Published in final edited form as:

J Mol Biol. 2011 May 20; 408(5): 909–921. doi:10.1016/j.jmb.2011.02.062.

WAXS studies of the structural diversity of hemoglobin in solution

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Abstract

Specific ligation states of hemoglobin are, when crystallized, capable of taking on multiple quaternary structures. The relationship between these structures, captured in crystal lattices, and hemoglobin structure in solution remains uncertain. Wide-angle x-ray solution scattering (WAXS) is a sensitive probe of protein structure in solution that can distinguish among similar structures and has the potential to contribute to these issues. We used WAXS to assess the relationships among the structures of human and bovine hemoglobins in different liganded forms in solution. WAXS data readily distinguished among the various forms of hemoglobins. WAXS patterns confirm some of the relationships among hemoglobin structures that have been defined through crystallography and NMR and extend others. For instance, methHb A in solution is, as expected, nearly indistinguishable from HbCO A. Interestingly, for bovine hemoglobin, the differences between deoxyHb, metHb and HbCO are smaller than the corresponding differences in human hemoglobin. WAXS data was also used to assess the spatial extent of structural fluctuations of various hemoglobins in solution. Dynamics has been implicated in allosteric control of hemoglobin and increased dynamics has been associated with lowered oxygen affinity. Consistent with that notion, WAXS patterns indicate that deoxyHb A exhibits substantially larger structural fluctuations than HbCO A. Comparisons between the observed WAXS patterns and those predicted on the basis of atomic coordinate sets suggest that the structures of Hb in different liganded forms exhibit clear differences from known crystal structures.

Perutz's masterful explanation (1) of hemoglobin cooperativity and physiological responsiveness on the basis of crystallographic structures of liganded and unliganded forms laid the intellectual foundation for a generation of scientists pursuing the relationship

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between protein structure and function. His identification of the liganded and unliganded forms of hemoglobin as the high-affinity R-state and low-affinity T-state of the Monod-Wyman-Changeux (MWC) model for cooperativity provided a self-consistent model for the behavior of this prototypical protein. Early crystallographic analysis of hemoglobin was a *tour de force*, requiring months of data collection. During the data collection, hemoglobin, originally crystallized in the oxy-form, oxidized to the met-Hb form, and the first structure of hemoglobin was of met-Hb. Eventual crystallographic analyses of the oxy- and carbonmonoxy forms of hemoglobin demonstrated that all liganded forms (including met-Hb) can adopt the same, R-state quaternary structure in crystals (2).

Nevertheless, the structural basis of hemoglobin cooperativity and physiological responsiveness has, in recent years, come under renewed scrutiny. The discovery of an alternate, R2, quaternary structure for liganded hemoglobin (3,4) raised questions about its structure in solution (5). Structural analysis suggests that the R state is intermediate in structure between T and R2 (6). Additional crystallographic investigations have revealed the existence of a nearly continuous distribution of quaternary structures spanning from the R to the R2 state (7), suggesting that the dimer-dimer interface acts as a 'molecular slide bearing' providing access to these multiple conformations. The possible influence of crystallization conditions and crystal contacts on the hemoglobin quaternary structure has been discussed extensively. The potential for crystal contacts to influence structure was clearly demonstrated when it was shown that in crystals of deoxy-Hb in the T-state, hemoglobin could undergo either oxygenation or oxidation without triggering a change in quaternary structure (8,9).

NMR studies indicate that the solution structure of HbCO A does not correspond to one of the crystallographically characterized structures, but rather is a dynamic intermediate of two or more of these structures (10, 11). Evidence has also been accumulating that suggests the existence of multiple forms for the unliganded, T-state structure (12, 13), although these are much more similar to one another than the R-state structures. It seems apparent now that hemoglobin exhibits an ensemble of structures in each of its physiological states and that crystallization selects different members of those ensembles depending on the conditions of crystal growth.

The nature of the T->R transition is now much less certain than it was 30 years ago (14). Based on crystallographic data, Perutz (1) and, in more detail, Baldwin and Chothia (15) carried out an analysis of the structural changes that occur as hemoglobin transits from the deoxy- to the liganded-state. In the liganded form, the environment of the heme is altered in ways interpreted as increasing its affinity for oxygen. To achieve this conformation, the $\alpha\beta$ -dimers rotate about 15° relative to one another and multiple small tertiary structural changes occur. Several hydrogen bonds near the C-terminus of each subunit are broken. In the absence of ligand, these hydrogen bonds may provide the energy needed to favor the unliganded quaternary structure. Much of the physiological responsiveness of hemoglobin can be explained in the context of this structural model. Nevertheless, the identification of multiple quaternary structures associated with the liganded state of hemoglobin has thrown this analysis into question.

The impact of structural dynamics on the oxygen binding properties of hemoglobin is also an area of intense current study. Molecular dynamics (MD) studies indicate increased fluctuations of the α -helices in deoxy-Hb compared to the liganded structures (16) and it has been proposed that these fluctuations control the coordination mode of the heme Fe with the proximal and distal histidine thereby regulating oxygen affinity (16). NMR studies have detailed clear differences in the dynamics of the deoxy- and liganded-forms of hemoglobin

(17–19) and increased mobility of the side-chains of the distal histidines has been directly correlated with decreased oxygen affinity (20).

The current status of our understanding of the structural basis of hemoglobin function is in considerable flux. The solution structures of hemoglobin in different liganded states are uncertain. The degree to which the dynamics of the protein varies among these states remains only partially characterized and the functional consequences of these changes remain unknown. Motivated by these uncertainties, 40 years after the structural basis of hemoglobin function seemed firmly established, we initiated a series of solution scattering experiments aimed at shedding some light on these questions.

Wide-angle x-ray solution scattering (WAXS) is an extension of SAXS in which data are collected to larger scattering angles. SAXS provides information about the global properties of a structure, can be used to calculate the radius of gyration (R_g) of a structure, and may be used to calculate a low-resolution envelope providing information about the shape of a molecule. Data at larger scattering angles contains information about the secondary structures and their arrangements (21). WAXS has proven highly sensitive to small structural changes in proteins (22) and, as we demonstrate here, represents a powerful approach for establishing structural relationships among different forms of hemoglobin in solution.

WAXS also provides an approach for characterizing structural fluctuations of proteins in solution. An increase in structural polymorphism results in readily predicted changes in solution scattering patterns, most strikingly a filling in of troughs and a decrease in peak amplitudes in the scattered intensity pattern (23). At least some polymorphism may be expected in any protein solution as a simple reflection of the breadth of the structural ensemble that the protein is exploring. WAXS can be used to make a quantitative estimate of the magnitude of these fluctuations. We have shown that some proteins exhibit substantial concentration-dependent changes in scattering intensity, and that these changes can be interpreted as reflecting a change in the magnitude of structural fluctuations (23). The amplitude of these fluctuations is greatest at low concentrations and, for many proteins, decreases progressively as the concentration increases up to roughly 50 mg/ml - above which the spatial extent of structural fluctuations remains approximately constant. Interactions among proteins - macromolecular crowding - appears to provide substantial damping of these fluctuations at higher protein concentrations. Reducing the pressure due to crowding by decreasing protein concentration provides an approach to assessing the degree of structural rigidity intrinsic to a protein under a particular set of solution conditions. For instance, proteins with multiple disulfide bonds often exhibit little concentration dependent change in scattering intensity, indicating little change in the magnitude of structural fluctuations as protein concentration changes. Conversely, large, multi-domain proteins may exhibit substantial concentration-dependent intensity changes (23). Measurement of the concentration dependence of WAXS data from a protein solution thereby provides a highly informative probe of the rigidity of the protein and the degree to which it requires a crowded environment to maintain that rigidity.

Results

Comparison of WAXS patterns from different forms of hemoglobin

WAXS patterns were collected as described previously (23) and detailed in Materials and Methods. Data were collected as a function of protein concentration from solutions of carbonmonoxy, deoxy- and met-Hb A; as well as from di- α -rHbCO (a variant in which the two α -chains are covalently linked by a single glycine residue (24,25)); from rHbCO (α V96W/ β N108K) (26); and from bovine Hb in the met-, CO-, and deoxy- forms. We

carried out detailed comparisons using WAXS patterns collected on samples with protein concentration of 50 mg/ml since we have shown previously (23) that scattering from hemoglobin at concentrations higher than 50 mg/ml is relatively invariant whereas scattering at lower concentrations exhibit changes that reflect increased levels of structural fluctuations. Data were collected at 4°C. Optical absorption spectroscopy data were taken immediately prior to X-ray measurements to assess the redox and ligand state of the hemoglobin solutions. Additionally, X-ray absorption spectroscopy measurements were made on some of the hemoglobin samples simultaneously with the X-ray scattering data. All x-ray scattering data were collected using a flow cell adjusted to limit x-ray exposure of any one protein to not more than 100 msec. Nevertheless, we remained concerned that met-Hb might photoreduce in the x-ray beam - thereby transforming into deoxy-Hb during x-ray exposure - and re-oxidizing after exposure. To assure that this was not happening, we collected x-ray absorption spectra of the samples in the vicinity of the iron edge (~7 keV) during scattering experiments using the flow cell. The x-ray absorption edge is expected to shift approximately 4 eV between the liganded and deoxy-Hb (27), a shift readily observable with the spectrometer used. However, there was no observable shift (<0.1 eV) in the iron absorption edge between HbCO and met-Hb, confirming that both remained liganded hemoglobins during x-ray exposure. Since absorption (and therefore photoreduction) is much greater at 7 keV than 12 keV, we would expect even less photoreduction using an x-ray energy of 12 keV which is the energy at which most of the diffraction data was collected.

WAXS patterns from hemoglobins include four distinct peaks in the region $0.02 < 1/d < 0.15 \text{ \AA}^{-1}$ (see Figures 1 and 2). The peak at $\sim 0.03 \text{ \AA}^{-1}$ corresponds to the $\alpha_1\beta_2$ distance ($\sim 33 \text{ \AA}$) and is the most variable feature in WAXS patterns from different hemoglobins. It may also vary as a function of protein concentration, an effect that has been interpreted as indicative of different degrees of fluctuation in the rotation of $\alpha\beta$ dimers relative to one another (23). The intensity of the peaks at 0.03 and $0.05 \text{ \AA}^{-1}$ are sensitive to changes in contrast which can be modulated by adding an electron dense solute, such as glucose, to the solution (28), thus demonstrating that they are indicative of the shape of the molecule. The peaks at 0.08 and $0.1 \text{ \AA}^{-1}$ are not sensitive to changes in contrast, and are indicative of internal structure. In particular, the peak at $1/d \sim 0.1 \text{ \AA}^{-1}$ corresponds to a spacing of $10 \text{ \AA}$ and is usually strong in α -helical proteins because α -helices tend to pack with a center-to-center distance of $\sim 10 \text{ \AA}$.

WAXS patterns observed for a variety of hemoglobins at 50 mg/ml are compared to HbCO A in Figure 1 and R-factors ($\sum(|I_1 - I_2|/I_1)$ - see materials and Methods) that provide a quantitative measure of the differences among these patterns are tabulated in Table 1. Figure 1a compares HbCO A to deoxy-Hb. The differences are subtle, but statistically significant (22). The R-factor for this comparison is 0.053 (5.3%). Figure 1b compares HbCO A to metHb A. As expected from crystallographic analyses, these patterns are very similar (R-factor of 0.023 (2.3%)). Figure 1c compares the WAXS patterns from HbCO A and rHbCO ($\alpha V96W/\beta N108K$); and Figure 1d compares WAXS data from HbCO A with that from di- α rHbCO. Comparison of Figures 1a and 1c shows that the pattern from rHbCO ($\alpha V96W/\beta N108K$), although very similar to that of HbCO A, also exhibits some features present in the deoxyHb A pattern. Its R-factor with deoxyHb A (0.040) is lower than that between HbCO A and deoxyHb A (0.053), indicating that the mutations have resulted in a shift in the structure towards that of the unliganded state. rHbCO ($\alpha V96W/\beta N108K$) has lower oxygen affinity than HbCO, suggesting that these observations reflect structural changes that modulate oxygen affinity. These results are also consistent with NMR results showing that this low-affinity mutant in the CO form can switch to a T-type structure without changing the ligation state in the presence of IHP and/or at lower temperature (29).

Figure 1d compares the WAXS pattern from HbCO A with that from di- α -rHbCO. There are small differences in the two patterns, especially in the vicinity of the quaternary peak at $0.03 \text{ \AA}^{-1}$ and the comparison has an R-factor of 0.044. Analysis of the crystal structure of di- α -rHbCO indicates that it cannot adapt the R- or R2- quaternary states of hemoglobin (26). The glycine cross-link cannot span the distance required in the R2-state of hemoglobin and the bond angles and torsion angles that would be required in a bridging glycine in an R-state would be severely strained.

To better demonstrate the differences among patterns from the various hemoglobins, we calculated difference intensity distributions. Figure 1e compares the differences between human HbCO A and deoxyHb A with the differences in patterns from HbCO A and rHbCO (α V96W/ β N108K). The differences are similar in character providing further evidence that rHbCO exhibits a structure intermediate between HbCO A and deoxyHb A. The positive peak at low scattering angles reflects the fact that HbCO has the lowest radius of gyration among these structures. Figure 1f includes a comparison of the differences between HbCO A and deoxyHb A with those from HbCO A and di- α -rHbCO. The patterns are similar to those in Figure 1e with the exception that the difference at $0.05 \text{ \AA}^{-1}$, prominent in Figures 1a, 1c and 1e is missing here.

Figure 2 compares WAXS patterns from analogous forms of human and bovine hemoglobins. Figure 2a compares bovine metHb with bovine deoxyHb and bovine HbCO. Except for intensity at the position of the trough at $0.025 \text{ \AA}^{-1}$, the patterns are very similar, and suggest that, in solution, the T-to-R transition in bovine hemoglobin involves a smaller structural shift than human hemoglobin. These observations appear consistent with the crystal structure of bovine HbCO which takes on a structure intermediate between the prototypical R and T states (30). Figure 2b compares patterns from human and bovine metHb, which are very similar, and Figure 2c compares human and bovine deoxyHb. In both cases, the intensity is shifted to the left for the bovine sample, and the trough at $0.025 \text{ \AA}^{-1}$ is somewhat filled in. The difference patterns in Figure 2d further demonstrate that the differences between met and deoxy hemoglobins observed in scattering from human hemoglobin are greatly suppressed in patterns from bovine hemoglobin.

Comparison of Calculated and Observed WAXS Patterns

Figure 3a compares the WAXS patterns calculated from atomic coordinate sets of crystalline HbCO A and deoxy-Hb A using an explicit atom representation of water (31). The patterns calculated from these two forms of hemoglobin exhibit relatively small differences that are similar in form to the differences seen between WAXS patterns collected from HbCO A and deoxy-Hb A (Figure 1a). Nevertheless, the calculated patterns are quite different from observed. Figure 3b compares the WAXS pattern from HbCO A at 50 mg/ml with that calculated from the atomic coordinates (2DN3) using an explicit atom representation of water (31). The calculated intensities include peaks in approximately the same positions as observed and, except for the peak at $0.03 \text{ \AA}^{-1}$, similar relative heights. The height of this peak is sensitive to changes in the quaternary structure of hemoglobin; amplitude of structural fluctuations; electron density of the solvent; and density of water in the hydration layer. In the presence of 47.5 % sucrose, the electron density of the solvent is close to that of the protein ($0.398 \text{ e/\AA}^3$) and the intensity of the peak observed at $0.03 \text{ \AA}^{-1}$ diminishes to near zero - as does the peak at $0.055 \text{ \AA}^{-1}$ (28). This result indicates that the peak is due to the shape of the molecule and not to internal structural features, and supports the idea that the discrepancies between calculated and observed, plainly visible in Figure 3b, are due to differences in quaternary structure that alter the overall shape of the molecule.

The computational method used generally reproduces the WAXS patterns to within experimental errors except for small discrepancies due to structural fluctuations (31). The

differences apparent in Figure 3b are a clear demonstration that the structure of HbCO A in solution does **not** correspond to the atomic coordinates used for the calculation. The coordinate set used corresponds to the R-state. The conformation of HbCO A in solution has been described as a dynamic intermediate of the R- and R2-states (10, 11). Consequently, we would not expect these two plots to correspond precisely. However, the observed data cannot be reconstructed as a linear sum of computational patterns generated from the R and R2 states. In all patterns computed from structures corresponding to the R or R2 quaternary state, the peak at $0.03 \text{ \AA}^{-1}$ is much larger than the observed one.

An explicit atom representation of water was used for the calculation of WAXS patterns (31) from atomic coordinate sets of hemoglobins. Previous computational methods, based on continuum models of water, have been unsuccessful at reproducing observed scattering patterns accurately in the wide-angle regime (32). This was due largely to the assumption of a uniform hydration layer and to the representation of the excluded volume. Although adequate for calculation of SAXS data, these assumptions break down when used for calculation of scattering to higher angles. By using an explicit atom representation of the water of hydration, both of these assumptions are circumvented. NAMD (33) was used to generate a 20 psec equilibration followed by a 100 psec molecular dynamics simulation during which 100 snapshots of the water of hydration were captured. During the MD simulation the protein was held rigid. The scattering due to the solvated protein was then calculated and the corresponding scatter from a comparable 'droplet' of water was subtracted. With no empirical adjustments, this method has produced scattering patterns of unprecedented accuracy in the length scale between 5 and 100 $\text{\AA}$. Figure 3c shows the results of this calculation for myoglobin. The only region in which calculated and observed deviate by more than the expected errors of observation is in the vicinity of the peak at $0.04 \text{ \AA}^{-1}$. This discrepancy appears to be due to fluctuations in the protein in solution that are not represented in the calculation which employed a rigid body model for the protein. The correspondence observed here for myoglobin is much more typical of the results we have achieved using this approach than the results reported for hemoglobin in Figure 3b.

Concentration dependence of WAXS Patterns from different hemoglobins

Studies of WAXS patterns as a function of protein concentration (21,23) indicate that at protein concentrations of less than 50 mg/ml, WAXS patterns of some proteins exhibit concentration-dependent intensity changes that reflect increasing structural fluctuations as the concentration decreases. Figure 4 provides evidence of this in HbCO A and deoxy-HbA and bovine deoxy Hb. Figure 4a includes WAXS patterns of HbCO A at concentrations of 60; 20 and 10 mg/ml. The patterns are almost identical except for a modest filling in of troughs at 0.02 and $0.04 \text{ \AA}^{-1}$, and a small decrease in the peak at $0.10 \text{ \AA}^{-1}$. These differences, although small, are highly reproducible. This trend is associated with enhanced fluctuations of the quaternary structure of hemoglobin that occur at low protein concentrations (23,34). Figures 4b and 4c contains the corresponding patterns for deoxy-HbA and bovine deoxy-Hb which exhibit much larger changes in intensity as a function of concentration, particularly in the case of bovine deoxy Hb. These results indicate that, in dilute solutions, the deoxy-forms of these molecules exhibit substantially greater polydispersity and may be undergoing substantially larger structural fluctuations than their carbonmonoxy counterparts.

The effect of concentration change can be tracked more clearly with the plots of *differences* in intensity. Difference intensity [$I(20 \text{ mg/ml}) - I(50 \text{ mg/ml})$] was used to compare the behavior of HbCO A with other forms of hemoglobin. Figure 5a compares the difference intensities for di- α -rHbCO (red curve) and HbCO A (black curve). Di- α -rHbCO exhibits relatively modest concentration-dependent intensity changes, the polydispersity (or dynamics) apparently being limited by the covalent linkage between the two α -chains. By

contrast, Figure 5b includes deoxy-Hb A which exhibits differences that are much larger than HbCO A. In this Figure the differences for deoxy-Hb A have been divided by 2.0 in order to ease comparison of the two plots. Figure 5c compares HbCO A with met-Hb A, which, although similar in magnitude, have somewhat different character. Figure 5d demonstrates that the differences observed for rHbCO (α V96W- β N108K) are very similar to those for HbCO A. Qualitatively, these difference plots share some aspects but differ in significant ways. One gets the impression of a molecular structure that undergoes fluctuations that are perturbed by these changes in the protein molecule, but retain many common features.

An alternate explanation for the concentration dependence is that at low concentrations the hemoglobin tetramer is dissociating into dimers. We can rule out that possibility on the basis of several observations. First, the di- α rHbCO cannot dissociate into dimers since the two α -chains are covalently linked. Although the concentration dependent differences are smaller for di- α rHbCO (Figure 5a), they are real and reproducible and have a form similar to HbCO A. Second, deoxy Hb is far more stable to dissociation than HbCO, yet the concentration-dependent intensity differences we observe for deoxy Hb are substantially larger than those observed for HbCO A. If dissociation were causing the intensity differences we would expect to observe the opposite effect.

Discussion

Comparison of hemoglobins in solution

We have compared WAXS patterns from a number of hemoglobins in order to assess the relationships among the structures of these molecules in solution. The WAXS pattern from metHb A is virtually identical to that from HbCO A, and both are distinctly different from that of deoxy Hb A, confirming the well known relationships among these structures as defined by crystallography. On the other hand, the WAXS patterns from bovine metHb and bovine HbCO, virtually identical to one another, are also very similar to that from bovine deoxyHb, suggesting that their structures are more similar to one another than their human counterparts. The crystal structures of bovine Hb exhibit differences from human that appear consistent with these observations. In bovine deoxyHb the N-termini and A-helices of the β -subunit are shifted 2.1 Å towards the molecular dyad as compared to those of human deoxy Hb A (35), a shift interpreted as being associated with the low intrinsic oxygen affinity of bovine Hb. A similar shift in the N-termini and A-helices has been observed in a crystal structure of bovine HbCO (36) which exhibits a number of quaternary and tertiary features more characteristic of T-state structures than liganded hemoglobin. This pattern is consistent with the WAXS observations reported here. However, in crystals, bovine HbCO can adapt alternate quaternary structures including an R2-like structure and two additional structures both described as intermediate between R and R2 (7). Our observations suggest that the most abundant conformation of bovine HbCO in solution is shifted slightly from the R-state towards the T-state.

The WAXS pattern from rHbCO (α V96W/ β N108K) appears intermediate between HbCO A and deoxyHb. The two mutations in this Hb variant are along the $\alpha_1\beta_2$ interface (α V96W) and the $\alpha_1\beta_1$ interface (β N108K). The mutant exhibits decreased oxygen affinity and cooperativity. These effects appear to be mediated by quaternary structural changes, since NMR studies indicate that the protein exhibits properties intermediate between R-state and T-state, and the protein is capable of switching quaternary state from R- to T-state even when liganded (29). The WAXS data reported here support this interpretation.

Di- α rHb exhibits a pattern strikingly similar to that of rHbCO (α V96W/ β N108K) (R-factor = 0.023). This similarity suggests that they adopt very similar quaternary structures in

solution. From a stereochemical point of view, there is strong reason to believe that this structure is neither the R nor R2 conformation. The quaternary structure of di- α -rHbCO is severely constrained by the presence of a glycine linker that spans from the C-terminus of one α -chain to the N-terminus of the other. The distances between the carboxyl oxygen of the C-terminus and the amino nitrogen of the N-terminus of the R and R2 structures are 3.30 Å and 11.15 Å, respectively (25). One glycine residue cannot span the distance required in the R2 state and the bond and torsion angles that would be required in a bridging glycine in the R state would be severely strained. This indicates that di- α -HbCO is not in the R- or R2-conformation in solution, and, given their similar WAXS patterns, by inference suggests that rHbCO (α V96W/ β N108K) is also in a different structure.

What is the structure of liganded hemoglobin in solution?

Comparison of the different quaternary structures exhibited by Hb in crystals indicates that the dimer-dimer interface of liganded hemoglobin has a wide range of energetically accessible structures that are related to each other by a simple sliding motion (7). The dimer-dimer interface acts as a “molecular slide bearing” that allows the two $\alpha\beta$ -dimers to slide back and forth without greatly altering the number or nature of the intersubunit contacts (7). But substantial evidence now suggests that, at least in the case of liganded Hb, the most abundant structure in solution is not represented by one of the available crystal structures (10, 11). In 1999, Tame published a paper entitled ‘What is the true structure of liganded hemoglobin?’ (5). We would argue that the question remains open and that, furthermore, the ensemble of structures that constitutes hemoglobin in any liganded state in solution remains poorly characterized. It is now possible using an explicit atom representation of the water of hydration (31) to accurately predict WAXS patterns based on atomic coordinate sets. But, calculations of WAXS patterns from hemoglobins based on atomic coordinate sets from crystals uniformly overestimate the intensity of the peak at $0.03 \text{ \AA}^{-1}$ compared to observed patterns (Figure 3b). Rotation of the $\alpha\beta$ -dimers relative to one another does not result in a substantial decrease in the predicted intensity of this peak. Movement of the $\alpha\beta$ -dimers closer to one another in the tetramer leads to a decrease in the predicted intensity, but preliminary studies have not arrived at a model with acceptable correspondence between the calculated and the observed intensities.

Oxygen affinity and structural fluctuations

There is now substantial evidence to suggest that under conditions that correspond to reduced oxygen affinity, Hb exhibits increased structural fluctuations. Yonetani and LaBerge (37) carried out molecular dynamics simulations that show that the fluctuations of helices of oxy-Hb are increased upon deoxygenation and/or binding 2,3-BPG - both of which lower the oxygen-affinity of Hb. They proposed that the coordination mode of the heme Fe with proximal and distal His is modulated by these helical fluctuations, resulting in the modulation of the oxygen-affinity of Hb. Abaturov et al (38) showed that the fluctuations (as detected by H/D exchange) are larger in the deoxy form than the liganded form. Extensive NMR studies are consistent with these observations (18). In particular, increased mobility of the distal histidines is closely correlated with lower oxygen affinity (20).

In this study, we have used the responsiveness of WAXS patterns to changes in protein concentration as a tool for estimating the amplitude of structural fluctuations at low protein concentrations. From this work, we conclude that deoxy-Hb exhibits greater amplitude fluctuations than the other forms of Hb studied here. These observations are consistent with the association of increased dynamics with lowered oxygen affinity which has been suggested by both MD (16, 36) and NMR studies (17–20). Nevertheless, it seems inconsistent with the crystallographic studies that have identified a narrow ensemble of

quaternary states in unliganded mammalian hemoglobins (RMSD 0.1–0.4 Å (20)), but an increasingly broad ensemble of quaternary states in liganded hemoglobins (RMSD 1.7–1.9 Å (20))).

Given the extensive crystallographic studies of hemoglobin, the close similarity of all crystalline T-state quaternary structures would seem to imply a narrow structural ensemble in solution; whereas the multiple distinct quaternary structures observed for liganded hemoglobins might imply sampling of a far broader structural ensemble. But the relationship between polymorphism in solution and polymorphism of crystallographic structures is still poorly understood. It could equally well be the case that the energy landscape for unliganded hemoglobin is smooth and broad resulting in a single 'selected' quaternary structure for many different crystallographic conditions; whereas the landscape for liganded hemoglobin might be rough and relatively narrow, making possible selection of multiple distinct quaternary structures during diverse crystallographic experiments. One might speculate that a broad, smooth energy landscape could provide a kinetic advantage for oxygen binding; whereas an irregular landscape may provide for conformations consistent with different levels of partial ligation.

Conclusions

We have used a comparison of WAXS patterns to study the relationships between different forms of hemoglobin in solution. These comparisons indicate that rHbCO (α V96W/ β N108K) and di- α rHbCO have structures in solution that are intermediate between HbCO A and deoxyHb A. Furthermore, deoxyHb A exhibits substantially greater structural fluctuations than HbCO A in dilute solution. This observation is consistent with recent arguments that increased dynamics is associated with decreased oxygen affinity in hemoglobin (18, 34, 38). Bovine hemoglobin exhibits a somewhat different WAXS pattern from human hemoglobin, with differences among the patterns of deoxy, met and CO smaller for bovine than human. Conversely, bovine deoxy and met exhibit substantially greater fluctuations in solution than their human counterparts. Given these facts, it is tempting to speculate that differences in dynamics may be relatively more important for control of oxygen affinity in bovine hemoglobin than in human.

Comparison of the observed WAXS patterns with those calculated from atomic coordinate sets suggests that the structure of Hb in solution is different from known crystal structures. This may help explain confusion over the molecular basis of cooperativity in hemoglobin which may have been exacerbated by crystallographic results that provide detailed information about quaternary structures that are stabilized by crystal contacts, but may not always represent the most abundant structures present under relevant conditions in solution. Due to the apparent pliability of hemoglobin in response to crystal contacts, the use of crystal structures to define structural transitions should be made with considerable care. A deeper understanding of the cooperative transitions should be informed by direct structural information obtained from the protein in solution. Based on the WAXS measurements presented in this paper as well as other results summarized by Ho and Yuan (14), a two-structure description of hemoglobin allostery is over-simplified and cannot account for the structures and dynamics of this interesting protein in solution.

Materials and Methods

Protein Preparation

Hb A was isolated from human blood sample was prepared in 0.05 M sodium phosphate buffer, pH 7.0, and equilibrated against CO to saturation. Di- α -HbCO were expressed in *E. coli* using a hemoglobin expression system in which both methionine aminopeptidase and

globin genes are co-expressed under the control of a strong promoter (39). rHbCO (α V96W/ β N108K) was expressed and prepared as described by Tsai et al. (29). Bovine met-hemoglobin obtained from (Calbiochem, EMD Biosciences, Inc., San Diego, CA) was diluted in 0.05 M sodium phosphate buffer, pH 7.0 (Biowhittaker, Cambrex, Rockland, ME) to final concentrations required for data collection. A low concentration sample was transferred to a cuvette for optical spectroscopy to determine the redox state of the heme. Optical spectra in the visible region indicated that CO was bound to the hemoglobin.

Deoxy-Hb was generated by reduction of met-Hb with Na-dithionite under a nitrogen atmosphere or by nitrogen gas exchange from previously prepared HbCO A. All solutions were prepared in glove bags purged with nitrogen gas. Phosphate-buffer saline (PBS) buffer was purged of oxygen by bubbling with nitrogen gas and then powdered Na-dithionite was added to make a 500 mM stock solution. Similarly, powdered met-Hb was added to purged PBS solutions and an aliquot of the stock Na-dithionite solution was added to make a final concentration of 50 mM Na-dithionite. A low concentration sample was transferred to a cuvette for optical spectroscopy to determine the redox state of the heme. Optical spectra in the visible region indicated the conversion of met-Hb to deoxy-Hb. Spectra recorded 50 minutes apart confirmed the stability of anaerobic deoxyHb samples. A glove bag was attached to the WAXS setup and all samples were prepared in a similar fashion to that described above. The Na-dithionite aliquot was added to the Hb solutions immediately prior to loading the sample in the WAXS setup. The time between mixing and collecting the WAXS data was less than 5 minutes.

WAXS Data Collection

All data were collected at the BioCAT undulator beamline (18ID) at the Advanced Photon Source (40). Data were collected on a WAXS camera (e.g. 41). The sample cell consisted of a thin-walled quartz capillary (1.5 mm I.D.) attached to a programmable pump (Hamilton Microlab 500 series) that was adjusted to deliver continuous flow through the capillary during data collection. The ambient temperature of the air surrounding the capillary and sample tubing was maintained at 4°C by attachment of an ethylene glycol bath to the brass capillary holder and through an outer layer of tubing surrounding the inner sample tubing during data collection. The x-ray scattering pattern was recorded with a MAR165 2kx2k CCD detector. The specimen to detector distance was approximately 170 mm and was calibrated using powder diffraction rings from either silicon or silver-behenate. The range of useful data was $0.008 \text{ \AA}^{-1} < 1/d < 0.4 \text{ \AA}^{-1}$ (where $1/d = 2 \sin(\theta)/\lambda$; and 2θ is the scattering angle). Selected datasets were collected using a SAXS camera with specimen-to-detector distance of 2 meters and useful data range of $0.001 \text{ \AA}^{-1} < 1/d < 0.05 \text{ \AA}^{-1}$. In most cases, 15–20 independent scattering patterns were collected for each sample.

The beamline is capable of delivering approximately 2×10^{13} photons/sec/100 mA of beam current. As previous experience on the BioCAT beamline has demonstrated that proteins under a variety of physical conditions are damaged after exposure times of a few tenths of a second to a few seconds at these intensity levels, in these experiments eight to thirty-two 20 μ m aluminum foils were used (depending on the concentration of protein in the samples) as x-ray beam attenuators to control the incident beam flux. Typically, a data-set consisted of a series of 2-second exposures with five from buffer, fifteen to twenty from protein solution and five from the empty capillary. Exposures from sample and buffer were alternated to minimize the possible effects of drift in any experimental parameter. Incident beam flux was monitored using nitrogen gas filled ion chambers. Integrated beam flux during each exposure was used to scale scattering from the protein solutions with scattering from the buffer solutions. Diffraction data were collected with continuous protein solution flow such that no protein was exposed to the attenuated x-ray beam for more than 100 ms. At these exposure levels, the effect of radiation damage on radio-sensitive test proteins is

undetectable. Standard deviations of the observed data were calculated, with error propagation formulae used to calculate their effect on the final estimate of scattering from protein.

Scattering Data Analysis

The two dimensional scattering patterns were circularly averaged with Fit2D (42, 43), and the resulting one-dimensional intensity distribution was plotted as a function of spacing $1/d$. The origin of the diffraction pattern was determined from the powder diffraction rings from silver-behenate and/or silicon powder. Intensity from the proteins was separated from that due to the buffer and the capillary using methods previously described (23). For clarity, in the Figures, only 10% of the data-points are represented by open circles. Although error bars representing the standard deviation of the 15–20 patterns collected for each sample are included, in many cases they cannot be seen because they are smaller than the size of the open circles representing the data.

Pair distribution functions, $p(r)$, were calculated using an indirect Fourier transform.

The radius of gyration, R_g , of each hemoglobin under different conditions was estimated both from SAXS data extending in to a spacing of $(1/d) \sim 0.002 \text{ \AA}^{-1}$ and from fitting of WAXS data that extended in to a spacing of $(1/d) \sim 0.008 \text{ \AA}^{-1}$ ($q \sim 0.048 \text{ \AA}^{-1}$). R_g for met-Hb samples at all protein concentrations were measured using both SAXS and WAXS data sets. Comparison of the two data-sets indicated that the estimates from the WAXS data did not deviate from the SAXS estimates by more than $\pm 0.2 \text{ \AA}$. All estimates of R_g for rHb ($\alpha V96W/\beta N108K$), HbCO A, di- α -rHbCO, and deoxyHb were subsequently made using WAXS data, and are referred to as "apparent R_g " values. Expected errors for the resulting R_g are $\sim \pm 0.2 \text{ \AA}$.

X-ray absorption spectroscopy

X-ray absorption spectroscopy measurements were performed by scanning the incident beam across the Fe K absorption edge (7112 eV) in fast continuous scan mode (30 seconds / scan), energy bin size of 0.5 eV, and 7020 – 7200 eV energy range. A silicon drift detector (Ketek SDD, 80 mm² active area, energy resolution 170 eV @ 5900 eV) was used to detect the Fe K α fluorescence. The SDD was located in standard configuration, 90 degree relative to beam direction (44) and was aligned to an opening on the sample capillary device. Data were collected during flow of Hb through the scattering volume.

Optical spectroscopy

Optical spectroscopy was used to confirm the redox state and presence of ligands bound to the hemoglobins in solution. A Cary 50 UV-Vis spectrophotometer (Varian, Inc.) recorded spectra covering the Soret and visible regions (400 nm to 800 nm). UV/VIS Spectroscopy cells (PerkinElmer, Inc.) with either 1.0 mm or 0.1 mm path length were used owing to the high optical density of the solutions. A flat baseline was obtained by correcting the instrumental baseline with the optical spectral of the buffer solution.

Acknowledgments

This work is supported by research grants from the NIH (R01GM-084614 to C. Ho; and R01GM-085648 to L. Makowski). We would like to thank Raul Barrea for assistance in the XAS measurements and Donald Caspar for illuminating discussions. BioCAT is a National Institutes of Health-supported Research Center RR-08630. Use of the Advanced Photon Source, an Office of Science User Facility operated for the U.S. Department of Energy (DOE) Office of Science by Argonne National Laboratory, was supported by the U.S. DOE under Contract No. DE-AC02-06CH11357

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Figure 1.

Comparisons of WAXS patterns from HbCO A with different hemoglobins collected from samples at 50 mg/ml at 4°C (a) Comparison with the pattern from deoxy-Hb A. (b) met-Hb A. (c) rHbCO A (α V96W/ β N108K), (d) di- α rHbCO. Differences among patterns are also displayed including a comparison of the changes in intensity going from HbCO A to deoxyHb A with the changes in going from (e) HbCO A to r HbCO (α V96W/ β N108K) and (f) HbCOA to di- α -rHbCO.

Figure 2.

Comparison of WAXS patterns from human and bovine hemoglobins collected from samples at 50 mg/ml at 4°C. (a) Comparison of patterns from bovine metHb, HbCO and deoxyHb. (b) Bovine metHb with human metHb A. (c) Bovine deoxyHb with human deoxyHb A. (d) A comparison of the differences between WAXS patterns from metHb and deoxyHb for human and bovine hemoglobins.

3a

3b

3c

Figure 3.

Computed and observed WAXS patterns from Hb. (a) Comparison of computed WAXS patterns from HbCO A (black) and deoxy-Hb (red). (b) Comparison of a computed WAXS pattern with that observed from HbCO A at 50 mg/ml and 4°C. (c) Comparison of a computed WAXS pattern with that observed from myoglobin.

Figure 4.

Dependence of WAXS patterns on protein concentration. Patterns from samples at 10, 20 and 50 mg/ml are shown for (a) HbCO A; (b) deoxy-Hb A; and (c) bovine deoxy HbA.

Figure 5. Differences between WAXS patterns at 20 mg/ml and 50 mg/ml. Each plot contains the differences from HbCO A (black) compared to that of another Hb (red). (a) di- α -rHbCO. (b) deoxy-Hb A divided by 2. The differences are so large for deoxy-Hb (see Figure 5), that they were divided by 2 in this figure in order to aid visual comparisons. (c) human met-Hb A. (e) rHbCO (α V96W/ β N108K).

Table 1

R-factors among hemoglobin WAXS patterns

R-factors providing a metric for assessing differences among WAXS patterns from hemoglobins at 50 mg/ml. R-factors were calculated using intensities from 0.020–0.150 Å⁻¹.

	HbCO A	rHb	di-α	metHbA	deoxyHbA	bovmetHb	bov deoxyHb
HbCO A	---						
rHb	0.047	---					
di-α HbCO	0.044	0.033	---				
met Hb A	0.023	0.050	0.054	---			
deoxy Hb	0.053	0.040	0.044	0.052	---		
bov metHb	0.068	0.086	0.103	0.058	0.102	---	
bov deoxyHb	0.057	0.063	0.078	0.053	0.070	0.045	---
bov HbCO	0.052	0.062	0.077	0.047	0.080	0.030	0.049