Movement of Fe with Respect to the Heme Plane in the R-T Transition of Carp Hemoglobin

AN EXTENDED X-RAY ABSORPTION FINE STRUCTURE STUDY

(Received for publication, December 19, 1985)

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Carp Hb undergoes a well known change in kinetics over the pH range 6-9. X-ray absorption spectroscopy, in conjunction with refined data analysis procedures, shows no difference in iron-ligand distances when carp HbCO is switched from R (high affinity) to T (low affinity) states. These distances are 2.015 ± 0.015 Å for the average iron-pyrrole nitrogen distance, 2.14 ± 0.04 Å for the iron-nitrogen (of histidine) distance, and 1.89 ± 0.05 Å for the Fe-C (of CO) distance. Examination of the region from 30 to 100 eV above the threshold, called the ligand field indicator region, reveals spectral differences, which when compared to model compounds suggest that the iron and the heme are less coplanar in the T-like forms. These results are consistent with the iron being 0.1 Å more out of the mean heme plane in both carp HbCO and carp Hb T states, relative to the R forms, and that the change in iron position on ligation to either T or R state is four times larger than that occurring with the quaternary switch.

One of the central problems in hemoglobin research is to assign, with the highest precision possible, those structural features that distinguish the high (R) and low (T) affinity states. X-ray diffraction studies on mammalian hemoglobins have revealed the major quaternary changes involving large scale motion of the α-β dimers relative to each other (1-3), and there has been considerable discussion concerning the movement of the Fe atoms during ligation and conformational changes (4-11). Extended x-ray absorption fine structure (EXAFS) spectroscopy is uniquely suited for probing structural changes around the Fe atom with higher precision than that provided from conventional x-ray diffraction on single crystals. We have begun an extensive EXAFS study of numerous hemoglobins locked into well defined R and T states to examine those structural features that correlate with kinetic and thermodynamic ligation parameters.

We report here EXAFS data for carp HbCO and find that the iron ligand distances for the pyrrole nitrogens and the axial ligands are identical within the experimental error for the low affinity (T) and high affinity (R) forms. But, x-ray spectral differences in the ligand field indicator region (LFIR) (12), when compared to the results from many other heme and globin models, suggest that the iron atom and the heme are less coplanar in the lower affinity form. One explanation is that the iron is out of the heme plane in the low affinity form by 0.08 ± 0.04 Å relative to the high affinity form. These conclusions are based on an empirical examination of known structures with similar spectral characteristics. We find a similar change (0.09 ± 0.04 Å) in the same direction for deoxy carp R and T forms, though the data are not yet of the same quality as the HbCO data. An alternative explanation involves ruffling of the heme so that in the T state form the heme carbons are, on average, further out of the heme plane.

Carp Hb was selected because of the extensive kinetic data already available (13-16) and because both deoxy and ligated forms can be stabilized as noncooperative R or T states by changes in pH and organic phosphate. Thus, at pH 5.6 (or pH 6 plus inositol hexaphosphate) the hemoglobin is locked into the T state, both in the deoxy and CO ligated forms. Similarly, the protein is in the R state at pH 9. This means, in contrast to studies on mutant Hbs, that we can compare data on the liganded form in two different quaternary states with no alteration in primary structure about the hemes or elsewhere in the protein. Presumably, these R and T states formed at the extremes of pH correspond to the R/T states at pH 7 during the normal functioning of the hemoglobin, though a simple rapidly equilibrating two-state allosteric model does not provide a good fit to the pH 7 kinetic results (17). Previous studies by EXAFS attempting to compare the R and T states were based on a comparison of human deoxy T state and deoxy-Hb Kemptsy (18, 19). In those studies, no differences in Fe-pyrrole nitrogen (Fe-Np) distances were found, consistent with our results on liganded forms. However, their analysis precluded the detection of any differences in axial ligand distances. Interestingly enough, the investigators did observe spectral differences in the low k region, corresponding to the LFIR, similar to those differences reported here, but attributed them to differences in background removal. Those studies, however, did provide convincing evidence that the differences in Fe-Np distances were 0.01 Å or less for the low and high affinity deoxy forms studied.

