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Structural Differences in Solution and Crystalline Forms of Met-Myoglobin†

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ABSTRACT: For several decades X-ray diffraction studies have been the paragon of biological structure studies at atomic resolution. Diffraction provides three-dimensional structure information, which is essential to our fundamental understanding of protein function. However, since X-ray diffraction cannot be done to atomic resolution on proteins in their native solution or membrane-bound state, the possibility exists that the conformations of the protein in crystals are slightly different from the conformations in solution, and attempts to interpret details of the structure may be misleading and without physiological relevance. In this paper, we show that this concern is justified for a familiar protein, myoglobin. Performing X-ray absorption fine structure experiments on both solution and crystalline met-myoglobin (met-Mb), we find significant differences in the local environment of the iron between the two states. Specifically, the average iron–nearest neighbor atom distance in the crystalline form is 0.05 Å shorter than that in the solution form, and the iron–nearest neighbor bond is more rigid in the crystalline met-Mb. Possible artifactual explanations for the differences have been ruled out.

It is generally believed that the function of a biological system directly depends on its three-dimensional structure. Most of our knowledge on protein structure at atomic resolution is obtained by X-ray diffraction on single crystal protein samples that are extracted from protein solution. Since the crystalline form of the protein is not the one performing physiological roles in vivo, the question of whether protein structures in solution and crystalline forms are really the same deserves a detailed inquiry. More than 20 years ago Chance et al. (1966) reported that the reactivity of a suspension of horse ferrimyoglobin microcrystals with azide is much slower (about 21 times) than the reactivity of the soluble protein with azide. This decrease was attributed to a loss of flexibility in the crystalline structure that may limit the probability of the conformational change induced by ligation. However, direct comparison of the structure between solution and crystalline forms of met-myoglobin (met-Mb)[1] had not been made.

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[1] Abbreviations: met-Mb, OH_2 (Fe^{III}) myoglobin; XAFS, X-ray absorption fine structure; 2-D NMR, two-dimensional nuclear magnetic resonance.

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X-ray absorption fine structure (XAFS) is a structural technique that can be performed in both solution and crystalline forms, an advantage over X-ray diffraction and 2-D (and higher dimensional) NMR. A recent XAFS study (Lin et al., 1990, 1991) reports that the zinc ions in concanavalin A have a different coordination number and average ligand distance when in solution and in the crystal. Our previous experiments on deoxymyoglobin (Zhang et al., 1991) also indicated that the first coordination sphere of heme iron may contain more than the five atoms determined by X-ray crystallography, suggesting that the structures may be different between crystalline and solution samples. This and the decreased reactivity with azide in the crystals (Chance et al., 1966) have led us to further investigations of the structure differences for the two forms of met-Mb using XAFS.

XAFS is a local structural probe for determining interatomic distance and coordination number around selected atomic species, such as metal ions in metalloproteins (Stern & Heald, 1983). XAFS also can provide information on the mean square displacement of atomic pairs via the XAFS Debye–Waller factor σ^2. The σ^2 is a measure of radial structural disorder, which has both static and thermally induced components that can be separated by studying the temperature dependence of σ^2. To a good approximation, the temperature dependence of σ^2 can be fit to a simple Einstein model (Stern & Heald, 1983; Sevillano et al., 1979), in which the bond-stretching modes are approximated by a simple harmonic oscillator, characterized by the XAFS Einstein temperature θ_E.

Sperm whale met-Mb was obtained from Sigma Chemical Co. The crude met-aquomyoglobin was purifed on DEAE column at pH 6.5 and concentrated to 6–8 mM on Amicon diaflow apparatus. The crystals were grown (Kendrew & Parris, 1956) at 4 °C and were dialyzed initially against 2.5 M ammonium sulfate (pH 6.7). When the microcrystals were seen under the light microscope, the ammonium sulfate concentration was changed to 3 M, and the sample was further dialyzed against it until the size of some of the crystals grew to 1 mm. The crystals are monoclinic and flat on (001) surface. The crystals were stored at −20 °C until the experiment.

