HbA and HbC with respect to CO geminate recombination kinetics (10-ns resolution). Geminate rebinding has been shown to be sensitive to both proximal (18) and distal (19) perturbations. Considering all of the above, a difference in heme environment between HbC and HbA is an unlikely mechanism to explain the increased fluorescence.

In summary, based on the increase in the intrinsic fluorescence of oxygenated HbC and the lack of differences in the heme environment as demonstrated by the visible resonance Raman spectroscopy and CO geminate rebinding kinetics (17), it is likely that differences exist between oxyHbA and oxyHbC in the microenvironment of at least one tryptophan. Trp<sup>37</sup>, a possible candidate since it contributes to the intrinsic fluorescence of Hb using 296 nm excitation (3–7, 20), can be elimi-
nated since the UV resonance Raman spectroscopy (Fig. 2) shows no HbA/HbC difference involving this residue. In contrast, a substantial intensity difference is apparent at the peak assigned to either Trp$^{14}$ or Trp$^{15}$ (which are not distinguishable by this technique). Given that the β6 mutation is on the same helix as Trp$^{15}$, we conclude that Trp$^{15}$ is the source of the change in signal in both the fluorescence and UV resonance Raman spectra.

Trp$^{15}$ is an A helix residue that lies in the crevice formed by the A and E helices and normally forms a hydrogen bond with Ser$^{77}$ of the E helix in both the deoxy and oxy structures (13). Based on the analysis of Rodgers and Spiro (13), the UV resonance Raman data presented here are interpreted as a weakening of this hydrogen bond in oxyHbC. In addition, the increased fluorescence intensity pattern for oxyHbC is consistent with Trp being farther away from the heme, with the A helix looser or more distant. Finally, as reported in the transient resonance Raman studies, there are no detectable differences in the heme environment between HbA and HbC in either the deoxy T state or liganded R state as reflected in the transient photoproduct spectrum. Therefore, the proximal heme pocket of the liganded R structure does not appear to be altered as a result of the Lys$^{6}$ substitution within the 10-ns timeframe.

The Raman data presented here, the observation that HbC and HbA are functionally identical, and the findings by Shapiro et al. (17) indicating no difference in the CO geminate recombination of HbC compared with HbA indicate that both the proximal and distal heme pocket architectures are indistinguishable.

**Fig. 3.** The high ($v_4$, reporting the porphyrin breathing motion) (a) and low ($v_{Fe-His}$, reporting the Fe-His stretch mode) (b) frequency 10-ns transient resonance Raman spectra of HbACO and HbCCO (1 mM heme) and the equilibrium deoxy forms. The buffer used was 0.05 M sodium phosphate buffer, pH 7.25. Excitation was at 435.7 nm. For a description of the instrument, see "Experimental Procedures." The purified proteins were stored under liquid nitrogen. Storage did not change the absorption spectra or scattered light intensities. To prepare the deoxy derivatives, the sample was degassed with vacuum/nitrogen cycles. HbCO was formed by saturating the protein solutions with gaseous CO. The ligation state of the hemoglobin, sample integrity, and stability were verified by absorption spectroscopy obtained before and after the resonance Raman experiments for each sample.
Fig. 4. Illustration of the A, E, and F helices of the oxyhemoglobin β-chain modified from the data base of Shaanan (22). Our results indicate that the Gluβ6 → Lys substitution of HbC results in a weakened hydrogen bond (Trpβ115-Serβ72) between the A and E helices, with a likely swinging away of the A helix from the E helix (direction indicated by the arrows). The view of the A, E, and F helices is similar to that presented by Rodgers and Spiro (13) to illustrate the α-chain helical movements of the HbA nanosecond transient intermediate.

...able in these two proteins. Hence, the perturbation of the A helix alteration is not transmitted to the critical heme interactions with the E and F helices.

We conclude that the weakened hydrogen bond between the A and E helices, reflected by UV resonance Raman spectroscopy, involving Trpβ115 of the A helix and Serβ72 of the E helix and induced by the Lysβ6 replacement, in turn induces a swinging away of the A helix from the E helix, with the E helix remaining unperturbed (Fig. 4). The absence of comparable differences for the deoxy derivatives could be due to the known increase in the tightness of packing between the EF corner and the N terminus in the deoxy T state, which could be sufficient to maintain the A helix in its standard configuration relative to the E helix.

This model is adopted as the simplest explanation that accounts for our observations and incorporates physical and chemical intuition. Other possible alternative mechanisms to explain the observed fluorescence difference are unlikely in light of the following. 1) A different heme orientation is ruled out by the lack of differences in the visible resonance Raman spectra and by the work of others (17). 2) Resonance energy transfer rate efficiency, this study exemplifies the energy transfer rate efficiency, this study exemplifies the resonance energy transfer rate efficiency, this study exemplifies the energy transfer rate efficiency, this study exemplifies the energy transfer rate efficiency, this study exemplifies the energy transfer rate efficiency, this study exemplifies the energy transfer rate efficiency, this study exemplifies...
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J. Biol. Chem. 1996, 271:372-375.
doi: 10.1074/jbc.271.1.372

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Additions and Corrections

Vol. 273 (1998) 9480–9485

The importance of two conserved arginine residues for catalysis by the Ras GTPase-activating protein, neurofibromin.

Beth A. Sermon, Peter N. Lowe, Molly Strom, and John F. Eccleston

Page 9482, first paragraph, last sentence: This line should read: “Furthermore, the fluorescence change with Ha-Ras-mantGMPPNP was not accelerated by p120-GAP.”

Vol. 271 (1996) 372–375

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Dr. Vidugiris’s name was spelled incorrectly. The correct spelling is shown above.

Vol. 273 (1998) 13037–13046

Identification of a nickel(II) binding site on hemoglobin which confers susceptibility to oxidative deamination and intramolecular cross-linking.

Joseph Levine, Michael Weickert, Maria Pagratis, Jeff Etter, Antony Mathews, Tim Fattor, Julie Lippincott, and Izydor Apostol

Page 13044, Sequence 2: This sequence showing cross-linked peptides was misaligned. The corrected alignment is shown below:

\[
\begin{align*}
A^*-H-L-T-P-E-E-K \\
| \\
NH \\
| \\
V-V-A-G-V-A-N-A-L-A-H-K-Y-H
\end{align*}
\]

SEQUENCE 2

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