Recent kinetic measurements on carp HbCO at low temperature (20) show that the R/T differences at the temperatures used in this EXAFS study, 170 K, are similar to those seen at room temperature. The HbCO form is particularly appropriate for EXAFS studies because of its stability and because of the possibility of carrying out EXAFS studies on photolyzed intermediates as a function of time and temperature for both R and T states, allowing us to probe structural changes at the Fe site in ligation intermediates.
EXPERIMENTAL PROCEDURES

Carp Hb was prepared as described elsewhere (17). For these studies the protein concentration ranged from 4 to 7 mM in Fe. At pH 6, the inositol hexaphosphate concentration was always in slight excess over the Hb tetramer. The buffer was typically 25 mM potassium phosphate. At pH 9, the buffer was typically 0.5 M borate. Measurements of pH were made on the concentrated Hb solutions at 20°C. EXAFS measurements were carried out at Stanford Synchrotron Radiation Laboratory (April 1984 and February and July 1985) and Cornell High Energy Synchrotron Source (November 1984 and May 1985). Hemoglobin II-2 at Stanford and C-2 at Cornell were used for all studies. Resolution at the Fe edge was 3 eV or better in all cases. For the carp HbCO EXAFS analysis, 15 scans of 2-eV intervals at 2 eV/point were averaged. LFIR ratio calculations were made from EXAFS data or were the average of 5 scans of 1-eV intervals at 2 s/point. All experiments were carried out at 160-170°C except the results for human oxy- and deoxy-Hb, which were carried out at 270°C.

EXAFS data were collected, averaged, and analyzed as described previously (21, 22). The background subtracted k^2 weighted data were Fourier-transformed to resolve the first and higher shell backscattering contributions. The first shell contributions represent the imidazole nitrogen of histidine, the carbon of CO, and the pyrrole nitrogen atoms. The higher shell contributions are dominated by the backscattering from the heme carbons.

Back-transformation of Fourier filtered first shell data with a window of 1.4 Å full-width half-maximum from k = 3.5 to 12.5 Å⁻¹ contains 7-8 independent degrees of freedom (21). These data were fit to Fe-N and Fe-C model data treated in the same fashion using a 2-atoms fitting procedure with the following parameters for each atom type: N, the number of ligands; r, their average distance from Fe; ΔEo, the Debye-Waller factor of the model minus that of the unknown; and ΔEo, the difference in threshold or the ionization energy for the 1s electron (21). Knowledge of the number of ligands of iron reduces the number of unknowns in the data to six. Full details of this fitting procedure for heme compounds are provided by Peisach et al. (23), Powers et al. (24), and Woolery et al. (25) and are not repeated here.

For the LFIR ratio calculation, raw data are averaged and normalized to a linear background. As described by Chance et al. (12), the heights of the two peaks (A and B in Fig. 1) in the region from 30 to 100 eV above the threshold are calculated from the baseline as shown. As such, all comparisons are made on spectra that have been treated in the same way. The LFIR ratio is the height of the first peak (A) divided by the height of the second (B). Although this method is crude, it quickly and efficiently provides a numerical estimate of the major spectral changes and serves as a starting point in the process of ultimately assigning these features precisely. Errors in the ratio arise from noise in the data, differences from preparation to preparation, and the presence of contaminants. Our error in the LFIR ratio as evidenced by the reproducibility from experiment to experiment with duplicate preparations is ±0.05 (for three preparations). This error is 4-fold larger than that expected from counting statistics alone.

Reflectance spectroscopy was used to examine the optical characteristics of all the samples in situ at low temperature before and after X-ray exposure. All samples showed the presence of minimal methemoglobin contamination, and the appropriate presence or absence of deoxy species was confirmed by examination of the near infrared region (26).

RESULTS

The filtered first shell data from the carp HbCO R and T forms are almost identical in amplitude and phase (not shown). When the higher shell data are Fourier-filtered and back-transformed (not shown) the amplitudes and phases are also almost identical, except in the ligand field indicator region. Fig. 1 shows the raw data in the LFIR in one experiment and the differences in peak heights of R versus T. The ratio of peak heights is 0.62 ± 0.05 for the R state and 0.76 ± 0.05 for the T state. The Fourier analysis shows clearly that these spectral differences correspond to changes in the backscattering signature of the higher shell ligands, not those of the first shell (27).

