Probing the Hemoglobin Central Cavity by Direct Quantification of Effector Binding Using Fluorescence Lifetime Methods*

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The solvent-accessible central cavity of hemoglobin A (HbA) contains functionally important binding sites for several classes of allosteric effectors that facilitate the lowering of oxygen affinity (1, 2). The β-subunit end of the central cavity contains a cluster of eight positive charges that interact with the negative charges of 2,3-diphosphoglycerate (DPG) (3). This site also binds a variety of other negatively charged effectors such as inositol hexaphosphate (IHP), inorganic phosphate, chloride, and polyglutamic acid. The other end (α-subunit) of the central cavity contains additional binding sites, particularly for chloride ions. Another class of potent effectors derived from clofibrate acid and bezafibrate (e.g. L35) bind near the middle of the central cavity with their negative charges projecting toward the α-subunit end (4–6). Binding of these effectors is also associated with a reduction in oxygen affinity. Study of these effectors is of practical interest since control of oxygen affinity is a necessary component for the design of acellular Hb-based oxygen carriers (7, 8).

Time-resolved fluorescence methods have been used to show that 8-hydroxy-1,3,6-pyrenetrisulfonate (HPT), a fluorescent analog of 2,3-diphosphoglycerate, binds to the central cavity of carboxyhemoglobin A (HbACO) at pH 6.35. A direct quantitative approach, based on the distinctive free and bound HPT fluorescence lifetimes of 5.6 ns and ~27 ps, respectively, was developed to measure the binding affinity of this probe. HPT binds to a single site and is displaced by inositol hexaphosphate at a 1:1 mol ratio, indicating that binding occurs at the 2,3-diphosphoglycerate site in the central cavity. Furthermore, the results imply that low pH HbACO exists as an altered R state and not an equilibrium mixture of R and T states. The probe was also used to monitor competitive effector binding and to compare the affinity of the binding site in several cross-bridged HbA derivatives.

X-ray crystallographic studies are important both in pinpointing effector-binding sites and in characterizing the geometry of the effector-bound site (2). However, other methods must be used to determine the structural and functional interactions that are important in solution and to perform titration studies for obtaining binding constants as a function of solution conditions and/or structural state. Functionally relevant synergistic and antagonistic effects among effectors are also best elucidated through solution studies. Functional characterization of hemoglobin suggests that synergistic and competitive activity can occur when combinations of effectors are bound (4). Of the various allosteric effectors, the interactions of DPG (the natural allosteric effector found in the red blood cell) and its analogs in HbA have been investigated the most extensively. However, the binding of DPG can only be measured indirectly through its effects on ligand reactivity and on spectroscopically accessible chromophores such as the heme groups. In this report, we present an extension of the use of a fluorescent analog of DPG to probe directly the hemoglobin DPG-binding site in a quantitative fashion.

Gibson and MacQuarrie (9, 10) showed that 8-hydroxy-1,3,6-pyrenetrisulfonate (HPT) can be used as a fluorescent analog of DPG. Utilizing the observation that the fluorescence signal from HPT is highly quenched when bound to HbA, they performed steady-state fluorescence intensity measurements and established that HPT has a lower affinity for the DPG-binding site than DPG or IHP. A subsequent study by that group (11), using HPT as a probe of the DPG-binding site, focused on the detection of ligand-binding intermediates occurring along the R to T transition pathway. In those initial experiments, the results were limited in large part by two aspects of the methodology. (i) To monitor changes in HPT fluorescence in the presence of the highly absorbing heme, low concentrations of Hb had to be used. As a result, dissociation of the tetramer produced αβ dimers in a concentration high enough to complicate data analysis. (ii) The use of steady-state fluorescence quenching as a means of obtaining titration data does not allow for probing of the HPT-bound species or for a direct determination of the fractions of both bound and unbound effector in a single measurement. In the present study, these limitations were overcome using a combination of front-face optical geometry and fluorescence lifetime measurements. The use of front-face excitation allows for the observation of HPT fluorescence upon binding to hemoglobin at high concentrations without inner-filter effects or substantial dimer formation. Front-face fluorescence techniques have been used previously to probe the highly quenched fluorescence from tryptophans within both hemoglobins and myoglobin (12).