The XAFS experiments were performed at beamline X9-A and X11-A at the National Synchrotron Light Source (NSLS) of Brookhaven National Lab (BNL). Si(111) double crystal monochromators were used at both beamlines, while harmonic rejection was attained by using a harmonic rejection mirror at X9-A and by detuning the monochromator at X11-A. The Fe K_α fluorescence emitted after absorption of the incident X-ray photons was measured with a 13-element Ge detector and/or a Stern/Heald ionization chamber. The XAFS experiments were performed on several forms of met-Mb: single crystal, polycrystalline, solution, and solution with high concentration of ammonium sulfate. The polycrystalline powder was measured in two forms: the "dry form" and the "wet form", depending on whether or not the sample was measured in the liquid for protein crystallization—the mother liquor. The dry crystalline powder and the solution sample were measured at several temperatures between 40 and 260 K. The single crystal sample and the wet crystals were measured at 300 K, while the solution sample with salt was measured at 120 K. Ethylene glycol, 20–30%, was usually added to the solution samples measured below room temperature to prevent formation of large ice crystals in the samples, which can distort the XAFS spectrum. The solution sample of met-Mb without ethylene glycol was also measured at the room temperature.

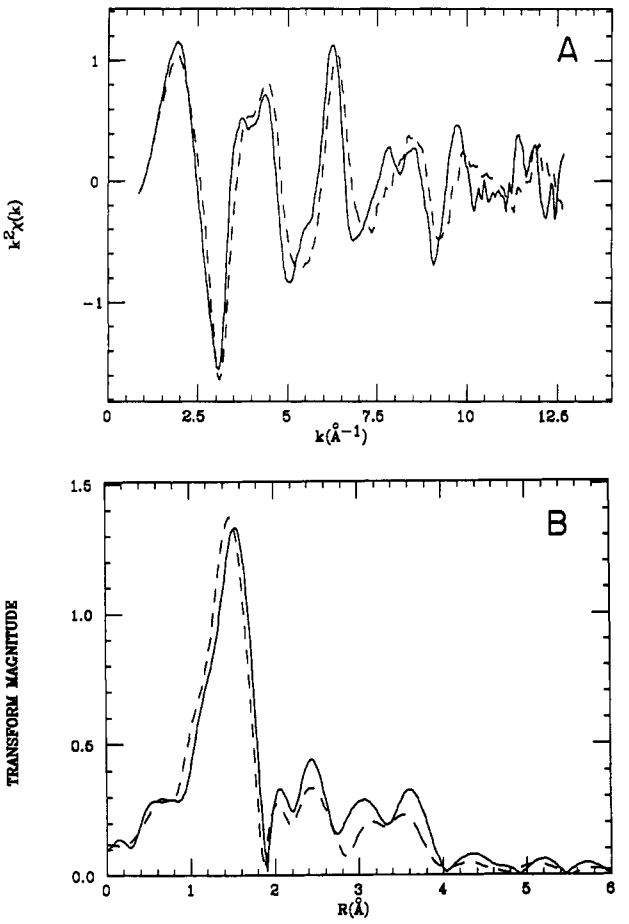

FIGURE 1: (A) Fe K-edge XAFS raw data for solution and crystalline met-Mb. The solid line represents the solution sample at 240 K, and the dashed line represents the polycrystalline sample at 260 K. The XAFS data are plotted as $k^2\chi$, where the XAFS oscillations χ were obtained by removing the atomic absorption background from the absorption spectra using a three region cubic spline fit, converting from energy space to k-space, and normalizing to unit edge step. (B) Fourier transforms of the XAFS raw data for solution and crystalline met-Mb. The $k^2\chi$ Fourier transform was performed from 1 to 12 Å^{-1}. The first coordination shell was isolated by inverse Fourier transform using a window from 0.75 to 1.95 Å in R-space. The polycrystalline sample was made by grinding single crystals to a fine powder and sieving to 200 mesh, and the powder was spread uniformly on Scotch tape. The final concentration of the solution sample was about 2–3 mM in 30 mM phosphate buffer.

A sufficient number of scans were collected for all samples to obtain a total of several million effective photon counts per point.

The XAFS data were analyzed in the standard manner [for example, see Stern and Heald (1983)]. Some parameters used in the data analysis can be found in the corresponding figure legends. Figure 1A shows the XAFS raw data for the dry polycrystalline sample and the solution sample after background subtraction with a cubic spline fit, E to k conversion, and normalization to the constant edge step. The spectra were Fourier transformed into R-space, which is shown in Figure 1B. Significant phase shifts can be seen between the solution and crystalline met-Mb XAFS spectra, which correspond to interatomic distance differences, especially for the first coordination neighbors as shown in the Fourier transform. The first neighbor peak was inverse transformed (Figure 2A) back to k-space for detailed analysis.

