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Quantification of Effector Binding to the Hemoglobin Central Cavity by Intrinsic and Extrinsic Steady-State Fluorescence

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The relative intrinsic and extrinsic fluorescence of hemoglobins are established markers of the R (oxy) $\rightarrow$ T (deoxy) transition and reveal site-specific conformational differences amongst hemoglobin (Hb) mutants. The Hb central cavity has been probed by binding the fluorescent analogue of 2,3-diphosphoglycerate, 8-hydroxy-1,3,6-pyrenetrisulfonate (HPT). An approach to quantify binding of HPT to HbC (β6 Lys) and HbA (β6 Glu), by steady-state front-face fluorescence spectroscopy using both the intrinsic and extrinsic emissions, is presented. When HPT specifically binds to Hb, efficient fluorescence energy transfer from the intrinsic fluorescence of Hb to HPT occurs, decreasing the intrinsic fluorescence of Hb that plateaus upon stoichiometric binding. HPT fluorescence is significantly but not totally quenched upon binding to HbA and HbC. HPT exhibits a molecular binding ratio of 2:1 to HbC, in contrast to HbA (1:1 binding). The apparent secondary binding site for HbC is weaker (K_{D1} = 25 μM vs. K_{D2} = 0.15 mM). Conformational alterations of HbC at the αα and ββ clefts of the central cavity are further supported by these data.

KEY WORDS: Hemoglobin; front-face fluorescence; 8-hydroxy-1,3,6-pyrenetrisulfonate; HbC; conformation.

INTRODUCTION

The essential function of hemoglobin (Hb) is to carry and deliver oxygen to all cells of the organism. Native Hb, a tetramer, consists of two α and two β chains arranged around a central water cavity (lined with polar residues) bisected by a molecular 2-fold axis. In the red blood cell, Hb oxygen affinity is modulated by 2,3-diphosphoglycerate (DPG), which binds reversibly to the central cavity of Hb. Upon DPG binding to the central cavity, oxygen affinity decreases, allowing for oxygen release to regions of low oxygen saturation. Under physiological conditions, DPG and Hb are present in normal human red blood cells at a stoichiometric ratio of 1:1 [1].

At the ββ entrance to the central cavity, there are positively charged groups of the β chains that comprise the specific binding site for DPG. The negatively charged groups of DPG form salt bridges with eight positively charged groups of the β subunits, including the α-amino group of β1 Val, the imidazole groups of β2 His and β143 His of both subunits and the α-amino group of β82 Lys of either subunit [2].

The natural allosteric effectors DPG, its analogs, inositol hexaphosphate (IHP) and 8-hydroxy-1,3,6-pyrenetrisulfonate (HPT), synthetic effectors, and chloride bind at specific sites at either end of the central cavity or in the middle of the cavity [3–15]. These allosteric effectors differentially regulate hemoglobin oxygen affinity by shifting the R (liganded) $\rightleftharpoons$ T (deoxy) equilibrium. In the quest for a hemoglobin-based thera-

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peutic oxygen carrier [16,17], an understanding of the structure and function of the central cavity has become paramount in designs to modulate hemoglobin oxygen affinity through the binding of synthetic allosteric effectors and/or site-specific mutagenesis/modification of central cavity residues [7–13,18–21]. Various spectroscopic methods coupled with crystallographic information have been successful in illuminating details of the central cavity structure. This article focuses on the application of intrinsic and extrinsic fluorescence spectroscopy to explore the solution-active structure of the Hb central cavity, and presents a means to quantify the binding of effectors using steady-state fluorescence spectroscopy.

β37 Trp at the α1β2 interface is a significant contributor to the intrinsic fluorescence of Hb that is sensitive to quaternary changes [i.e., T (deoxy) → R (oxy) transition] and serves as a reporter for structural/conformational changes at this critical region [22,23]. Binding of DPG or IHP to the central cavity amplifies the pH sensitivity of the intrinsic Hb fluorescence during the R → T transition [24,25]. Furthermore, the intrinsic fluorescence also serves as an indicator for dissociation/aggregation studies [26].