Fluorescence lifetime measurements of HPT in the presence of HbA reveal two distinct and easily resolved lifetime components that allow unambiguous determination of the fractions of
bound and free ligand under various solution conditions (see below). In contrast, conventional methods of saturation equilibrium binding analysis require a full titration data set complete with a plateau in the binding curve to quantify bound and free ligand (13). In addition, quantitative analysis of steady-state measurements comparing binding data under different solution conditions (pH and/or ionic strength) would be hampered due to the variation in the extinction coefficient of HPT at the excitation wavelength and would therefore require a correction factor to account for this dependence. Furthermore, as will be shown below, although the fluorescence intensity of the bound chromophore is significantly diminished compared with that of the free chromophore, the quantum yield is not negligible. This residual intensity complicates steady-state titration measurements that do not normalize to the intensity of fully bound ligand. Aside from obviating the need for these corrections, lifetime measurements of the bound species can also be used to characterize (structurally and dynamically) the binding site as a function of solution conditions or protein modifications. In particular, the fluorescence spectrum of the bound ligand can be obtained, independent of a large background of free ligand, using time-gated spectroscopy. Last, fluorescence lifetime measurements can assist in the determination of multiple binding sites and in the identification of nonspecific binding.

Lifetime fluorescence methods are applied here to the study of effector binding at low pH to the carboxy forms of both native HbA and three intramolecularly cross-bridged derivatives of HbA. Liganded hemoglobin was chosen for several reasons. First, the affinity for DPG-binding site effectors of deoxy derivatives of HbA is very high. Consequently, as long as the HbA remains in the deoxy state, there is not likely to be substantial variation in the binding properties of the effectors as a function of protein modification. The greatly reduced affinity of liganded Hb for these effectors increases the dynamic range associated with the parameters describing the interaction between the effector and the protein.

A second reason for studying the liganded derivatives is that there has been considerable ambiguity as to the nature of the interaction of effectors with the liganded protein. Most of the work on the role of effectors in regulating ligand binding is predicated on the assumption that the effect arises from preferential binding of the effector to the T state structure. There is, however, considerable evidence that DPG and IHP do indeed bind to the liganded ferrous forms of HbA (14–17). What is not clear and is still being explored is whether the effectors bind exclusively to the small fraction of liganded T state species, thereby shifting the equilibrium from R toward T as binding progresses, or whether the effectors bind with reduced affinity to the R state liganded species, resulting in an altered R state.

The present study directly addresses the issue of whether HPT and IHP bind stoichiometrically to liganded HbA. These data are then used in conjunction with earlier spectroscopic studies to assess the nature of effector-bound liganded HbA. In addition, the HPT-based technique is used to evaluate how three different site-specific cross-linkers influence the DPG-binding site in liganded HbA.

MATERIALS AND METHODS

Sample Preparation—Human adult hemoglobin was purified as described previously (18). A hemolysate was prepared from whole blood obtained from an AC or AA individual using the freeze-thaw technique to remove membranous material. The hemolysate was dialyzed against 10 mM sodium phosphate (pH 6.8), and the hemoglobins were separated on a CM-52 column using a double gradient (10 mM sodium phosphate (pH 6.8) to 50 mM sodium phosphate (pH 8.3)). Purified HbA was collected and verified using cellulose-acetate gel electrophoresis and isoelectric focusing. The hemoglobin was concentrated and dialyzed against 50 mM bis-tris with 100 mM sodium chloride (pH 7.35) and stopped on a Sephadex G-25 column equilibrated with the same buffer. This purified material was then placed on a second Sephadex G-25 column equilibrated with 50 mM Hepes (pH 7.35) and concentrated and equilibrated with the buffer of choice using aCentricon-10 concentrator (Amicon, Inc.). The hemoglobin was stored frozen under nitrogen until used. HbACO was prepared by gently flowing chemically pure CO over the surface of the HbA solution. Deoxy-HbA was obtained by gently passing pure nitrogen or helium into an anaerobic vessel containing the HbA solution, which was then anaerobically transferred to the cuvette.

Complete conversion to the CO or deoxy forms was verified by the absorption spectrum.

Three forms of intramolecularly cross-bridged HbA were used in this study. An αα cross-linked HbA was prepared by reacting deoxy-HbA with bis(3,5-dibromosalicylic)malonate as described by Walder et al. (19). The cross-link introduced is between the α-amino groups of Lys-o99 and is within the central cavity. Two other derivatives, with cross-links between residues of the β-chains, were also prepared. Bis(3,5-dibromosalicylic)sebacate reacts with deoxy-HbA at slightly alkaline pH to introduce intramolecular cross-linking between the α-amino groups of Lys-b82 as described by Bucci et al. (20). This selective ββ cross-linking reaction has been optimized for the preparation of pure cross-linked material.2 Deoxy-HbA (1–2 mM) in 50 mM borate buffer (pH 9.1) was reacted with bis(3,5-dibromosalicylic)sebacate at 37 °C for 3 h. At the end of this reaction, glycyglycine was added in 10-fold excess over the cross-linker to scavenge the excess reagent. The reacted HbA was dialyzed against 10 mM Tris and 20 mM glycine (pH 8.3). The cross-linked HbA was purified by ion-exchange chromatography using a Bio-Rad Macroprep High Q anion exchanger. The column was equilibrated with the dialysis buffer, and the proteins were eluted by starting sodium chloride (30, 75, and 200 mM) in the same buffer. The ascending side of the 75 mM NaCl elution peak was used in this study, and ~95% of the material was cross-linked. Intramolecular cross-linking of HbA between the Cys-β93 residues was carried out using bis(maleimide)/polyethylene glycol 2000 (bis-Mal/PEG2000). HbA0o (0.5 mM) in phosphate-buffered saline was reacted with 2-fold molar excess of the cross-linking reagent at room temperature for 1 h. Under these conditions, nearly quantitative (>99%) intramolecular cross-linking occurred. The cross-linked material was separated from the excess reagent by gel filtration and then further purified by CM-cellulose chromatography. The cross-link results in a slight increase in the oxygen affinity of the protein. The cross-linking reagents bis(3,5-dibromosalicylic)sebacate and bis-Mal/PEG2000 were prepared by Paul Smith of Bioaffinity Systems (Rockford, IL).