Least-squares fitting and the ratio method (Stern & Heald, 1983; Bunker, 1983) were used for quantitative data analysis of the first coordination shell. A 0.05 ± 0.01 Å difference in average interatomic distance was found by directly comparing

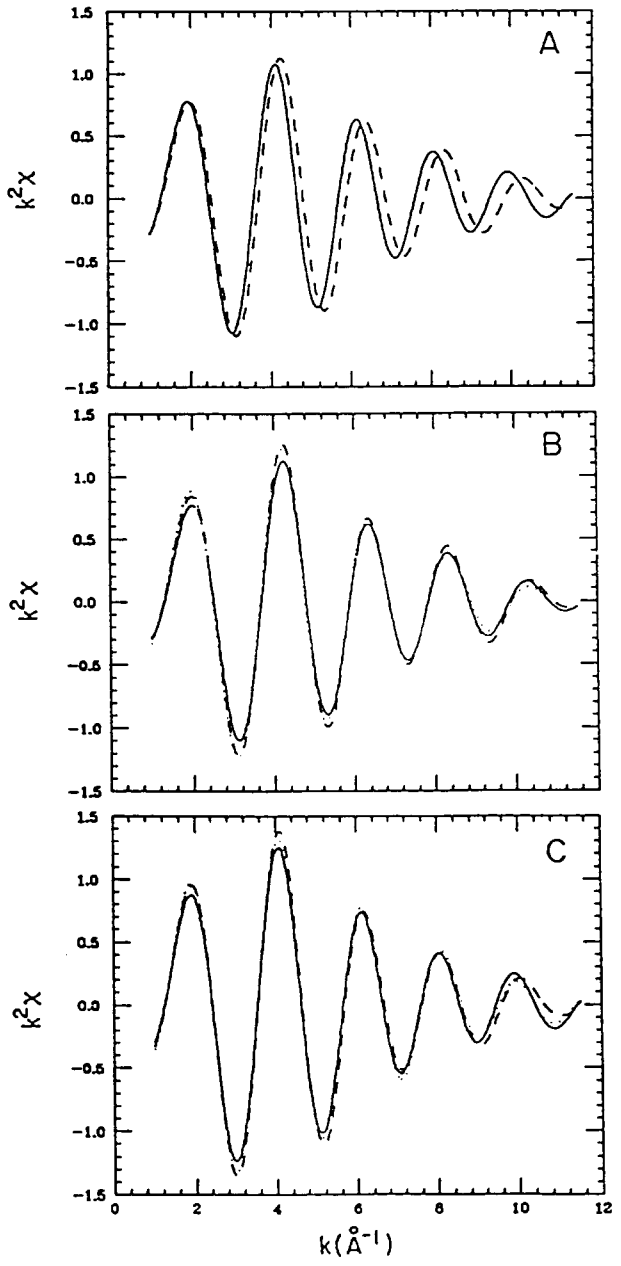

FIGURE 2: Isolated first shell χ data. (A) Comparison of the Fourier filtered $k^2\chi$ data of solution met-Mb at 240 K (solid line) and polycrystalline met-Mb at 260 K (dashed line). The difference in phase corresponds to 0.05 Å in average interatomic distance of the iron first neighbor atoms. (B) Comparison of the $k^2\chi$ data between polycrystalline met-Mb at 260 K (solid line), single crystal met-Mb at 300 K (dashed line), and the wet form of the polycrystalline met-Mb at 300 K (dotted line). The "wet" polycrystalline sample was obtained by adding the "mother liquor" to the crystalline powder. The single-crystal spectrum shown in a linear combination of two different crystal orientations to approximate the isotropic average. The size of the single crystal was 1 mm × 0.5 mm. (C) Comparison of the χ data between solution met-Mb at 130 K (solid line), solution met-Mb with 2.5 M ammonium sulfate at 130 K (dashed line), and the solution sample without ethylene glycol at 300 K (dotted line). The pH is 6.5 for both samples. No observable precipitation occurred during the measurement.

the solution met-Mb with the crystalline met-Mb. Before the fitting method was used, the transferability of model compounds was checked. Fitting of the Fe(II) dianion of *meso*-tetraphenylporphyrin-1-methylimidazole with Fe(II) 1,10-phenanthroline gave the average distance and coordination number for the first coordination shell to about 0.01 Å and within 10% compared with the known structure, respectively.