In 1971, MacQuarrie and Gibson [5,6] first employed HPT as a fluorescent analogue of DPG. Based on steady-state fluorescence intensity measurements, it was demonstrated that HPT fluorescence is highly quenched when bound to deoxy HbA, and HPT has a lower affinity for the DPG-binding site than DPG or IHP. They also quantitatively compared the time courses of HPT release and CO combination during the reaction of CO with deoxy Hb. Subsequent studies have been carried out to characterize the Hb allosteric transition and conformational changes using the binding of HPT to hemoglobin [27–33]. Earlier studies [5,6,27,28,30], using right-angle optical steady-state fluorescence measurements, used a low Hb concentration in order to measure the HPT fluorescence in the presence hemes with their large extinction coefficients over a wide wavelength range. At low Hb concentrations (≤0.3 g%) dimers predominate, complicating the analysis. However, these complications are eliminated using front-face fluorescence measurements [29,31–33], wherein a Hb concentration level of 0.5 g% and greater may be used, ensuring the predominance of tetramers. Front-face fluorometry provides advantages in the study of highly absorbant samples, such as heme proteins, in that the significant inner-filter effect in standard fluorescence measurements is significantly reduced. It not only permits for the detection and analysis of the intrinsic fluorescence of a heme protein, but also serves as a powerful tool to analyze bound extrinsic fluorescent probes (for a review of front-face fluorescence, see Hirsch [34,35]).

HPT fluorescence is partially quenched upon binding to Hb via mechanisms involving resonance energy transfer to the heme [27–30]. Unfortunately, in the presence of hemes and aromatic amino acids, resonance energy transfer calculations become complex. This is primarily due to the involvement of energy transfer from Trp to HPT, from HPT to the heme, in addition to the conformational changes of these sites induced by central cavity effector binding. However, the partial quenching of HPT fluorescence when bound to Hb permits direct detection of both the fluorescence emission of the probe and the intrinsic fluorescence emission of hemoglobin, applicable for relative binding studies. Thus, direct monitoring of HPT steady-state fluorescence provides a straightforward means to explore the hemoglobin central cavity, and has been useful in relative comparisons demonstrating: (1) structural alterations distal from the site of mutation, such as HbS (β6 Glu → Val) and HbC (β6 Glu → Lys) [33]; and (2) central cavity differences of crosslinked hemoglobins designed as potential therapeutic oxygen carriers [29].

Based on the HPT bound and unbound lifetime difference, Gottfried et al. [29] using time-resolved fluorescence coupled to front-face optics has quantified the binding of HPT to CO liganded HbA at pH 6.35. It is established that an altered R-state Hb is induced at this pH, capable of binding IHP or HPT [36,37]. Lifetime methodologies have certain advantages in that it can clearly differentiate multiple specific and nonspecific bindings of HPT in a single measurement. Another fluorescent DPG analog, 1,3,6,8-pyrenetrisulfonate, was shown to be advantageous for time-resolved fluorescence studies probing the DPG pocket: this application revealed a long range communication from the positively charged substitution in the middle of the central cavity of Hb Presbyterian (β108 Asn → Lys) to the DPG binding pocket at the entrance to the ββ cleft [21]. However, lifetime measurements typically require highly sophisticated and costly instrumentation coupled to a laser.

This report presents an approach to quantify HPT binding to Hb using steady-state front-face fluorescence and linear least square analysis. New insight into HPT binding to the mutant HbC is compared HbA. This is of significance in furthering the understanding of molecular mechanisms driving R-state HbC crystal formation that occurs in red blood cells of humans expressing β^C globin.

EXPERIMENTAL

Normal and CC blood were obtained from human donors under NIH guidelines and IRB (AECOM) approved protocols. HbA was purified from AA hemolysates followed by DE-52 anion exchange chromatography as previously described [33]. HbC was separated from CC hemolysates on a CM-52 column using a double gradient (pH 6.8, 0.01 M and pH 8.3, 0.05 M sodium phosphates). The hemolysates were dialyzed against the starting buffer overnight before running the column. Purity of HbA and C was confirmed by isoelectric focusing. The Hbs were then concentrated, dialyzed against pH 7.35, 0.05 M bis-Tris with 0.1 M sodium chloride, and further stripped of low molecular weight effectors on a Sephadex G-25 column equilibrated with the same buffer. The eluted Hb was concentrated and chromatographed again on a second Sephadex G-25 column equilibrated with 0.05 M pH 7.35 HEPES for studies in the absence of chloride. The concentrated Hbs were frozen in liquid nitrogen and stored at −135°C until use.

A stock solution of HPT was prepared in 0.05 M HEPES, pH 6.35. A sodium salt of inositol hexaphosphate (IHP) was obtained from Sigma. A 200 mM IHP stock solution was passed through an Amberlite column and recycled as described previously [38]. All reagents used were of spectroscopic grade.

Steady-state front-face fluorescence spectroscopy was performed with a SLM 8000 photon-counting spectrophotometer equipped with a front-face cuvette holder. A concentration of 0.8 g% Hb was used, a condition in which the tetramer predominates. Fluorescence emission was recorded from 300 nm to 450 nm, using 280 nm excitation. The variability of fluorescence intensity measurements was less than 2%. The emission maximum variation is ± 1 nm.

All data analysis was performed in Sigmaplot 7.0 by using scatter plot coupled with simple linear regression.

RESULTS AND DISCUSSION

Fluorescence Spectra

A traditional titration analysis was used to determine the binding constant and stoichiometry for the association of HPT to Hb by recording both the intrinsic fluorescence of Hb and the extrinsic fluorescence of HPT in one spectral scan (ex. 280 nm, em. 300–450 nm). As previously shown, HPT has multiple transitions with both electronic and vibrational character [29]. Although the absorption spectrum of HPT undergoes considerable change as a function of pH, there is a near-UV transition (near 300 nm), which exists in both acidic and basic conditions. Therefore, excitation at 280 nm was selected, which also excites the intrinsic fluorescence of Hb.

In general, COHb has a relative intrinsic fluorescence emission maximum at ~322 nm. In the presence of HPT, this emission decreases mainly due to the fluorescence resonance energy transfer from Trp to HPT. Figure 1 shows the steady-state fluorescence emission spectra of COHbA and COHbC in the absence and presence of HPT (1:1 molar ratio). While there are no spectral differences (e.g., wavelength or shape change), the intrinsic fluorescence intensity decrease induced by the binding of HPT is smaller in HbC than in HbA. Interestingly, the mutant HbC has a much smaller extrinsic HPT fluorescence emission (em. 385nm) compared to HbA. These observations are attributed to the solution-active conformational differences between HbC and HbA [33] that are discussed further in this paper.

HPT Fluorescence Intensity Change as a Function of HPT Titration

The fluorescence intensity of HPT (em. 385 nm) increases when aliquots of HPT are added to the COHb solution. It is noteworthy that HPT fluorescence exhibits

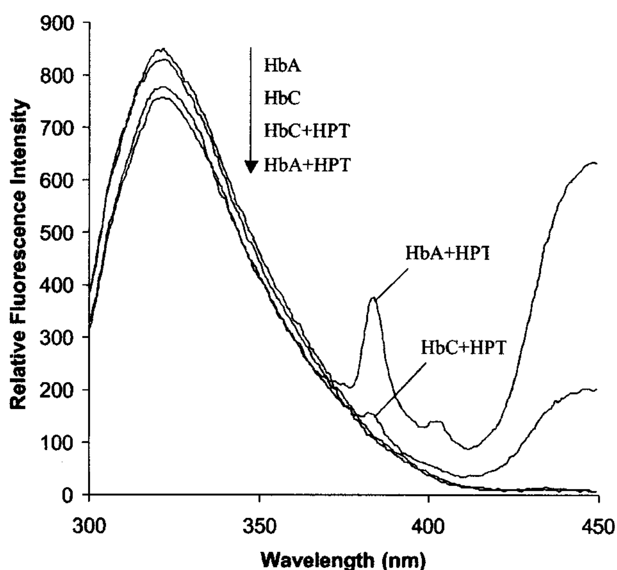

Fig. 1. Uncorrected steady-state fluorescence emission spectra of COHbA and COHbC in the absence and presence of HPT (1:1 Hb/HPT molar ratio). Excitation at 280 nm, with 4nm excitation and emission bandpasses. COHb, 0.125 mM in tetramer, pH 6.35, 50 mM HEPES, HPT, 0.125 mM. The arrow denotes the order of decreasing intensity at ~322 nm for the respective hemoglobin in the absence and presence of HPT.

a progressive increase without a plateau when R-state Hb is titrated by HPT at the concentrations employed [29] and (Figs. 2 and 3). This could complicate the quantitative determination of HPT binding to Hb using steady-state fluorescence of HPT, since the conventional methods of saturation equilibrium binding analysis require a plateau in the titration curve to quantify the bound and unbound HPT. However, when coupled to earlier findings [5,6,27–33], the different fluorescence quantum yield of HPT observed upon titration (Figs. 2 and 3) correlates to the specific and nonspecific binding that provides the basis for quantifying HPT binding.

Plotting HPT relative fluorescence as a function of HPT concentration, followed by linear least squares analysis, generates two linear fits that intersect at one point (Fig. 2). The second linear fit has a greater slope (599.85) than the first one (288.35), indicating a second and different binding microenvironment with a higher fluorescence quantum yield of HPT. The slope values are not absolute, but rather relative to the arbitrary y-axis values.

Since HPT has been demonstrated to bind to the central cavity of COHbA at the ββ cleft at pH 6.35 [29], we hypothesize that the smaller slope of the COHbA titration curve (first fit) corresponds to the specific HPT binding in the central cavity, while the higher slope (second fit) is linked to that of non-specific HPT binding or free HPT in the solution, or the combined contributions of nonspecific binding and free HPT. Thus, the intersecting point in the titration curve indicates stoichiometric binding: based on the concentration of Hb (0.125 mM) used in this study, COHbA clearly has a 1:1 binding to HPT in the central cavity (Fig. 3).

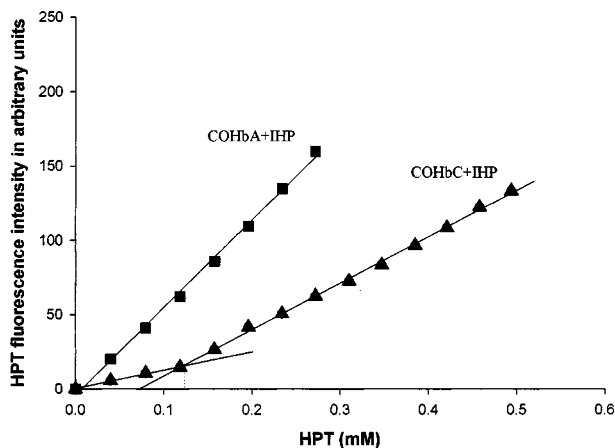

Fig. 3. HPT fluorescence intensity change upon HPT titration of COHbA+IHP (squares) and COHbC+IHP (triangles) in the presence of IHP. The titration data were linearly fit as described. The equations and the correlation coefficients (R^2) for each of the linear regressions are: F = 589.42 C − 4.20 (R^2 = 0.997) for COHbA; F = 122.69 C + 0.33 (R^2 = 0.996) for COHbC at the first fit; F = 312.93 C − 22.66 (R^2 = 0.998) for COHbC at the second fit. F refers to HPT fluorescence intensity; C refers to HPT concentration in mM. COHb, 0.125 mM in tetramer; IHP, 0.125 mM; pH 6.35, 50 mM HEPES. Excitation was at 280 nm and the emission was collected at 385 nm.

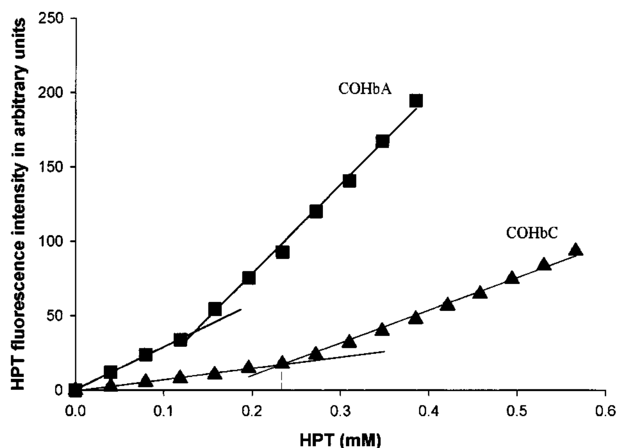

Fig. 2. HPT fluorescence intensity change upon HPT titration of COHbA (squares) and COHbC (triangles). The titration data treated by linear least square analysis generate two linear fits intersecting at one point. Points of intersection are marked by a vertical dashed line drawn to X-axis. The equations and the correlation coefficients (R^2) for each of the linear regressions are: F = 288.35 C + 0.37 (R^2 = 0.998) for COHbA at the first linear fit; F = 599.86 C − 41.40 (R^2 = 0.995) for COHbA at the second linear fit; F = 75.47 C − 0.48 (R^2 = 0.993) for COHbC at the first fit; F = 220.35 C − 34.27 (R^2 = 0.990) for COHbC at the second fit. F refers to HPT fluorescence intensity; C refers to HPT concentration in mM. COHb, 0.125 mM in tetramer, pH 6.35, 50 mM HEPES. Excitation was at 280 nm and the emission was collected at 385 nm.

The above hypothesis is further examined by adding IHP at a 1:1 Hb/IHP ratio to the HbA solution prior to HPT titration. Previous studies have suggested two IHP binding sites in R-state HbA (oxy, NO, or CO liganded), with the higher affinity binding in the ββ cleft and the lower affinity binding in αα cleft [39,40]. It follows that IHP would predominantly bind to the high affinity site at the central cavity ββ cleft when a 1:1 IHP/Hb is added to Hb. Also, IHP is known to have a higher binding affinity to COHb than HPT and efficiently displaces HPT from the central cavity of Hb in solution experiments [31–33]. It can be assumed that when the central cavity ββ cleft is occupied by IHP, there will be no specific binding of HPT at this site.

As shown in Figure 3, titration of HPT to the 1:1 IHP/Hb solution results in one single linear titration fit. It is noteworthy that the slope of the linear fit in Figure 3 (slope, 589.42) is similar to the greater linear slope shown in Figure 2 (slope, 599.85) second fit for HbA, indicating the same microenvironment of HPT in both cases. Therefore, it is concluded that the smaller slope

in Figure 2 for HbA corresponds to HPT binding to the central cavity ββ cleft. The intersecting point of the titration is used to calculate the binding stoichiometry of HPT. The 1:1 HPT/COHbA binding derived from Figures 2 and 3 is consistent with the previous report of Gottfried [29] and the early studies of Gibson [5] showing a 1:1 Hb/HPT binding in deoxy state, since COHbA at pH 6.35 has been demonstrated as an altered R-state that that binds IHP and also HPT [36,37].

The intersecting point shown for COHbC (Fig. 2) indicates that COHbC has a 1:2 Hb/HPT binding stoichiometry. Similarly, this is further examined by titration of HPT into the 1:1 IHP-bound COHbC solution. For COHbC, titration still results in two linear fits with one intersecting point (Fig. 3), demonstrating that HPT binds at a secondary site in a 1:1 ratio following the occupation of the central cavity DPG site by IHP. The existence of a secondary binding site in HbC could be due to the altered central cavity in this mutant (discussed below).

Hb Intrinsic Fluorescence Intensity Change upon HPT Titration

As mentioned above, binding of HPT to COHb is associated with a decrease in the intrinsic fluorescence of Hb due to fluorescence resonance energy transfer from Trp to HPT. The decrease in intrinsic fluorescence intensity appears to plateau when HPT approaches its stoichiometric binding to Hb (Fig. 4). Consistently, HPT exhibits a 1:1 binding to tetrameric HbA as a function of intrinsic florescence changes. The dissociation constant K_D of HPT, denoted as the midpoint response from the intrinsic fluorescence titration curve, is 30 μM for COHbA. This value is in reasonable agreement with 19 μM obtained by Gibson [5] with the difference, if real, perhaps originating from different experimental conditions (pH, buffer).

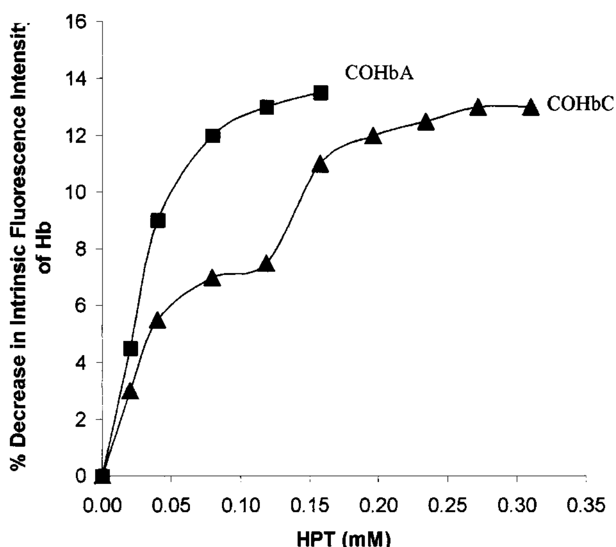

Fig. 4. Intrinsic fluorescence intensity change of COHbA (squares) and COHbC (triangles) upon HPT titration. COHb, 0.125 mM in tetramer; pH 6.35, 50 mM HEPES. Excitation was at 280 nm and the emission was collected at 385 nm.

Under conditions inducing an altered R-state, HbC also shows differences in the intrinsic fluorescence change upon HPT titration. In contrast to HbA, HbC clearly has two binding sites for HPT with the first binding site having a smaller K_D value or higher affinity (K_{D1} = 25μM) than that of the second site (K_{D2} = 0.15mM). Figure 4 shows that the first plateau occurs at approximate 1:1 HPT binding to tetrameric Hb, and the second one is reached at 2:1 HPT/HbC. All these agree with binding ratios derived from the HPT fluorescence titration data shown in Figure 2. Moreover, the difference in HPT extrinsic fluorescence emission intensities (em. 385 nm) for HbC and HbA at a 1:1 molar ratio (Fig. 1) may be understood in terms of the similar but not identical apparent K_D and/or differences in conformational orientation upon binding.

To confirm that the decrease in the Hb intrinsic fluorescence intensity mainly results from the *specific* binding of HPT to the central cavity of Hb, an HPT titration with oxy HbA at pH 7.35 is performed. It is established that compared to deoxy HbA, oxy HbA at pH 7.35 has a smaller central cavity at the ββ cleft and does not specifically bind HPT or other organic phosphates. Results show almost no decrease in the intrinsic fluorescence of oxy HbA at pH 7.35 in the presence of HPT [29]. Similarly, under the conditions investigated (COHb at pH 6.35), the presence of HPT free in solution or the nonspecific binding of HPT, induced by initial binding 1:1 IHP to COHbA, was found to cause a small decrease in the intrinsic fluorescence intensity. This small decrease in the intrinsic fluorescence intensity does not plateau to at least 0.5 mM HPT. The lack of a plateau in the titration is consistent with the observation of nonspecific binding [29]. All of the above imply that specific binding results in the most efficient and significant fluorescence resonance energy transfer from the Trp to HPT, giving rise to the quenched intrinsic fluorescence intensity of Hb.

Since the specific binding has a much higher binding affinity than the nonspecific one, HPT binds predominantly to the specific binding site during the first stage of titration. Although nonspecific binding causes a small decrease in the Hb intrinsic fluorescence intensity, it does not contribute to the *initial* significant decrease in the Hb intrinsic fluorescence intensity as shown in Figure 4. The initial decrease is due to specific binding. Therefore, the

existence of nonspecific HPT binding does not interfere with the specific binding site K_D determinations demonstrated here.

Possible Location of the Secondary HPT Binding Site(s) for COHbC

The 2:1 binding of HPT to tetrameric COHbC with different affinities discussed above raises a question regarding the location of the secondary binding site. The positively charged surface β6 Lys residue is unlikely to be a potential secondary binding site for COHbC because a binding ratio of 3:1 (e.g., the ββ cleft and two β6 Lys sites) would be predicted. Our binding ratio of 2:1 does not support secondary binding at the β6 site. We have tested and confirmed that the first binding site for HPT is in the central cavity ββ cleft DPG binding site for both COHbA and COHbC. A potential candidate for the secondary HPT binding site in HbC is the αα cleft, previously suggested as a secondary binding site for IHP in HbA [39,40]. The αα cleft is an established chloride binding site containing positively charged groups that include the α-amino group of Val −1α and the guanidine residue of Arg 141α of both α chains. This hypothesis also appears to be consistent with an earlier study showing that the dipyridoxal 5-sulfate specifically reacts with liganded HbA at the N-termini of α-chains [41].

Consistent with the above, previous studies of ours [42] indicate that not only is the central cavity ββ cleft altered in COHbC compared to COHbA, but that the αα cleft is altered. It is possible that this alteration may be such that the dimension of the altered R-state HbC central cavity αα cleft is large enough to accommodate HPT. While HbA has been shown to bind IHP and dipyridoxal 5-sulfate at the αα cleft [41], we postulate that HPT would not bind secondarily to the HbA central cavity at the αα cleft due to the larger molecular size of HPT compared to IHP and dipyridoxal 5-sulfate.

SUMMARY

The details and consequences of central cavity alterations coupled to effector modulation in the β6 mutants warrant further exploration. The link between the reported central cavity differences in the β6 mutants and their propensity to form higher-ordered structures remains to be uncovered.

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