HPT was obtained from Eastman Kodak Co. and used without further purification. Stock solutions for titrations were prepared in 50 mM Hepes (pH 6.35). It should be noted that there is a significant change in the absorption spectrum of HPT upon changing the pH from 6 to 8 (9). However, excitation in most cases was at 310 nm, where the effect is small, and no pH dependence of the lowest energy (505 nm) emission spectrum or fluorescence lifetime was observed (see below). The sodium salt of IHP was purchased from Sigma. A 200 mM aqueous IHP solution was passed and recycled for 1 h through a dry Amberlite column as described previously (21). This solution was frozen and stored in aliquots until needed. 20 mM stock solutions used for titration were prepared with the desired buffer. L35 was obtained as a generous gift from Drs. I. Lalezari and P. Lalezari, and a stock solution was made by dissolving L35 in the desired buffer to a final concentration of 20 mM.

Steady-state Fluorescence—Front-face fluorescence spectra were measured using an SLM 8000 photon-counting spectrophotometer equipped with a front-face accessory. HbA solutions of 0.6–1 g/100 ml (0.10–0.16 mM in tetramer) were prepared for titration with the corresponding effector. The met (Fe3+) content was <5%. For a review of front-face hemoglobin fluorescence measurements, see Hirsch (12).

Time-resolved Fluorescence—The time-correlated single photon-counting system was described previously (22). Considerable assistance in optimization was obtained using the methods described by Holtom (23). The light source was a mode-locked, frequency-doubled neodymium:YAG laser that synchronously pumped a dye laser with rhodamine 6G as the active medium. The cavity-dumped dye laser output was frequency-doubled and used visible to the near-ultraviolet. A KDP crystal was used to generate light in the near-ultraviolet. The cavity-dumped dye laser output was frequency-doubled and used in the near-ultraviolet. A KDP crystal was used to generate light in the near-ultraviolet.
fluorescence decay. In this manner, spectra with time resolution of
wavelength that fall into a predefined temporal region of interest in the
monochromator while counting only those emission events at each

where

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the S2 state.

in the near-UV region (280–310 nm), which excites primarily
cr exciting transitions with both electronic and vibrational character. At pH
8.0, it is assumed that the band at 455 nm represents the

absorption of the protonated HPT. Under acidic conditions, the
solution was slowly stirred using a miniature magnetic bar to reduce
artifacts from photodamage.

The fluorescence, collected at right angles to the illuminating light,
was collimated, passed through a sheet polarizer (at the magic angle)
and a cutoff filter, and then focused onto the entrance slit of a 1/8-meter
subtractive double monochromator. The monochromator output was
directed onto the surface of a microchannel plate photomultiplier tube,
and a cutoff filter, and then focused onto the entrance slit of a 1/8-meter
subtractive double monochromator. The monochromator output was
directed onto the surface of a microchannel plate photomultiplier tube,
and the signal was measured using standard NIM electronics (22). The
instrument response function was typically 35 ps fwhm. Deconvolution
of the emission decay curves and fitting to a sum of exponentials
(Equation 1),

\[ R(t) = \sum_i a_i \exp(-t/\tau_i) \]  

(Eq. 1)

where \(a_i\) and \(\tau_i\) are the normalized pre-exponential and decay time
components, respectively, were achieved using the Marquardt nonlin-
ear least-squares method. Determination of the quality of fit was judged
from the value of the reduced \(\chi^2\), the runs test, the residuals, and the
autocorrelation of the residuals (25). Estimation of error in the fitting
parameters was done using the support plane method (\(\chi^2\) surface) (26).