Table I: Average Interatomic Distance, Coordination Number, and Mean Square Displacement for the First Coordination Shell of Met-Mb[a]

	samples	N	R (Å)	σ^2 (Å^2)
XAFS	solution	5.9 (5)	2.05 (1)	0.003 (2)
	polycrystals	5.9 (5)	2.00 (1)	0.002 (1)
	solution (salt)	6.3 (5)	2.04 (1)	0.003 (2)
	single crystal	6.2 (5)	2.00 (1)	0.003 (1)
X-ray diff.	single crystal[b]	6	2.05	0.002
	single crystal[c]	6	2.00	<0.001

[a] All the samples used for XAFS measurements are described in the text and in the figure legends. The σ^2's for X-ray diffraction are structural disorders, while the XAFS σ^2's are referenced to the model compound. The value in parentheses is the error in the last digit. [b] See Takano (1977). [c] See Kuriyan et al. (1986) and references cited therein.

Table II: Debye–Waller Factor and Interatomic Distance Differences at Different Temperatures and the Corresponding Einstein Temperatures for Solution and Polycrystalline Met-Mb[a]

sample	T_1/T_2	$\Delta\sigma^2$ (Å^2)	Θ_E (K)	ΔR (Å)
solution	40/240	0.0016 (4)	460 (40)	0.003 (10)
crystalline	60/260	0.0005 (2)	680 (80)	0.002 (10)

[a] The temperature dependence of the XAFS Debye–Waller factor measures the bond-stretching modes provided that no structural change has occurred. Therefore, the Einstein temperature, which approximates all the modes with a single-frequency harmonic oscillator, is a measure of the bond strength. The value in parentheses is the error in the last digit.

Least-squares fitting, using Fe(II) 1,10-phenanthroline and Fe(III) acetylacetonate as nitrogen and oxygen reference compounds, respectively, was carried out for one-distance and two-distance structural models. The two distances from the two-distance model fit are not very well separated (≈0.1 Å), and the goodness of the fit is about the same as that for the one-distance model. The difference of Debye–Waller factors σ^2 is small for both solution and dry polycrystalline met-Mb at the low temperature, indicating a small disorder in the displacement of the atoms. The results for the single-distance model are listed in Table I compared with X-ray crystallographic results.

The change of the XAFS Debye–Waller factor σ^2 and the interatomic distance with temperature were determined by the ratio method (Bunker, 1983). The Debye–Waller factor difference $\Delta\sigma^2(T)$ as a function of temperature T was fitted to the Einstein model. The Einstein model is expected to be a good approximation since the first shell ligands are a mixture of five nitrogens and one oxygen at more or less the same distance. The XAFS Einstein temperature obtained from the fit is a measure of the Fe–ligand bond strength. The Debye–Waller factor and interatomic distance differences for the solution and polycrystalline met-Mb are listed in Table II along with the Einstein temperature. The Einstein temperature for the polycrystalline met-Mb (680 ± 80 K) is much larger than that for the solution met-Mb (460 ± 40 K), indicating the active site structure of met-Mb in polycrystalline form is more rigid than it is in the solution form (note that the XAFS Einstein temperature is related to the bond-stretching frequency and is larger than the Debye temperature from X-ray diffraction). No significant changes of the average first coordination shell distance were found for both crystalline and solution met-Mb at different temperatures (Table II).

We also measured the intensity (height) of the preedge bump, 3d "pip", relative to the absorption edge step. The 3d "pip" is caused by the electron transition from 1s state to unoccupied 3d states, namely quadrupole transition. However,

the unoccupied states with p-character can be mixed in with the "3d" states when the Fe environment is not inversion symmetric, resulting in enhanced 3d "pip" intensity (Shulman et al., 1976). The 3d "pip" intensity for the crystalline and solution met-Mb are 0.056 ± 0.003 and 0.68 ± 0.004, respectively, suggesting that the Fe ion environment in solution met-Mb is more unsymmetric.

Several potential artifactual explanations for the structure difference were ruled out: damage to the crystal by grinding, nonrandom orientation of the polycrystals, and the effect of humidity on the crystals. A single crystal was measured at several orientations relative to the X-ray polarization vector. An approximation to the isotropic average was generated by taking a suitable linear combination of the spectra obtained at different orientations. This spectrum and the spectrum of the "wet form" of the polycrystalline are shown in Figure 2B along with the "dry" polycrystalline spectrum, and the fitting results for the single crystal sample and the polycrystals are listed in Table I. No significant spectrum differences (i.e., within 0.01 Å and 10% in the distance and coordination number, respectively) can be detected among the different forms of the crystaline samples. This test shows that grinding of the crystals and humidity have no substantial effects on the active site of the protein.