The fitting of the R and T filtered first shell data to models using a two-atom type procedure (22, 23) is shown in Table I. For 5/1 amplitude ratios, which reveal axial ligand contributions, distances of 1.88 and 2.14 Å are seen for carp T while similar distances of 1.90 and 2.15 Å are seen for carp R state. The 4/2 amplitude ratio fits, which reveal average Fe-N distances, differ by less than 0.01 Å, with both at approximately 2.01 Å. The small differences observed are at the lower limit of the possible error in these measurements. Certainly, within the noise, and possibly within the current limits of the
technique, no difference in average distance from iron to any of its first shell ligands (>0.02 Å) can be seen. The use of a 3-atom procedure (24, 25), certain in the knowledge of atomic type and number of atoms in the first coordination sphere, allows the assignment of the two possible axial ligand distances to their respective atomic types, in this case the nitrogen of histidine and the Fe-His distance and the carbon of CO and the Fe-C distance. The procedure is a consistency test, judging which combination of possible solutions, when taken together, are contained in the data. Solutions G and H in Table I show the effect of switching the axial distances and the respective models for the carp T data. Solution H, with an Fe-C distance of 2.14 Å and an Fe-N distance of 1.88 Å is unreasonable as evidenced by its large \( \Delta \sigma^2 \) and \( \Delta E_0 \), illustrated in boldface type. The results for the R state are nearly identical. A full description of the physical limits of these parameters, the methods of error analysis, and the accuracy of these procedures when compared to the results of crystallography are described elsewhere (24, 25). The minimum solution and error analysis for both the R and the T forms, given by the 3-atom procedure (24, 25), is an Fe-N distance of 2.015 ± 0.015 Å, a Fe-histidine (Fe-N) distance of 2.15 ± 0.04 Å, and a Fe-C distance of 1.89 ± 0.05 Å. These results are the averages for the tetramers and are not necessarily those of individual \( \alpha \) or \( \beta \) chains, but their \( N^*/r^* \) average.

The error estimates are to be used when comparing this structure to other EXAFS and crystallographic results; as discussed above, the differences between the R and T forms are probably less.

**DISCUSSION**

The x-ray absorption data on carp Hb presented here define for the first time the precise iron-ligand distances in carp HbCO. We also show that the R-T differences in iron-ligand distance are ±0.02 Å for the axial ligands and less than 0.01 Å for the iron-pyrrole nitrogen average distances. Previous resonance Raman results on liganded Hbs also show little or no differences in structure (for review, see Refs. 28 and 29), consistent with the results observed here. However, Friedman et al. (30) have seen quaternary structure-dependent differences in the Fe-His stretching frequency 20 ps subsequent to photolysis. The photolyzed R state was at a much higher frequency than the T state, indicating less tilt in the proximal histidine in the R state photolyzed form. Our results here show that in the liganded state the R form has the iron more in the heme plane and structurally is further from deoxy than the T form. Friedman also saw small differences in the deoxy R and T Fe-His stretching frequencies, with the R state again at a higher frequency. It is possible that the extra strain exhibited by the T state form pulls the iron more out of the heme plane than in the R form.

Although we see little change in first shell ligand distances, the higher shell and raw data do show reproducible, measurable differences in x-ray absorption spectra in the low \( k \) region for the two forms. Examination of the LFIR ratio changes in a number of globin compounds and heme models suggests that the changes in the LFIR ratio may be correlated with changes in iron geometry. It is possible that LFIR changes arise from focusing effects, which are important when backscattering atoms are in line with the absorbing atom. Specifically, for bis(mimidazole)-\( \alpha,\beta,\gamma,\delta \)-tetraphenylporphinatoiron(III) chloride (31), where the iron atom lies in the mean heme plane the Fe-Ct-Ct angle is 142°. For high spin forms, like \( \mu \)-oxo-bis(\( \alpha,\beta,\gamma,\delta \)-tetraphenylporphinatoiron(III)), the iron comes out of the mean heme plane by 0.5 Å reducing the angle to 114° (32). Teo (33) has reported focusing effects for angles greater than 75°. These effects modulate the amplitude and phase of the peaks in the ligand field region, producing the spectral changes seen.

In Fig. 2 we plot the LFIR ratio values versus the Fe-out of plane (Fe-Ct) distances for a number of heme models and globin compounds. Data for the 5-coordinate models (points 1-4) evidence the effect of Fe-Ct on the LFIR, which is considerable. For each model, a decline in the measured Fe-Ct is accompanied by a reduction in the LFIR ratio. The Fe-Ct distances of a number of globin compounds are also known and are plotted in Fig. 2. The least squares slope and standard error for all 11 points correspond to a change of 0.17 ± 0.03 in the LFIR parameter for a change of 0.1 Å in the Fe-Ct distance. The minimum error in the LFIR measurement implies an error of at least 0.04 in the Fe-Ct estimation, assuming an LFIR ratio error of ±0.05. The correlation error added to the uncertainty in the LFIR ratio measurement gives a total error of ±0.07 Å. In addition, errors inherent in the measured Fe-Ct distances, departures from linearity in the effect, and differences in axial ligand geometry and distance may affect the slope of the line and the interpolations below. Alternatively, differences in ruffling of the heme, so that large numbers of atoms are out of the mean heme plane in the T form, could account for some or all of the x-ray spectral differences between the quaternary states of carp HbCO.

We also plot in Fig. 2 the LFIR parameter values for several hemoglobins for which the Fe-Ct values are unknown. The interpolation suggests changes in the Fe-Ct distance of 0.08 and 0.09 Å for carp HbCO and carp Hb, respectively, for R-T quaternary changes. Previously, it has been reported (34), from a comparative study of numerous noncooperative hemoglobins, that there is no sharp dividing line between R and T states. Rather, there is a virtual continuum that spans some three orders of magnitude in \( K_a \) for CO ligation. It has been shown that the log of the CO association rate constant \( (l')^2 \) correlates quite well with the \( \Delta G^c \) for CO ligation. The log \( (l') \) ordering for the various proteins also correlates with the

![Fig. 2. Correlation of the LFIR ratio (y axis), with the Fe-out of plane (Fe-Ct) (x axis) distances of various heme and hemeprotein compounds.](image)
LIFIR parameters shown in Fig. 2. Thus, the lowest affinity noncooperative and kinetically homogeneous hemoglobin we have investigated, that from Urechis, provides a convenient upper-bound (1.0) on the LIFIR scale for T state HbCO. Leghemoglobin HbCO provides a lower limit for R state HbCO of 0.60. It can be seen that the difference in LIFIR values for carp HbCO R and T spans 37% of this range. From Fig. 2 of Ref. 34, the log span for R-T HbCO corresponds to 38% of the corresponding distance for leghemoglobin HbCO-Urechis HbCO. However, some of these differences could be due to changes in the heme pocket structures for these extreme forms.

Our preliminary work on the deoxy system shows that carp T state is not quite so “T” as is human deoxy, an assignment consistent with the ordering of the T states based on oxygen dissociation rate constants (34). From human-carp hybrid hemoglobin studies, it has been suggested (35) that it is the peculiar rigidity of the carp α chains that leads to a reduced R-T difference in carp Hb compared to human Hb. In Fig. 2, the arrows marked A and B correspond to the magnitude of changes of the LIFIR ratio associated with CO ligation to the T and R quaternary states, respectively. It can be seen from Fig. 2 that the structural changes in Fe-Ct associated with CO ligation exceed those associated with the quaternary conformational change. To state the matter somewhat differently, movement of the iron from 0.52 to 0.14 Å into the heme plane upon ligation of CO can occur without a switch from the T to R quaternary state, which requires only movement from 0.52 to 0.43 Å into the heme plane. This strongly suggests that quaternary changes are not rigidly coupled to large scale motions of the Fe atom into and out of the heme plane, but must be coupled to other changes as well, perhaps on the distal side or the periphery of the heme.

In summary, the R/T differences found in carp Hb are most clearly correlated with geometric changes around the Fe atom, consistent with motion into and out of the heme plane (4). However, other geometric changes (ruffling or histidine tilt) which increase the focusing consistent with the differences in affinity for the various Hb forms studied may also contribute to the LIFIR ratio changes. Both ligation and the transition T-R lead to movement of the iron into the heme plane, but the inferred change in Fe-Ct for the R-T conformational change is only about 23 ± 6% of that generated by ligation to either T or R state. Careful examination of other heme proteins displaying more extreme R/T behavior may well show additional significant changes in Fe-ligand relationships.

Acknowledgments—We would like to thank Prof. R. Klucas and Dr. L. Saari for a sample of pure leghemoglobin, Dr. J. Freedman and Dr. C. Kumar for their helpful advice, and J. Freedman, M. O’Dell, Y. Zhou, A. Naqui, and S. Khalid for their technical assistance.

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