Time-gated fluorescence spectra were obtained by step scanning the
monochromator while counting only those emission events at each
wavelength that fall into a predefined temporal region of interest in the
fluorescence decay. In this manner, spectra with time resolution of
\(~100\) ps can be measured.

RESULTS AND DISCUSSION

Probe Characterization—The absorption spectra of HPT at
pH 8.0 and 6.35 (Fig. 1) reveal the presence of multiple tran-
sitions with both electronic and vibrational character. At pH
8.0, it is assumed that the band at 455 nm represents the
lowest energy electronic transition, \(S_0 \rightarrow S_1\), of the deproto-
nated species (42). The shoulders at higher energy near 490
and 375 nm are attributable to transitions of excited vibra-
tional states within this electronic band or to ground-state
absorption of the protonated HPT. Under acidic conditions, the
positions of these features remain unchanged, but their rela-
tive intensities are altered. In both acidic and basic solutions,
there is a near-UV transition around 297 nm, which is assigned
to a higher energy electronic transition, \(S_0 \rightarrow S_2\). All fluo-
rescence measurements in this report were made with excitation
in the near-UV region (280–310 nm), which excites primarily
the S2 state.

Because we measured spectroscopic parameters of HPT under
different solution conditions (e.g. pH), we wished to ensure that
there were no anomalous properties of the probe chromo-
morph that would lead to misinterpretation of the data.
Consistent with the observations of Gibson and MacQuarrie (9,
10), there is considerable change in the absorption spectrum of
HPT as a function of pH. Consequently, this could hamper
quantitative analysis of equilibrium ligand binding from
steady-state spectroscopy when comparing solutions at differ-
ent pH values, due to the variation in the extinction coefficient
at the excitation wavelength. In contrast, lifetime measure-
ments may be used to quantitate the binding of HPT to Hb
without the need for correction factors. The shape and peak
wavelength of the HPT emission spectrum are independent
of protonation state in this pH region (Fig. 1). Note, however,
the presence of a shoulder near 440 nm in the spectrum at pH 6.35.
As will be argued below, this emission originates from the
pH-dependent higher energy transition.

Time-gated fluorescence spectra of bound \((\tau < 200\) ps) and
free \((\tau > 2\) ns) HPT (see below) do not differ significantly. This
observation is crucial to ensuring that the fractional popula-
tions of these species can be extracted from the pre-exponential
amplitudes in a multiexponential decay fit without correcting
for spectral changes. This requirement for invariance as a
function of binding is applicable as well to the extinction coef-
ficient, which is also unchanged due to binding of the HPT
chromophore to the protein.

Experimental constraints of the present study necessitated
the use of near-UV excitation, which populates a higher energy
state of HPT, as opposed to direct excitation in the lowest
energy band around 450 nm. S2 excitation is typically followed
by relaxation to the S1 state, which fluoresces with high quan-
tum yield. Fig. 2 presents the fluorescence decay curves of free
HPT in solution at pH 6.35 for excitation at 310 nm and
emission at 510 nm. The fitting parameters for both one- and
two-exponential decay models at high and low pH are given in

4This pH dependence has interesting implications for the use of HPT
as a probe of electrostatics within the Hb central cavity. In future
experiments, we intend to measure the excitation and emission spectra
of bound HPT to determine the apparent pH at the DPG-binding site
(42).
Effect of Binding to Hemoglobin

Table I. Fluorescence decay parameters of HPT

| Solution | [HbA]/[HPT] | $a_1$ | $\tau_1$ | $a_2$ | $\tau_2$ | $a_3$ | $\tau_3$ | $\chi^2$ |
|----------|-------------|-------|----------|-------|----------|-------|----------|---------|
| pH 8.0   | 0           | 1.0   | 5.64 ns  | 0.824 | 5.62 ns  | 0.955 | 5.36 ns  | 1.1     |
| pH 6.35  | 0           | 0.83  | 0.045    | 1.73 ns| 0.955    | 5.36 ns| 1.1      | 1.1     |
| pH 8.0   | 0.88        | 0.578 | 28.1 ps  | 0.040 | 1.72 ns  | 0.382 | 5.30 ns  | 1.1     |
| pH 6.35  | 0.45        | 0.879 | 26.3 ps  | 0.034 | 307 ps   | 0.087 | 5.12 ns  | 1.2     |

Table I. It can be seen that the excited state lifetime ($\tau_2 = 5.6$ ns) is the same at the two pH values and is in agreement with that previously reported (43). In addition to the fluorescence decay, there is a rise time ($\tau_1$), denoted by the negative pre-exponential parameter, with a time constant of $\sim 100$ ps. Although it is difficult to quantify the amplitude of this component using a simple sum of exponentials decay model, it is clear that the rise time has a larger amplitude, relative to the decay, at pH 6.35. Measurements of decay curves at emission wavelengths around 450 nm (see Fig. 1) also result in lifetimes around 100 ps (data not shown). In addition, a time-gated spectrum of the early time fluorescence ($t < 200$ ps) shows bands at 445 and 505 nm, which are assigned to emission from the absorption transitions at 400 and 450 nm, respectively. A time-gated spectrum of the long-lived fluorescence shows only the reddest (505 nm) emission. This assignment explains the apparent difference in rise time amplitude and the intensity of the 440 nm emission band, depending on pH, since the pH alters both the ratio of S2 to S1 absorption at the excitation wavelength of 310 nm and the proportions of protonated and unprotonated HPT fluorescence (see Fig. 1). All of this evidence indicates that this rise time is consistent with the rate of relaxation from a higher excited state (electronic or vibrational) to a vibrationally thermalized S1. Alternatively, this decay component may represent the time for photo-initiated proton transfer. The practical implications of this rise time on the quantitative determination of HPT binding to HbA are discussed below.

An additional potential complication due to the excitation at 310 nm results from the effect of energy transfer from the intrinsic Trp residues of Hb to HPT. Because this excitation transfer occurs only to bound HPT chromophores that lie within the Förster transfer radius, it essentially increases the apparent extinction coefficient of bound HPT by a factor proportional to the product of the donor (Trp) extinction coefficient at the excitation wavelength and the energy transfer efficiency from Trp to HPT under the conditions of the measurement. This increased apparent extinction coefficient would overly weight the fraction of bound chromophores in the decay analysis. In the present case, however, there is reason to assume that this effect is negligible. First, the extinction of Trp at 310 nm is small, $\epsilon \sim 20$ M$^{-1}$ cm$^{-1}$ (27), compared with that of HPT, $\epsilon \sim 4500$ M$^{-1}$ cm$^{-1}$ (28). Second, in a separate experiment that monitored the Trp lifetime in the absence and presence of saturating HPT, the energy transfer efficiency was estimated to be no more than 10%. Consequently, systematic error due to energy transfer is considered to be small.

Probe Binding to Deoxy-HbA—In steady-state fluorescence measurements, the addition of HPT to deoxyhemoglobin (50 mM Hepes (pH 7.35)) results in a decrease in the intrinsic tryptophan fluorescence intensity and the loss of HPT emission (Fig. 3A). The addition of HPT to HbCO (above pH 7) results in little or no Trp fluorescence change and the retention of a measurable HPT signal (Fig. 3B). Furthermore, the Trp fluorescence decrease observed with deoxyhemoglobin upon addition of HPT is reversible upon oxygenation of the solution. These results are as expected since studies by Gibson and MacQuarrie (9, 10) demonstrated that HPT binds with much higher affinity to deoxyhemoglobin than to oxyhemoglobin. HPT has also been shown by those workers to bind to the T state of hemoglobin with 1:1 stoichiometry (9, 10). However, the measured dissociation constant is sensitive to pH and ionic strength. HPT is displaced from deoxy-HbA with the same stoichiometry by DPG and IHP, with binding affinities in the order IHP & DPG > HPT.

Probe Binding to HbACO—Binding of HPT to HbACO under slightly acidic and alkaline conditions was monitored using the fluorescence at 505 nm. The HPT fluorescence intensity is much more than an order of magnitude lower in the presence of HbACO at pH 6.35 compared with pH 8.0. The decrease is attributed to energy transfer to the heme, and this is confirmed by lifetime measurements showing a picosecond lifetime for bound HPT and a nanosecond lifetime for free HPT (Fig. 4 and Table I). Using the lifetimes, it becomes possible to measure directly the fraction of HPT bound and thereby to establish its dissociation constant. Thus, Equation 1 can be rewritten more explicitly as follows (Equation 2),

$$I(t) = a_b \exp(-t/\tau_b) + a_s \exp(-t/\tau_s) + a_f \exp(-t/\tau_f)$$  (Eq. 2)

where the subscripts $b$, $n$, and $f$ refer to the bound, nonspecifically bound (see below), and free fractional populations of HPT, respectively (and correspond to the subscripts 1, 2, and 3 in Table I).

As can be seen from Table I, in addition to the lifetime components representing free and bound HPT, there is an intermediate lifetime ($\tau_2 = 1$ ns, but with large uncertainty due to its small intensity contribution) whose fractional population varies between 4 and 7%. Titration data also reveal that this component is only mildly dependent on HPT concentration (for a given concentration of HbACO). We interpret this artifact as

Unpublished results (D. S. Gottfried and J. M. Friedman) indicate that one component of the loss of Trp fluorescence intensity with HPT binding is due to a decrease in the population of the long-lived nanosecond lifetime component of the Trp emission, accompanied by a comparable increase in the population of the highly quenched picosecond species. Recent results from this laboratory suggest that this may be due to a damping of hemoglobin conformational dynamics that increase the Trp-heme distance when the HPT ligand is bound, rather than an increase in the rate of energy transfer from the tryptophans to HPT (22).
arising from nonspecific binding of a fraction of the HPT to the surface of the Hb. This results in some fluorescence quenching by the hemes, but less than that for the more tightly bound, site-specific fluorophores. In all quantitative analyses, this component is excluded from the fractions of bound and free HPT. This highlights another advantage of time-resolved measurements for equilibrium binding studies in that distinct molecular species can be identified, isolated, and quantitatively measured.

The rise time component of the fluorescence decay for near-UV excitation of the unbound HPT described above ($\tau \sim 100\ p$s) is similar to the picosecond decay of the bound species. However, these cannot be distinguished in the fitting of the decay curve to a simple sum of exponentials. It is our observation that the negative amplitude merely cancels a portion of the positive amplitude for the picosecond (bound) lifetime. This implies that the fraction of bound HPT measured is a lower limit to the true value. This complication may become significant only when the amount of unbound HPT is much larger than that of the bound species, and data presented here are not corrected for this effect.6

A titration of HbACO at pH 6.35 with HPT by monitoring the amplitudes of the picosecond and nanosecond lifetime components as described above is shown in the binding curves of Fig. 5. Nonlinear fitting to the single-site binding expression (Equation 3)

$$B = B_{\text{max}} \cdot F/(K_d + F)$$  

(Eq. 3)

was performed (Fig. 5B). In Equation 3, $B$ and $F$ are the bound and free ligand concentrations, respectively; $B_{\text{max}}$ is the maximal bound ligand concentration; and $K_d$ is the equilibrium dissociation constant. Fitting parameters of $B_{\text{max}} = 93.3 \pm 2.5 \ \mu\text{M}$ and $K_d = 8.6 \pm 0.5 \ \mu\text{M}$ ($R^2 = 0.99$) were found. This value of $B_{\text{max}}$ implies that the binding stoichiometry is nearly 1:1 ([HbACO] = 100 \ \mu\text{M}). A linearized Scatchard-type plot is shown in Fig. 5C. The measured value of $K_d$ is similar to that obtained by Gibson and MacQuarrie (9, 10), who noted variations in binding affinity dependent on sample preparation and specific solution conditions. The fluorescence lifetime-based titration data are consistent with a single binding site for HPT, and at the relatively high concentrations of Hb used in the study, there is a minimal contribution from $\alpha\beta$-dimeric species. This result indicates that low pH HbACO binds HPT stoichiometrically, forming a single homogeneous population of effector-bound protein.

**Competitive Effector Binding by IHP**—By monitoring the fraction of HPT bound to HbA, the result of adding other allosteric effectors can be measured. The steady-state emission spectrum of HPT/deoxy-HbA (pH 7.35) in the absence and presence of IHP is shown in Fig. 6. The increase in HPT fluorescence is indicative of release of HPT upon addition of IHP. This observation is consistent with the earlier results of Gibson and MacQuarrie (9). Fluorescence lifetime measurements allow the fraction of bound HPT, for a given initial ratio of [HPT] to [HbACO], to be monitored as a function of a second added effector. The results for IHP are shown in Fig. 7A. The stoichiometric displacement of HPT from HbACO by IHP, with a sharp end point at 1 eq of IHP/tetramer, is consistent both with HPT binding at the DPG-binding site and with IHP having a much higher affinity for that site relative to HPT. Mac-

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6 Future experiments will be performed using pyrene-based probes that lack the hydroxyl group responsible for the photo-induced proton transfer. This will eliminate the presence of the rise time component in the decay curves. Direct excitation of the lowest energy transition will further reduce any complications due to excitation of and energy transfer from tryptophan.
Quarrie and Gibson (10), using a steady-state measurement, obtained a similar result with both IHP and DPG. Although it is possible that HPT binds at a different site and is released from HbACO due to an IHP-induced conformational change, this explanation is very unlikely. IHP binding to HbACO at low pH induces changes that show up in the heme resonance Raman spectrum (16, 17) and the steady-state ultraviolet resonance Raman spectrum,7 which indicate a shift toward a more T-like tertiary structure. Thus, if the HPT-binding site were different from the IHP-binding site, the IHP-bound HbA would be expected to bind HPT more tightly, based on the difference in binding affinities between the T state deoxy and R state CO derivatives of HbA. It follows with a high degree of certainty, therefore, that HPT and IHP are both binding to the same DPG-binding site.

Nature of IHP-bound HbACO—Earlier studies have demonstrated that the addition of IHP to solutions of liganded HbA (CO and O₂) has a clear influence on the structure (16, 17) and reactivity (15) of the liganded species. Significantly, IHP decreases the yield of geminate recombination on the picosecond and nanosecond time scales (29, 30), but not to the extent seen in the T state species (31). Upon addition of IHP, the visible resonance Raman spectra of the transient forms of HbA generated within 30 ps (32) and 10 ns (16, 17) of photodissociating the parent HbACO show a decrease in the frequency of the iron-proximal histidine stretching mode from 230 cm⁻¹ to 226 cm⁻¹. This shift was shown to originate from the β-chains (17). The corresponding frequency for T state photoproduct is ~220–222 cm⁻¹.

Both the geminate rebinding and Raman studies are interpretable in terms of two models. One possibility is that IHP binds to a T state fraction of the liganded Hb molecules. The Raman and geminate rebinding results then suggest a mixture of IHP-bound T state liganded Hb and IHP-free R state liganded Hb. A second possibility is that there is a single population of IHP-bound liganded Hb having a strained R state structure, resulting from IHP-induced conformational changes in the direction of the R to T transition. The results presented here show that, at low pH, the addition of a slight excess of IHP results in a single homogeneous population of IHP-bound HbACO. In conjunction with the Raman and geminate rebinding results, this indicates that the second model is much more likely. IHP-bound HbACO remains in a strained or altered R state, in contrast to the HbANO derivative, which is readily and unambiguously switched to the T state upon addition of IHP at low pH (33, 34) as reflected in the 222 cm⁻¹ frequency for the iron-histidine Raman band of the 10-ns photoprodut (16, 35).

Competitive Effector Binding by L35—X-ray crystallographic studies reveal that the clofibric acid derivative L35 binds well away from the DPG-binding site at a position within the central cavity in the region of Lys-α99 and extending to the C terminus of the α-subunits (4). If effector binding at either site does not globally alter the Hb structure, it is expected that simultaneous occupation of the sites by the two effectors can occur. If central cavity communication is important or multiple binding sites for L35 exist, then synergistic or antagonistic effects may result. Fig. 7B demonstrates that the addition of L35 to a solution of HbACO partially saturated with HPT does not cause additional binding or release of the HPT below [L35]/[HbA] = 2.5. At higher concentrations, however, displacement of the HPT begins to be observed. L35 is a potent clofibrate-derived allosteric effector that exerts a considerable pull toward the T state when bound to HbACO. This effect has been observed directly by monitoring

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7 S. Huang and J. M. Friedman, unpublished results.
the iron-His stretching frequency of the photoprod-
uct occurring within 10 ns of photodissocia-
tion of HbACO in the presence of L35. The spectr-
um of the photoproduc-
t, which reflects the influence of the initial ter-
ary/quaternary structure of the ligand-bound globin upon the five-coordinate, high-spin heme in the photoproduc-
t, shows an iron-proximal histidine stretching fre-
cency shifted toward the T state value.\(^8\)

The results of the present study indicate that, in excess, L35 can cause HPT to be displaced from HbACO. Since L35 binding to its site in the interior of the central cavity results in a more T-like structure, one would anticipate an increase in HPT binding upon addition of L35 (synergistic effect). The observed decrease in HPT binding coincident with the addition of excess L35 is most easily explained if one assumes that L35 can also weakly bind in the vicinity of the DPG-binding site. A secondary binding site for L35 has been observed along the length of the G helix of the \(\beta\)-subunits in x-ray crystallographic studies (4). It is plausible that this site is sufficiently close to the DPG site to alter HPT binding either through electrostatic effects or through a local conformational change. In either case, occu-
pancy of this second site reduces HPT binding.

\(^8\) E. S. Peterson and J. M. Friedman, unpublished results.

**FIG. 7.** Competitive binding of HPT with IHP (A) and L35 (B) to HbACO (pH 6.35) from lifetime measurements. The sample was initially bound with HPT, \([\text{HPT}]/[\text{HbA}] = 1.4\) for A and \([\text{HPT}]/[\text{HbA}] = 1.2\) for B, and the competitive effector was added. In both A and B, the left vertical axes show the fractional saturation of HPT (from the pre-
exponential value of the short decay component), and the right vertical axes show the change in HPT average lifetime \(\langle \tau \rangle = \Sigma \alpha_i \tau_i\) as the fraction of unbound effector increases. Both measures indicate complete displacement of HPT by 1 mol. eq. of IHP, while the fraction of bound HPT was stable until a large excess of L35 was added.

**FIG. 8.** Relative binding of HPT to HbACO and three cross-
linked HbACO derivatives at pH 6.35. In all cases, \([\text{HPT}]/[\text{HbA}] = 1.1\). The fraction bound is the normalized amplitude of the short lifetime \(\langle \tau \rangle\) component in the HPT decay. Error bars represent the one S.D. confidence interval and were obtained from a rigorous error analysis using the support plane method (26).

**Effector Accessibility in Cross-bridged Derivatives of HbA**

In the quest for stable tetrameric, low affinity hemoglo-
ins, cross-bridged hemoglobin derivatives have been synthe-
sized by a number of methods (7). Cross-bridges have been construc-
ted between residues lying at different locations or depths within the central cavity. The accessibility or affinity of the DPG-binding site toward effectors in the presence of such modifications may be determined by HPT binding studies as shown by a comparison of the fraction of bound HPT for wild-
type HbACO and three cross-bridged derivatives (Fig. 8). In these measurements, the ratio of HPT to Hb tetramer was fixed at 1:1. In all cases, the amount of nonspecifically bound HPT was constant at 3–5%.

The \(\alpha_{99}\)-cross-linked sample (Fig. 8, XL\(\alpha_{99}\)) shows the smallest change in HPT binding compared with HbA. This cross-
bridge is located well into the interior of the central cavity, and the spacer arm is short and rigid, which should preclude any direct steric or electrostatic effect on the binding of HPT to the DPG-binding site. Earlier functional (36, 37) and spectroscopic (38) studies of this cross-bridged Hb were inter-
preted as showing that the \(R\) state is destabilized. If so, en-
hanced HPT binding is anticipated. The present results, which reveal no increased effector binding, are more consistent with the interpretation that the cross-bridge influences local, but not global, tertiary structure as suggested by Ferrone and co-
workers (39).

The \(\beta_{82}\)-cross-linked Hb (Fig. 8, XL\(\beta_{82}\)) shows a marked decrease in the binding of HPT relative to native HbA and the \(\alpha\alpha\)-cross-linked species. The location of the cross-bridge is di-
rectly across the DPG-binding site, and Lys-\(\beta_{82}\) directly con-
tributes to the binding of effectors at this site (40). Thus, both the location of the cross-bridge and the site of attachment of the cross-bridging reagent favor a decrease in effector binding at the DPG-binding site. The presence of bound HPT, however, indicates that binding is not completely blocked in this deriv-
ative. This may be related to the flexibility of the spacer arm of the sebacate cross-bridge between the \(\beta\)-chains. The results are also consistent with a reduced binding affinity due to the loss of the contribution of the positive charges of the Lys-\(\beta_{82}\) side chains to the stability of the bound complex.

The bis-Mal/PEG2000 derivative of HbACO (Fig. 8, XL\(\beta_{93}\)) is a newly developed cross-bridged Hb (41) and shows a sub-
stantial increase in the binding affinity for HPT relative to native HbACO. Functional studies of this Hb have shown that it retains both cooperativity and near-normal oxygen affinity despite the fact that the site of attachment is Cys-\(\beta_{93}\). Most simple modifications of this site produce high affinity hemoglo-
bins with drastically reduced or eliminated cooperativity. Visible and ultraviolet resonance Raman spectroscopy studies\(^9\) on bis-Mal/PEG2000 HbA reveal that, upon switching from the deoxy to the carboxy derivative, the \(\alpha_\beta\)-interface undergoes transitions that are very similar to those that occur when HbA undergoes the \(T\) to \(R\) quaternary switch. There are indications from the UV resonance Raman spectrum that the presence of the PEG enhances the hydrogen bonding between the penultimate tyrosine (residue \(\beta 145\)) and its hydrogen-bonding partner on the FG corner in the CO-ligated derivative. An increase in this interaction is expected to impart \(T\)-like properties to the \(R\) state of the liganded form of the protein. Visible Raman spectroscopy supports this interpretation in that the spectrum of the 10-nsec photoproduce of the CO derivative shows a drastically lowered iron-proximal histidine stretching frequency, which is indicative of increased \(T\)-like strain in the \(R\) quaternary state. Also consistent with these findings is the observation that guanine rebinding is reduced in this derivative compared with HbA and the two other cross-bridged hemoglobins. The geminate yield is expected to decrease for the more \(T\)-like CO-bound derivatives of HbA. The emerging picture for the bis-Mal/malate yield is expected to decrease for the more \(HbA\) and the two other cross-bridged hemoglobins. The geminate rebind to HbACO at \(pH\) 6.35 have also set the stage for the \(T\) state of the liganded form of the protein. Visible Raman spectroscopy shows that the CO binding to HbACO at \(pH\) 6.35, but not at much higher \(pH\); (ii) HPT is in quaternary and tertiary structure. The extent of HPT binding can be correlated with other spectroscopic measurements of the \(T\) state and that this binding

\(^9\) E. S. Peterson, S. Huang, and J. M. Friedman, manuscript in preparation.

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