Other questions are whether the differences between crystal and solution met-Mb are due to the presence of a high concentration of ammonium sulfate that is used to induce protein crystallization or due to the addition of ethylene glycol in the sample to prevent the formation of large ice crystals, since the salt ions and other molecules may bind to the protein and cause structural distortion. A met-Mb solution sample with about 2.5 M ammonium sulfate, which is nearly the concentration used for producing the single crystals, were measured with controlled pH (pH 6.5 for both samples). The first shell data are plotted in Figure 2C for the solution sample with salt and are compared with the data for the solution sample without salt; the fitting results are listed in Table I. Only a slight difference was observed, which corresponds to 0.01 Å in interatomic distance and less than 10% in coordination number, which is within experimental error for this sample. This test shows that no significant structural differences are observed between the soluble samples with and without a high concentration of salt. Measurement was also made for a solution sample without ethylene glycol, and the spectrum is shown in Figure 2C. The similarity of the XAFS spectra with and without ethylene glycol shows that the addition of ethylene glycol does not change the active site structure of the protein significantly.

Damage to the protein by intense radiation (Chance et al., 1980) might be another artifactual explanation for the differences observed. The radiation damage was monitored by optical spectra and by XAFS itself. XAFS scans were compared according to time sequences during the data collection. A slight spectral change toward that of crystalline met-Mb was observed for the solution sample with salt over the time interval of several hours of data collection. However, the change (corresponding to about 0.01 Å in the first shell interatomic distance) is very small compared to the differences observed between solution and crystalline met-Mb. Optical spectra of the solution sample after 1 h of X-ray radiation with about 10^{10} photons per second at the iron K-edge shows that about 12% of the sample has been reduced to deoxymyoglobin at temperature of 33 K (not shown). The reduction saturates to a level of about 15%. This amount of reduction would not give a significant first shell data change, especially since the average first shell distance of the reduced form (deoxy-Mb) is similar to that of met-Mb (the average first neighbor distance is about 2.06 Å for deoxy-Mb).

We can compare our crystalline results with the X-ray crystallographic results. At 2.0-Å resolution, the refinement by Takano gave interatomic distances for the nearest neighbors of iron (Table I) of 2.13, 2.04, and 2.00 Å for Fe–His, Fe–pyrrole nitrogen, and Fe–O (water), respectively, which is about 2.05 Å on average (Takano, 1977). It has been pointed out (Kuriyan et al., 1986) that anisotropy and anharmonicity can introduce significant position errors through crystallographic refinement. The recent X-ray crystallographic results by Kuriyan et al. (1986) at 1.5-Å resolution on met-Mb obtained an average distance of 2.00 Å for the first coordination shell distance, which is consistent with our result.

The interatomic distance change from crystalline to solution met-Mb implies a dramatic difference of the heme environment between the two forms of the protein. It is conceivable that the Fe–O (water) distance is elongated somewhat in the solution state. However, the 0.05-Å average distance change cannot be fully accounted for by the change of one or two ligands, since this would introduce a large static disorder, which was not observed. Thus, it is reasonable to assume the four Fe–pyrrole nitrogen distances changes significantly from solution to crystal. If we interpret this change to be caused by doming of the heme iron for the solution met-Mb but not for crystal met-Mb, the Fe ion would move substantially out of the heme center in solution met-Mb. The slightly larger 3d "pip" intensity of the solution state over the crystalline state suggests that the coordination environment of Fe ion is less symmetric in the former, which is consistent with the notion that the increased doming occurs in solution met-Mb. This local structural change at the active site is probably induced by the global structure change of the protein driven by the forces causing crystallization. The structural change may be responsible for the reactivity difference between solution and crystalline met-Mb with azide.

In summary, we have observed a significant interatomic distance change of 0.05 Å for the first neighbor of iron between crystalline and solution forms of met-Mb. Control experiments ruled out possible explanations that our obaservations are artifacts. The temperature dependence of the Debye–Waller factor indicates that the nearest neighbor bonds for crystalline met-Mb are more rigid than that for the solution met-Mb. These results, together with the previous results on concanavalin A and deoxy-Mb, lead us to conclude that, although X-ray diffraction provides an excellent starting point for understanding protein structures, there is a possibility that the structure in vivo may be different and caution should be used when trying to explain the detailed mechanism of protein function.

Acknowledgments

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CORRECTION

Fluorescence Analysis of Tryptophan-Containing Variants of the LamB Signal Sequence upon Insertion into a Lipid Bilayer, by C. James McKnight, Maria Rafalski, and Lila M. Gierasch*, Volume 30, Number 25, June 25, 1991, pages 6241–6246.

Page 6245. An incorrect figure has been published. The correct Figure 4 follows: