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STRUCTURAL DYNAMICS OF PHOTOLYZED MYOGLOBIN
AT LOW TEMPERATURES
BY X-RAY ABSORPTION FINE STRUCTURES

by
Tsu-yi Teng

A thesis submitted
in partial fulfillment of the
requirements for the degree
Doctor of Philosophy

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Houston, Texas
April, 1987
ABSTRACT

STRUCTURAL DYNAMICS OF PHOTOLYZED MYOGLOBIN AT LOW TEMPERATURES
BY X-RAY ABSORPTION FINE STRUCTURES

by

Tsu-yi Teng

X-ray absorption fine structures of photolyzed carboxymyoglobin were measured at 4 K and at 40 K. The latter was a 10-s-resolved measurement following photolysis. The goal of the project was to elucidate the dynamic pathways through which ligand binding couples to protein conformation changes. The project involved five major tasks: 1) We constructed an integration spectrometer for x-ray absorption capable of 100-μs-resolved measurement. 2) Heme protein samples have to be photolyzable on the one hand and possess a high (Fe) fluorescence yield on the other. This is very difficult to achieve with solution samples. The problem was solved by embedding concentrated (~10 mM) myoglobin in thin (~20 μm) dry polyvinyl alcohol (PVA) films. Carboxymyoglobin embedded in PVA film has the same properties as in frozen buffer solution as proven by optical absorption, kinetics of CO recombination following photolysis and x-ray absorption spectra. 3) A cryostat was constructed for x-ray absorption measurement, which was also equipped with optical windows for flash photolysis and optical monitoring of the degree of photodissociation in the sample. 4) Synchrotron radiation experiments were performed at Stanford Synchrotron Radiation Laboratory and at National Synchrotron Light Source at Brookhaven. 5) Data analyses showed that the
photolyzed carboxymyoglobin (Mb*) is structurally different from either carboxymyoglobin (MbCO) or deoxymyoglobin (Mb): the Fe-C(O) distance is 1.9 Å, 2.17 Å, ∞ Å for MbCO, Mb* and Mb respectively; the corresponding Fe-N_imidazole distances are 2.2 Å, 2.18 Å, 2.1 Å and Fe-N_porphyrin distances are 1.97 Å, 1.98 Å, 2.06 Å. At 40 K, from 1 s to 10 s following photolysis, 40% of photodissociated CO has recombined with Fe, and the rest of the sample is indistinguishable from Mb*.

The structure of Mb* described above is in disagreement with a previous report by Powers et al. (Biochemistry 23, 5519 (1984)).
ACKNOWLEDGMENTS

This project was done by a team work effort. Mr. W. H. Liu and J. A. Buchanan have developed the earliest schema of the software and hardware for the time resolved spectrometer, and Mr. X. F. Wang made all necessary electronics. During the exhausting duration of this experiment, my colleagues G. A. Olah and S. C. Wei shared the joys of success and sadness of failure along with me.

As for the general uses of the synchrotron radiation, we always got help from the host of the beam lines. They are Dr. G. S. Brown of SSRL, Dr. J. C. Phillips of NSLS X-21A, and Mr. J. P. Kirkland of NSLS X-23B.

Personally, I would like to thank Dr. G. B. Bunker of ISFS for kindly supplying the EXAFS analysis software, Prof. S. H. Chen of MIT for advice and assistance in doing neutron scattering, and Dr. J. S. Lin of ORNL for taking the x-ray scattering data. Above all, I would like to thank my adviser, Dr. H. W. Huang, for his encouragement and patience while I worked on this project.

Finally, I would like to thank my wife, Yu-chen, for all her understanding and support.
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INTRODUCTION

It has been recognized for some time that the equilibrium structure of a protein does not fully explain its observed properties and functions. The well known example is hemoglobin: Perutz and Mathews[1] first noted that its equilibrium structure leaves no room for an oxygen molecule to reach the buried heme binding sites. The same observation also applies to the oxygen storage protein myoglobin. It seems certain that the ligand gains access to the interior of hemoglobin or myoglobin as a result of thermal motion of the protein structure. Thus, study of structural dynamics is essential for understanding the function of proteins. In the case of hemoglobin, structural changes in the molecule are also the underlying mechanism for the so called cooperative ligand binding. A (human) hemoglobin is a tetrameric protein consisting of two $\alpha$ subunits and two $\beta$ subunits. Each subunit has an oxygen binding site. The phenomenology of oxygen binding in hemoglobin is described, to the first order approximation, by a two-state allosteric model[2].

Within this description there is an $O_2$ concentration-dependent equilibrium between two distinct quaternary structures of the protein. At low $O_2$ concentration, hemoglobin is primarily deoxygenated and the low-affinity 'tense' quaternary structure is favored. Increasing the $O_2$ concentration and hence the degree of saturation shifts the equilibrium to the high-affinity 'relaxed' quaternary structure. As a result, the saturation curve as a function of the $O_2$ pressure is s-shaped or sigmoidal, and the binding process is called cooperative. The difference between the binding energy of the first and the last oxygen is called the energy of
cooperation and is about 0.15 eV. The physics problem posed by hemoglobin is: What is the mechanism of interaction which generates this free energy of cooperation? An essential part of this question is understanding the mechanism through which the overall protein structure couples to the local environment of binding sites.

This project was motivated by the opportunity of using the recently advanced synchrotron radiation techniques to study the sequence of local structural changes in heme proteins initiated by ligations in order to elucidate the dynamic pathways relating ligand bindings to protein conformation changes. As a first step toward this goal, I measured and studied time-resolved x-ray absorption spectra of photolyzed carboxymyoglobin at low temperatures. The iron K-edge extended x-ray absorption fine structure (EXAFS) of a heme protein contains information about distances of atoms within 3.2 Å from the iron atom. A set of time-resolved EXAFS of a photolyzed sample is a series of EXAFS, each measured at a chosen time after photolysis. Thus the time-resolved spectra can be used to study the sequence of the structure change taking place in and near the heme regions following ligand photodissociation.

This project involved five major tasks: 1) Construction of time-resolved x-ray absorption spectrometer, 2) preparation of heme protein samples suitable for x-ray absorption and photolysis simultaneously, 3) Control of sample during x-ray experiment, including temperature, photolysis, and monitoring of the degree of photodissociation in sample, 4) synchrotron radiation experiment, and 5) data analysis.

An integrating x-ray absorption spectrometer capable of 100 μs time-resolution was built in our laboratory a few years ago. It was motivated by the fact that the conventional fluorescence EXAFS spectrometer using photon counters saturate at 10^6 counts per second. Since the intensities of synchrotron radiation
beams had been improved steadily, a spectrometer which could make use of the full intensity was clearly needed. The spectrometer was designed to measure a spectral point at one energy at a time. So the sample has to be photolyzed for each energy point. Although this procedure seems to be tedious and time consuming, it is really the only way to perform time-resolved fluorescence EXAFS measurement. The often mentioned (but so far unsuccessful) energy dispersion method, which in principle measure the entire absorption spectrum in one shot, is applicable to the transmission method only; therefore it is useless for most biological samples which due to the low concentration of absorbing atoms are measurable only in the fluorescence mode. The details of the integration spectrometer have been described in two papers\[3,4\] and in my Master's thesis\[5]\; I will not repeat them here.

Samples for this experiment present a special problem. On the one hand, it is desirable to have them thick and concentrated in order to have a high x-ray fluorescence yield. On the other hand they need to be thin and dilute so that they can be completely photolyzed. The obvious solution is to make them thin and concentrated, and use several of them simultaneously but spaced out for photolysis. Such a sample is practically impossible to make from solutions. We decided to embed concentrated carboxymyoglobin (MbCO) in thin dry polyvinyl alcohol films. After repeated trial and error, I finally found a procedure for making such film samples which satisfied four important criteria: 1) the properties of MbCO in dry film are exactly like those in frozen buffer solution as proven by optical absorption, kinetics of CO recombination following photolysis and x-ray absorption spectra, 2) the transparency of film sample are as good as solution, 3) high concentration of MbCO ( > 10 mM) is possible, and 4) the film can be made thin ( < 50 μm). We believe that this film sample is very important for x-ray absorption studies of heme
proteins.

With the currently available synchrotron radiation facilities (the best beam gives $10^{12}$ photons/sec-ev), it is impractical to perform time-resolved EXAFS of heme proteins with a time resolution shorter than 1 ms. However the recombination time for photolyzed MbCO in room temperature is milli-seconds. Fortunately the recombination process slows down at low temperatures (in fact the recombination time is infinity at 4 K). For practical reasons explained in Chap. VI, we chose to do the experiment at 4K (metastable measurement) and 40 K (10-s-resolved measurement). The cryogenic requirement presented another difficulty for sample control. The task was to design a cryostat suitable for x-ray absorption measurement that simultaneously provides optical windows for flash photolysis and for optical monitoring to determine the degree of photodissociation of the sample. Because the design could not optimize all the requirements, the cryostat was remade several times until all the requirements were satisfied.

Because of the repeated delays in the construction of the National Synchrotron Light Source (NSLS) at Brookhaven and the scarcity of beam time at Stanford Synchrotron Radiation Laboratory (SSRL) in the last few years, our experiments were performed over several years. Preliminary experiments, mainly for the testing of the integration spectrometer and for testing of PVA film sample, were performed at SSRL beamline VII-3 and NSLS beamline X-21A. The 4 K and 40 K experiment were performed at NSLS X-23B. Synchrotron radiation facilities have not reached the mature stage. Every beamline has its own distinct character which influences the experimental data. As a result it is difficult to compare the data taken from two different beamlines or even from the same beamline at different times. Fortunately the final results (the atomic distances) seems to be quite independent of
where the data were taken.

The theory of EXAFS is well established and is relatively simple. There are basically two types of methods for analyzing the data. One method is based on the Fourier transforms of the fine structures and the other is based on model-fitting. Based on the experience of many authors, I composed a Fourier method for analyzing our data. For the purpose of comparison I also obtained a program from Dr. G. Bunker of the Institute for Structural and Functional Studies (ISFS), University of Pennsylvania. Every set of data was reduced by these two program and I found that the results are consistent. I found that the photolyzed state of MbCO at 4 K is structurally different from either MbCO or Mb: CO is displaced from its bound position, where the Fe-C distance is 1.9 Å, to 2.17 Å, but the iron is only slightly displaced from the MbCO position (Fe-N_{porphyrin} distance changes from 1.97 to 1.98 Å, whereas in Mb the distance is 2.06 Å). At 40 K, from 1 s to 10 s after complete photolysis, 40% of the photodissociated CO molecules have recombined with Mb; the rest of the sample was indistinguishable from Mb*.

This 40 K result is completely new. The 4 K experiment has been reported by Chance et al and Powers et al [6,7]. They reported a CO movement to 1.97 Å and a Fe-N_{porphyrin} stretching to 2.03 Å. This disagreement should stimulate further investigation on this important problem of heme protein structural dynamics.
2. see e.g., Hopfield, J. J., in 'Collective Properties of Physical System' pp.
   (1974)
3 Huang, H. W., Liu, W. H., Teng, T. Y., and Wang, X. F., Rev. Sci. Inst. 54,
   1488 (1983)
4 Liu, W. H., Wang, X. F., Teng, T. Y., and Huang, H. W., Rev. Sci. Inst. 54,
   1653 (1983)
7 Powers, L., Sessler, J. L., Woolery, G. L., and Chance, B., Biochemistry 23,
   5519 (1984)
I. SAMPLE

I-1. Myoglobin and Hemoglobin

Myoglobin and hemoglobin are respiratory heme proteins, that is, proteins able to undergo a reversible reaction with molecular oxygen by virtue of a prosthetic group containing ferro porphyrin (protopheme or, more simply, heme). 'Hemoglobin' (denoted by symbol Hb) is the name given to these proteins when they are present in the blood of vertebrates and 'myoglobin' (Mb) to those present in the smooth or striated muscles of all animals. In a protopheme there are six coordination sites about an iron atom (Fig. I-1), four of which are occupied by the pyrrole nitrogen atoms of the porphyrin ring. The fifth ligand is the nitrogen atom of an imidazole, the side chain of the 'proximal histidine', which probably anchors the heme to the protein. The sixth site is available to bind an extra ligand. The structure of a protopheme is formed by the four pyrrol rings connected by methine bridges and substituted by different groups in the external positions (Fig. I-2). The myoglobin and hemoglobin molecules are much more complex. In the case of Mb, the heme is surrounded by a single polypeptide chain of 140-160 residues with a total molecular weight of 16,000 to 18,000[1]. Hb consists of two pairs of two slightly different polypeptide chains called α- and β-chains each resembling a Mb[2]. The way in which a single polypeptide chain folds into a globular form is called the tertiary structure. The way in which the subunits are put together in a protein is called the quaternary structure.
Figure I-1 Iron coordinate in a heme. Distances are in Å.
Figure I-2  Structure of heme. The central iron is surrounded by four pyrrol rings. The amino acid chain is connected to the imidazole.
**TABLE I-1  AMINO ACID COMPOSITION IN SPERM WHALE MYOGLOBIN**

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<th>SEQUENCE</th>
<th>1-30</th>
<th>31-60</th>
<th>61-90</th>
<th>91-120</th>
<th>121-153</th>
<th>Total</th>
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<td>3</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>6 (7)</td>
<td>17</td>
</tr>
<tr>
<td>Arg</td>
<td>2</td>
<td></td>
<td>1</td>
<td>1</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Asp</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
<td>2 (3)</td>
<td>6</td>
</tr>
<tr>
<td>Asn</td>
<td></td>
<td></td>
<td>2</td>
<td>(1)</td>
<td></td>
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</tr>
<tr>
<td>Glu</td>
<td>3</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>2</td>
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<td>Gln</td>
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<td>1</td>
<td>2</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Gly</td>
<td>3</td>
<td>3</td>
<td></td>
<td>5 (4)</td>
<td></td>
<td>11</td>
</tr>
<tr>
<td>His</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>5</td>
<td></td>
<td>12</td>
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<tr>
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<td></td>
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<td>5</td>
<td>1</td>
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</tr>
<tr>
<td>Leu</td>
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<td>3</td>
<td>6</td>
<td>2</td>
<td>3</td>
<td>18</td>
</tr>
<tr>
<td>Lys</td>
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<td>6</td>
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<tr>
<td>Phe</td>
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<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Va</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
<td>8</td>
</tr>
</tbody>
</table>

* This Table was made by combining informations from\textsuperscript{[1,4-8]}. Figures in parentheses in the 6\textsuperscript{th} column indicate a slightly difference between \textsuperscript{[8]} and \textsuperscript{[1,4-7]} at the 121\textsuperscript{st} residual; it has little effects on evaluating of the molecular weight of Mb (they are 17,810 and 17,802 amu, respectively.)
Myoglobin and hemoglobin used as our samples are from sperm whale muscles and human blood, respectively. The former has the shape of a flattened prism having the overall dimensions of 45 x 35 x 25 Å \([3]\). If one estimates its volume by assuming it forming an ellipsoid with semi-axes of 22.5, 17.5 and 12.5 Å, one obtains 2.1 \times 10^4 \text{Å}^3 as its volume. The polypeptide chain of sperm whale Mb consists of 153 amino acid residues of which their sequence and stereo-structure are well known \([1,4-8]\). The molecular weight of sperm whale Mb evaluated from its composition is estimated to be 1.78 \times 10^4 \text{amu} (see Table I-1). The hemoglobin molecule is roughly spherical with a dimensions of 64 \times 55 \times 50 \text{Å}. The molecular weight of hemoglobin is 6.45 \times 10^4 \text{amu}\([2]\).

A hemoglobin or myoglobin molecule with a bound ligand \(X\) is denoted by \(\text{HbX}\) or \(\text{MbX}\), respectively. The ligand can be chemically removed; in this case the molecule is denoted by deoxyHb or deoxyMb, or simply Hb or Mb.

Since heme proteins can store and transport oxygen and electrons as well as catalyze oxidation-reduction reactions, it is not surprising that they have been the subject of extensive studies. Perhaps the most important and certainly the most interesting property (from the point of view of physics) of Hb is the cooperative way in which it binds ligands. Let \(Y\) be the fraction of hemes having ligand bound to them. For Mb, with one heme per molecule, the binding follows a hyperbolic curve

\[
Y = \frac{KP}{1 + KP} \quad (1-1)
\]

where \(K\) is a constant and \(P\) is the partial pressure of ligand molecules. For Hb, with four hemes per molecule, the binding follows a curve described by
\[ Y = \frac{Kp^n}{1 + Kp^n} \]  \hspace{1cm} (1.2)

where \( n \) is called the Hill constant and is found to be greater than one (if the ligand is oxygen, \( n = 3 \)), indicating that the hemes in a single Hb do not bind ligands independently. There must be interactions between the hemes within each Hb even though the iron atoms are approximately 35 Å apart. Indeed, the binding energy of the last heme is 0.15 eV more than that of the first\(^2\).

It is now well known that the cooperation manifested in the binding of ligands to Hb is mediated by conformational changes of the protein\(^9\). The binding of ligands to the hemes of Hb initiate a sequence of propagating structural events which culminates in a change of quaternary conformation. The changes in the protein in turn mediate the affinities of ligand binding. The structural changes in Hb and Mb caused by ligand binding have been studied by photodissociating the ligands and examining the resulting metastable states by various spectroscopies, including transient optical absorption \(^{10-14}\), near infrared absorption\(^{15,16}\), magnetic circular dichroism \(^{17}\), resonance Raman \(^{18,19}\), EPR\(^{20,21}\), and Mossbauer \(^{22,23}\).

It was found that immediately after the photolysis of HbX or MbX there are photodissociated metastable states, denoted by Hb* or Mb*, respectively, which have spectroscopic properties distinct from those of chemically prepared deoxy states. It is believed that in these photolyzed states, the deoxy heme is constrained in a metastable configuration due to an unrelaxed protein tertiary conformation and consequently the heme-globin interaction is manifested in the form of a structural distortion near the heme-globin interface.

In order to understand how ligand bindings initiate tertiary and
quaternary conformation changes in heme proteins, it is desirable to study the structural changes taking place in and near the heme. As a local structure probe, EXAFS is ideal for this purpose. The iron-edge EXAFS of deoxyhemoglobin has been reliably used for determining the distances of atoms from the iron atom with a standard deviation less than 0.02 Å within a 3.2 Å radius[24]. Within this range there are five nitrogen (four of them on the porphyrin ring, and one in the proximal imidazole) and ten carbon (eight of them in the porphyrin ring, and two in the imidazole ) atoms around the iron. Accompanying the radius of each shell around the central iron atom, the EXAFS analysis also gives a Debye-Waller factor which expresses the thermal and structural disorder of the atoms in that shell. The EXAFS of photolyzed MbCO in its low temperature steady state (Mb*) has been studied by Chance et al[25]. However this is an extremely difficult experiment and there has been controversy about their conclusions. Therefore another independent measurement is worthwhile.

I-2. Solution Sample vs. Film Samples

In order to study the metastable photolyzed hemoglobin and myoglobin with the x-ray absorption spectroscopy, i.e. the extended x-ray absorption fine structures (EXAFS) and near edge structures, the samples must meet the competing requirements of being transparent to light and having the protein concentration high enough so as to give a sufficient Fe fluorescence yield. This is difficult to achieve in the form of liquid solution sample.

To appreciate the sample problem we list in Table I-2 the x-ray and optical absorption lengths of the solution sample and the percent of x-ray absorption by
iron at the iron K-edge (7.2 keV). The values of x-ray absorption were calculated based on the published atomic absorption coefficients\[^{28}\]. If we assume the

**TABLE I-2 ABSORPTION CHARACTERISTICS OF CARBOXYMYOGLOBIN SAMPLES**

<table>
<thead>
<tr>
<th>THICKNESS</th>
<th>CONCENTRATION</th>
<th>X-RAY ABSORPTION OF IRON (7.2keV)</th>
<th>X-RAY ABSORPTION (542nm)</th>
<th>OPTICAL ABSORPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>minimum</td>
<td>(mm)</td>
<td>(mM)</td>
<td>(%)</td>
<td>(mm)</td>
</tr>
<tr>
<td>0.1</td>
<td>0.015</td>
<td>1.6</td>
<td>7.4</td>
<td></td>
</tr>
<tr>
<td>SOLUTION</td>
<td>0.5</td>
<td>0.076</td>
<td>1.6</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.15</td>
<td>1.6</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>0.70</td>
<td>1.6</td>
<td>0.15</td>
</tr>
<tr>
<td>PVA FILM</td>
<td>0.02</td>
<td>1.0</td>
<td>1.9</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>0.86</td>
<td>1.9</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>10.</td>
<td>1.4</td>
<td>1.9</td>
<td>0.070</td>
</tr>
</tbody>
</table>

* x-ray absorptions were calculated based on the atomic absorption coefficients\[^{24}\]; light absorptions were measured with a spectrophotometer. The sample preparation was as given in Sec.II in text.

incident x-ray is completely absorbed, then the total Fe K-shell fluorescence yield is proportional to the fraction of x-ray absorbed by the iron atoms in the sample.
The fourth column in Table I-2 gives the values of this fraction. The absorption lengths shown here are the thicknesses of 10% transmission. The optical absorption lengths are the measured values. Because of the uncertainties in sample densities, the x-ray values are only accurate to about 10%, which are consistent with our measurements. In practice we found that it is difficult to achieve 100% photolysis (with a 100-joule xenon flash) in samples with \( C \cdot l > 0.4 \text{ mM} \cdot \text{mm} \), where \( C \) is the heme concentration and \( l \) is the sample thickness. A 2 mm thick 0.2 mM solution sample practically absorbs all the (7 keV) x-ray incident on it, but only 0.03% is absorbed by Fe, which translates to a Fe fluorescence yield of \( 10^{-4} \) (the atomic fluorescence yield of Fe is 0.34). Since a typical EXAFS analysis requires a statistical accuracy of \( 10^6-10^7 \) photon counts per data point, such a low fluorescence yield is impractical for time-resolved experiments. Actually, the problem of photolysis of solution samples is worse than what is indicated in Table I-2. In order to study photolyzed MbCO with a long ligand recombination time ( \( > 1 \text{ sec} \)), the sample must be kept below 100 K. It is well known that a solution sample usually cracks at low temperatures and when that happens its light transmission diminishes\(^{10}\).

To overcome these difficulties, we have successfully made suitable samples with heme proteins embedded in dry polyvinyl alcohol (PVA) films. The idea is to prepare very thin but highly concentrated samples. Each sample is concentrated enough to afford substantial x-ray absorption, but thin enough to permit good light transmission. The absorption length and the percentage of x-ray absorption by an iron atom of the film sample are also listed in Table I-2. Compared with a solution sample of the same concentration, a film sample has about the same optical quality but a better x-ray fluorescence yield. Since a film sample can be made much
thinner than the liquid ones, even a 10 mM protein concentrated film can be completely photolyzed. For x-ray absorption experiments, we space out a number of such films in the direction of the incident x-ray, so that each film can be independently illuminated. The x-ray absorption or the x-ray fluorescence yield is then adjusted by the total thickness of the films to obtain the maximum yield of Fe fluorescence. This is equivalent to a thick and concentrated solution sample segmented into thin layers; each is thin enough for photolysis and together they give a large x-ray fluorescence yield. Note that there is no low temperature problem for PVA film. The film does not crack at all. Its light transmission remains the same from 300 K to 4 K. As a result we obtain a Fe fluorescence yield of 0.014 from 100% photolyzable film samples.

However, the exact nature of the chemical environment of heme proteins in the solid PVA matrix is unknown. Therefore it is important to examine the properties of the film samples and compare them with the standard solution samples. We found that the ligand recombination kinetics and the x-ray absorption spectra of the heme proteins in PVA film are the same properties as in frozen buffer solution. Also, the film samples are more stable than the solution samples and easier to handle. In the next two sections I will present the method of making such film samples and the results of measurements which demonstrate the properties described above.
1 Antonini, E. and Brunori, M., 'Hemoglobin and Myoglobin in Their Reactions with Ligands', North-Holland, Amsterdam (1971)
4 Edmundson, A. B., and Hirs, C. W., J. Mol. Biol. 5, 663 (1962)
5 Edmundson, A. B., Nature 205, 883 (1965)
8 Takano, T., J. Mol. Biol. 110, 537 (1977)
9 Perutz, M. F., Nature 228, 726 (1970)
II. METHOD OF MAKING FILM SAMPLE

Myoglobin was chosen as our first sample because of its importance in protein biophysics and also because it is easy to prepare. Hemoglobin, which has a more complicated structure, is the logical follow-up in the study of protein dynamics. Carbon monoxide was used as the ligand of our samples because it has the highest value of quantum yield in photolysis compared with other small molecular ligands.

A fresh buffered aqueous ferric myoglobin solution was made from crystalline myoglobin (Type 2, sperm whale Mb, iron contents 0.29%) purchased from Sigma Chemical Inc. (St. Louis, MO). It was dissolved in a 0.1 M buffer (pH 7.6) and centrifuged at 10 krpm for 10 minutes at 4 °C. A commonly used phosphate buffer, when used at high concentration, causes PVA polymers to form globular particles in the film. That makes the film opaque. Among other buffers\(^1\), we found Heps (4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid, pKa 7.55 at 20 °C) to be the best for making transparent films. Polyvinyl alcohol, \((-\text{CH}_2\text{CH(OH)})_n\text{\textend{itemize}}\), was dissolved in the same buffer by boiling, then cooled to room temperature. A wide range of molecular weights of PVA (2,000 - 115,000) are available from Aldrich (Milwaukee, WI), J.T.Baker (Phillipsburg, NJ) and Sigma; all make good films. The general rule is that the higher the molecular weight the more viscous the solution is, which makes it harder to mix a high concentration of protein. On the other hand Mb molecules tend to precipitate in PVA of low molecular weight. We have consistently made good
samples with molecular weight in the range of 14,000 to 40,000. The Mb solution of various concentration and the PVA solution were mixed to obtain a buffered aqueous Mb-PVA solution with the resultant concentration of Mb up to 1.5 mM and PVA about 10% (w/v). Higher Mb to PVA ratios led to myoglobin precipitation.

The Mb-PVA solution was rendered anaerobic by stirring in a nitrogen atmosphere for about an hour at 4 °C. Reduction of ferric Mb was accomplished by adding freshly prepared sodium dithionite solution into the anaerobic sample solution. The dithionite concentration was five times the Mb concentration. More than this is not necessary and, may alter the pH value of the solution or optical quality of the film. We tried other possible reduction agents, such as L-Ascorbic acid and sodium borohydride\textsuperscript{[2]}, and found that they were no better than dithionite as far as the quality of film was concerned.

After reduction, CO was introduced into the flask while continually stirring the solution. Equilibration took one hour for CO saturation. All these steps took place in a specially designed flask which kept the samples from coming into contact with oxygen and allowing the gaseous atmosphere to be replaced several times during reduction and equilibration. The temperature was always maintained at about 4 °C. At least four times during the process, at the completion of centrifugation, at the completion of reduction and twice during equilibration with CO, a small amount of sample was taken to check its absorption spectrum. These spectra (taken with a Varian Cary 17 spectrophotometer) were in agreement with published data\textsuperscript{[3]}.  

To make a film, MbCO-PVA solution was spread on a leveled plate, which was placed in a glove box filled and pressurized with CO at room temperature, and
allowed to dry. The ratio of the solution thickness to the dry film thickness is about 7 to 1. Our film samples were typically 20 to 50 μm thick. The drying time for these films was 1 to 2 days. The films were stored in a freezer under CO atmosphere or kept in liquid nitrogen. Apparently the dry film is relatively impermeable to oxygen and carbon monoxide.

III. CHARACTERISTICS OF FILM SAMPLES

III-1. Light Absorption Spectra

The optical spectroscopy was used for three occasions:

1) For comparing a film sample with a standard buffered sample.

The spectrum of MbCO in Hepes buffer was compared with that in the commonly used phosphate buffer; they are essentially identical in the entire visible range (400 nm to 700 nm). Figure III-1 shows the characteristic α- and β-bands of MbCO in a phosphate buffer, in a Hepes buffer and in a PVA film made with Hepes buffer. Low absorbance beyond 600 nm indicates that the transparency of the film is as good as the solutions. On the contrary, the PVA film sample made with phosphate buffer (the top spectrum in Fig. III-1) has a lower transparency and its α-band is rounded.

2) For quality control of sample making.

This has been mentioned briefly in the previous section. The extinction coefficients of our MbCO-PVA film are (14.3 ± 0.1) mM⁻¹.cm⁻¹ at 542 nm (β-band) and (13.1 ± 0.2) mM⁻¹.cm⁻¹ at 578 nm (α-band). (The uncertainties are mainly due to the errors in the measurement of the thicknesses of the films.) A peak of the ferric Mb spectrum at 635 nm is useful for checking the residual ferric Mb during the reduction process and for detecting possible deterioration of stored samples.
Figure III-1. Light absorption spectra of myoglobin samples. Liquid samples were 0.6 mM MbCO in buffer and their spectra were taken with a 1.0 mm path length cell; film samples were made from the corresponding buffered MbCO-PVA solution (see Sec.II) and their thicknesses were about 100 μm. Solid line (---): MbCO in 0.1 M phosphate buffer (pH 7.6). Dashed line (--------): MbCO in 0.1 M Hepes buffer (pH 7.6) with 10% (w/v) PVA. Single dot line (-----): MbCO in PVA(Hepes) film. double dot line (----): MbCO in PVA(Phosphate) film.
Figure III-2. Radiation damage of samples measured by optical absorption. The solid lines are spectra taken from fresh samples; the dashed lines are spectra of the same samples taken after exposed to synchrotron radiation (7 - 8 keV, 5 \times 10^{10} \text{ photons s}^{-1} \text{ for two hours at room temperature}).

a) Carboxyhemoglobin (HbCO) in PVA (Hepes) film (heme concentration about 8 mM, film thickness about 75 \mu m). b) Carboxymyoglobin (MbCO) in glycerol/phosphate buffer (3:1, v/v) (heme concentration 0.2 mM, sample thickness about 2 mm). The dashed spectrum of HbCO film was taken on the 80th day after x-ray exposure. The film had been stored in a freezer and it was exposed to air.
3) For checking the radiation damage.

Radiation damage due to x-ray exposure changes the transparency and the spectral amplitude of a sample. For example Fig. III-2 shows the spectra of a film sample and a solution sample, before and after x-ray exposure. Both samples were exposed to synchrotron radiation (7 to 8 keV, 2 eV bandwidth, $5 \times 10^{10}$ photons s$^{-1}$) for about two hours. The damage seemed to decrease the transparency and reduce the peak heights, but the overall shape of the spectrum did not change. (For example, we did not detect a peak at 635 nm characteristic of the presence of ferric Mb as usually happens to a deteriorated sample.) The peak heights of the solution sample decreased by 50%, while the corresponding decrease of the film sample was less than 10%. In this respect the film samples are similar to frozen samples. It is well known that one mechanism of radiation damage is the creation of free radicals which attack the proteins. It is thought that in frozen samples as well as film samples, the free radicals are immobile and are less effective in damaging the protein molecules.

III-2. Kinetic Spectra

The kinetic behavior of ligand recombination following photolysis is a well defined property of MbCO. The transient optical absorption is the standard method for measuring this property. The method is based on the fact that the ligand molecule can be flashed off from its binding site and that the absorption spectra of the ligated and deligated proteins are different.
Figure III-3. The set up for transient optical absorption measurement. The film sample is placed in the cryostat so that its temperature can be controlled from 4 to 300 K. The monitoring light is introduced in and brought out via fiber optics. A xenon flash light mounted at the bottom of the cryostat applies the energy needed for photodissociation of the ligand. A combination of mirror and filters which cuts both red and violet light from the flash light protects the sample and reduces radiation heat. The photomultiplier detects the transmitted light through the sample at a chosen wave length controlled by a narrow band pass interference filter. The signal is then sent to either a commercial waveform digitizer or to our data acquisition system.
Figure III-4. A photograph of the cryostat, xenon flash light, monitoring light, photomultiplier detector, and the fiber optics used as a part of the transient optical absorption spectrometer. See Fig. III-3 for a detailed descriptions. The background of the photo shows electronics of the time-resolved x-ray absorption spectrometer.
It was Gibson[1] who first demonstrated the use of flash-photolysis methods in the study of reactions of hemoglobin and myoglobin with ligands. After that, the kinetic studies of photolyzed myoglobin at different temperatures and with different viscosities of solution or different metallic substitutes have been carried out[2-8].

Myoglobin embedded in a PVA film was first studied by Austin et al.[4]. They examined the effects of the solvent on the kinetics of ligand rebinding to myoglobin after photodissociation. Their studies showed that the kinetic properties of MbCO embedded in a solid PVA film is essentially the same as that of MbCO embedded in frozen buffer solution.

I set up a transient optical absorption photometer for the purpose of checking the sample in 1983[9]. The kinetic behavior of different MbCO samples (in phosphate buffer, glycerol water and PVA films) were examined from 77 K to room temperature by using this apparatus. The results were in agreement with published data. In order to monitor the recombination kinetics during time-resolved EXAFS measurement, the apparatus was modified. The sample holder was replaced by a specially designed cryostat. Both the in and out monitoring light paths were provided by fiber optics. And an interference narrow band filter was used instead of the monochromator for the ease of handling (see Figures III-3 and 4). As a trade-off it lost about half of the light intensity and forfeited the tunability of the monitoring light.

This new apparatus was mainly used to check MbCO-PVA films at low temperatures. Figures III-5, III-6 show the kinetics of ligand recombination following photolysis of our sample at different temperatures. Two sets of
Figure III-5. Kinetics of ligand recombination following photolysis. The MbCO concentration in the PVA film was 9 mM (film thickness about 40 μm). The optical absorption \(1 - \frac{I}{I_0}\) was measured at 439.5 nm. Time equaled zero when the xenon flash (duration 100 μs) was triggered. Sample temperatures: a) 10 K; b) 60 K; c) 80 K; d) 100 K; e) 120 K. Signals were amplified and recorded by a commercial waveform digitizer.
Figure III-6. Kinetics of ligand recombination following photolysis. Sample temperatures are: a) 120 K; b) 100 K; c) 80 K; d) 60 K. Other experimental conditions are the same as described in Fig. III-5 except that the light absorption signals were treated by our integrator-A/D-converter in a 1 ms/point integration time. Compared with Fig III-5, we see that our data acquisition system has a better signal-to-noise quality than the waveform digitizer.
curves are displayed which were taken from the same film by a commercial waveform digitizer (LeCroy WD8256) (Fig. III-5) and by our integration spectrometer (Fig. III-6). It is evident that our spectrometer is superior to the former in the signal-to-noise ratio.

Assuming that there are only two states involved in the photolysis process, we have the kinetic expression

\[ \text{MbCO} \xrightleftharpoons[ hv ]{ } \text{Mb} + \text{CO}, \quad (\text{III}-1) \]

where we assume all CO molecules are photodissociated at \( t = 0 \). The intensities of light transmitted through the sample before and immediately after dissociation are given by the two equations

\[ I(t<0) = I_o \cdot \exp(-\epsilon_{\text{MbCO}} C l) \quad (\text{III}-2) \]
\[ I(t=0) = I_o \cdot \exp(-\epsilon_{\text{Mb}} C l) \quad (\text{III}-3) \]

where \( I_o \) is the incident intensity, \( t < 0 \) denotes the time before photolysis, \( \epsilon_{\text{MbCO}}, \epsilon_{\text{Mb}} \) are the extinction coefficients of bound and free myoglobin, \( C \) is the heme concentration, and \( l \) is the path length. By introducing \( N(t) \), the fraction of myoglobin molecules that have not rebound CO at time \( t \) after photolysis, we can derive the light intensity at any time \( t \) after flash

\[
I(t) = I_o \cdot \exp\{-\epsilon_{\text{MbCO}}[1-N(t)] C l \} \cdot \exp\{-\epsilon_{\text{Mb}}N(t) C l \}
= I(t<0) \cdot \exp[-N(t) (\epsilon_{\text{Mb}} - \epsilon_{\text{MbCO}}) C l]. \quad (\text{III}-4)
\]

Notice that the measured values of intensity can be related to the change in
absorbance $\Delta A(t)$,

$$\Delta A(t) = \log \left( \frac{I(t<0)}{I(t)} \right) \quad (III-5)$$

at a suitably chosen wavelength combining Eqs. (III-2), (III-4) and (III-5), we have

$$N(t) = \frac{\Delta A(t)}{\left( \epsilon_{Mb} - \epsilon_{MbCO} \right) C l} \quad (III-6)$$

<table>
<thead>
<tr>
<th>TABLE III-1. FRACTION OF UNBOUND MYOGLOBIN AFTER PHOTOLYSIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>$N(t)$ (%) at time $t$</td>
</tr>
<tr>
<td>$t = 1 \text{ ms}$</td>
</tr>
<tr>
<td>T (K)</td>
</tr>
<tr>
<td>$&lt; 10$</td>
</tr>
<tr>
<td>$40$</td>
</tr>
<tr>
<td>$60$</td>
</tr>
<tr>
<td>$80$</td>
</tr>
</tbody>
</table>

The values in parentheses are taken from [4] Fig. 4, their sample is a phosphate buffered MbCO embedded in PVA film with very low concentration.

I chose the monitoring light with the wavelength 439.5 nm, where the difference in extinction coefficient between MbCO and Mb is the largest, 66
mM$^{-1}$-cm$^{-1}$. Table III-1 compares our data compared with Austin et al.$^{[4]}$ (at low Mb concentration and with phosphate buffer). One can see that they are in good agreement. These data are important in the design and analysis of time resolved experiment (see Chs. VI, VII).

III-3. EXAFS and Near Edge X-ray Absorption Spectra

Although the general principle of molecular orbital transitions involved in the optical absorption is understood, the precise interpretation of the absorption spectra for a system as large as MbCO is very difficult$^{[10]}$. Similar remarks apply to the ligand recombination kinetics$^{[11]}$. On the other hand, EXAFS can be analyzed quantitatively$^{[12]}$. Since we will deal with its theory and data analysis method later, here we only show evidence that the film sample has the same EXAFS as the solution (Fig. III-7). This indicates that the PVA matrix does not disturb the structure of the heme and its immediate surrounding. The quantitative analysis of near edge x-ray absorption spectra is much more difficult$^{[13]}$. However, it is known that the edge spectrum is sensitive to the chemical environment of the absorbing atom, including the geometric distribution of the surrounding atoms and the charge distribution. For example, there is a considerable difference in the near edge spectrum between MbCO and deoxyMb (Fig. III-8). Again we found that MbCO in PVA has the same near edge spectrum as MbCO in solution (Fig. III-8).
Figure III-7. EXAFS spectra of MbCO samples. X-ray absorption spectra (starting from 50 eV above the Fe K-edge) were measured by iron fluorescence and plotted against the photoelectron wave number k (after background removal). Solid line: 10 mM MbCO solution sample in a mylar cell, thickness 2 mm. Dashed line: 12 mM MbCO in PVA film (thickness about 50 μm); four films were used together for the EXAFS measurement.
Figure III-8. Near edge x-ray absorption spectra of myoglobin samples. The x-ray absorption coefficient was measured by iron fluorescence as a function of the incident x-ray photon energy around the iron K-edge. The MbCO samples were the same as described in Fig. III-7 (solid line and dotted line). A near edge spectrum of freshly prepared deoxymyoglobin (10 mM deoxyMb in Hepes buffer solution, thickness 2 mm) was also plotted as dot-dashed line (-----) for comparison.
III-4. Density, Water Content and Chemical Composition

It is interesting to know the physical properties and chemical compositions of our MbCO-PVA films. For this purpose, I have systematically measured the density and water content of the films. Basically, this was a gravimetric analysis in which the weight ratio of the film and the solution from which the former was made was compared to the weight fraction of total solid substance in the liquid which were used for making the film. The gravimetric method is very accurate for a film with an area about 50 cm$^2$ and a thickness about 40 μm, which weights around a few hundred milligrams. The statistical error for a set of ten measurements was less than 0.1% in all processes.

The inaccuracy in the density determination was mainly due to the nonuniformity in the thickness of the film. For more than 50 films measured for this purpose, the density of MbCO-PVA film was determined to be $(1.1 \pm 0.1)$ g · cm$^{-3}$. An average of all available data from films made for various purposes within a four year period gave a density of $(1.0 \pm 0.2)$ g · cm$^{-3}$ which agreed with the measurement made on the 50 films.

It was found that the water content of a film depends on the humidity. In dry nitrogen or vacuum, the water content of a pure film is essentially zero; on other hand, it can be up to 8% by weight, if the film was exposed to air with 90% humidity. Figure III-9 demonstrates this behavior. The weight ratio of a film and its origin solution ($W_{\text{film}}/W_{\text{solution}}$ in %) vs the Mb concentration in the film ($C_{\text{Mb}}$ in mM) for seven samples of different myoglobin concentration are
Figure III-9. The water content of MbCO-PVA films vary with humidity exposure. Seven batches of different Mb concentrations MbCO-PVA films have been studied. The changing ratios of $W_{\text{film}}/W_{\text{solution}}$ represent the change in water content in the film. We also see that freshly made films (x) contain more water than films that have been evacuated (*, after 15 min pumping). Leaving the film under vacuum for a long time (+, 10 hr; 0, 24 hr) can get rid of almost all the water in the film. The squares are evaluated ratios of solid substances and the total weight of that batch; they represent the $W_{\text{film}}/W_{\text{solution}}$ ratios of 'absolutely dry' films.
presented. At high humidity, the films weigh more than they would if they consist only of solid substances. The weight decreases if the films are placed under vacuum. Almost all the water can be extracted out of the film if it is left under vacuum for a long time (10 hrs or more).

A typical MbCO-PVA film of 12 mM Mb concentration, exposed to air of 60% humidity, has the following chemical composition Mb : PVA : Water : Hepes : Dithionite \(\approx 1.0 : 3.4 : 1.2 : 1.5 : 0.05\) by weight\(^{[14]}\). Bragg \textit{et al} \(^{[15]}\) reported that the hydration of a crystallized hemoglobin is about 0.3 (gram water per gram protein). Following this line, Urnes\(^{[16]}\) estimated the hydration of a myoglobin crystal would be around 0.2 to 0.3 gm/gm which means that there are \(3 \times 10^2\) water molecules bound with one protein. In the myoglobin solution, Urnes\(^{[16]}\) estimated the hydration to be around 0.3 to 0.4 gm/gm. For a 10 mM solution, this implies that one myoglobin would associate with \(4 \times 10^2\) water molecules along with additional \(6 \times 10^3\) free ones. In the above mentioned example of MbCO-PVA film there are \(1.2 \times 10^3\) water molecules per myoglobin molecule. Since the water content varies with the humidity of its environment, it is unlikely that the majority of hydration water molecules are tightly bound to the protein. We believe that PVA polymers provide a hydrophilic environment similar to water and indeed replace water.

Finally we made a quick study on the distribution of myoglobin in film. Preliminary experiments of small angle neutron scattering (SANS)\(^{[17]}\) and small angle x-ray scattering (SAXS)\(^{[18]}\) on film samples were performed at the National Center for Small-Angle Scattering Research (NCSASR) of the Oak Ridge National Laboratory (ORNL) which employed a 30-m SANS instrument and 10-m
SAXS camera\textsuperscript{[19]}. Our film samples showed strong scattering signals at low angles (Fig. III-11) which were definitely not due to scattering from individual myoglobin molecules as those observed in very diluted solution\textsuperscript{[20-24]}.

Microscopic inspection reveals that MbCO molecules aggregate in our film sample (Fig. III-10). The strong SAXS and SANS signals were most likely due to this aggregation. Initial analysis of SAXS and SANS data showed that the aggregation of myoglobin molecules in PVA film is not likely a fractal\textsuperscript{[25,26]}. A few SAXS and SANS data are displayed in Figs. III-11 to III-13. These figures show that there is a lot of work to be done if one wants to know how the myoglobin molecules are embedded in the PVA matrix, and how protein influences the polymer configurations.

\textsuperscript{*} I thank Prof. S. H. Chen of MIT for advice and assistance in doing neutron scattering, and Dr. J. S. Lin of ORNL for taking x-ray scattering data.
Figure III-10. Microscopic photograph of a MbCO-PVA film shows granule structure of protein distribution.
Figure III-11. Small angle neutron/x-ray scattering data of MbCO-PVA film sample. Data was taken at the National Center for Small-angle scattering Research of the Oak Ridge National Laboratory, plotted as scattering cross section vs scattering amplitude. Upper curve: x-ray scattering (not normalized); lower curve: neutron scattering (normalized), combined from two set of measurement with different Q ranges.
Figure III-12. The Guinier plot of the x-ray data in Fig. III-11 shows no linear relation between $\lg(x)$ and $Q^2$. This implies that the origin of strong scattering may be due to clustering of myoglobin or polymer molecules.
Figure III-13. Small angle scattering data with a linear fit. In the simplest theory, the linear fit would give the radius of the scatterer at the reflection point and, the slope would present the fractal dimensionality, if the scatterers are fractal. Our data has a slope larger than 3 which means that most likely the myoglobin groups as revealed by microscopy in the PVA film are not a fractal aggregation.
1 Gibson, Q. H., *J. Physiol.* 134, 112 (1956)
17 see for example, Bacon, G. E., *‘Neutron Scattering in Chemistry’*, Butterworth, London (1977)
18 see for example, Guinier, A., and Fourent, G., *‘Small-Angle Scattering of X-Rays’*, John Wiley & Sons, New York (1955)
19 *‘National Center for Small-Angle Scattering Research User’s Guide’*, by Solid
State Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee (1980)


22 Schoenborn, B. P., Nature 224, 143 (1969)


IV. EXAFS THEORY

The theory of EXAFS was established in the seventies\textsuperscript{[1-3]}. The fine structure of an x-ray absorption spectrum was interpreted as the result of the modification of photoelectron wave function at the origin due to backscattering by its neighboring atom. The following is an outline of the theory of Lee and Pendry\textsuperscript{[3]}.

IV-1. The Derivation of $\chi(k)$

In the energy range under consideration (1 keV to 20 keV) x-ray absorption is mainly due to photoelectric effects. The transition probability for the excitation of a deep core level, which can be treated by the time-dependent perturbation theory of the quantum mechanics\textsuperscript{[4]}, is give by the Golden Rule

$$w = \frac{2\pi}{\hbar} \left| \langle f | H'_{\text{I}} | i \rangle \right|^2 \delta(E_i + \hbar \omega - E_f), \quad (IV-1)$$

where $| i \rangle$ and $| f \rangle$ are initial and final states, $E_i$, $E_f$, and $\hbar \omega$ are initial, final and photon energies, and $H'_{\text{I}}$ is the interaction energy between electron and the EM field. In the dipole approximation Eq. (IV-1) becomes

$$w = \frac{2\pi}{\hbar} \left| \langle f | \varepsilon \cdot \vec{r} | i \rangle \right|^2 \delta(E_i + \hbar \omega - E_f), \quad (IV-2)$$

where $\varepsilon$ is the polarization vector of the electric field.
The initial state is the unperturbed atom whereas the final state consists of a core hole and an excited electron. If the atom were isolated then the final electron state would be an outgoing spherical wave and \( w \) is a smooth function of the photon energy above threshold. In a molecule or condensed matter the atom is surrounded by other atoms and the outgoing wave function would be diffracted by the neighboring atoms, resulting in reflected waves which modifies the final state.

Let's calculate the modification of the final state. The unperturbed final state can be described as an outgoing spherical wave centered at the origin, i.e. the position of the absorbing atom\(^5\).

\[
\psi_0(\vec{r}) = \sum_{l,m} A_{lm} h_l^{(1)}(kr) \ Y_{lm}(\Omega(\vec{r})). \tag{IV-3}
\]

Where \( h_l^{(1)} \) is the Hankel function of the first kind and \( Y_{lm} \) is spherical harmonic of \( l \) and \( m \), and \( \Omega(\vec{r}) \) is the polar angle associated with vector \( \vec{r}(\theta, \phi) \). We first expand \( \psi_0 \) in spherical harmonics about a scattering atom sitting at \( \vec{R}_j \)

\[
\psi_0(\vec{r}_j) = \sum_{l,m} B_{lm} \ Y_{lm}(\Omega(\vec{r}_j)) \ Y_{lm}(\Omega(\vec{r}_j)) \tag{IV-4}
\]

where

\[
B_{lm} = \sum_{l',m'} \sum_{l,m} A_{lm} 4\pi i^{l'-l} (-1)^{m'+m} h_l^{(1)}(kR_j) \]

\[
\times Y_{l'-m'}(\Omega(\vec{R}_j)) \int Y_{lm} Y_{lm'} Y_{l'-m'} d\Omega. \tag{IV-5}
\]

\( B_{lm} \) can be written as a vector produced by the matrix product of

\[
B = \sum_{lm} R^{lm} \ t_r(\vec{R}_j) A_{lm} \tag{IV-6}
\]

where \( R \) is a matrix

\[
[R^{lm}]_{l',m'} = 4\pi i^{l'-l} (-1)^{m'+m} \int Y_{lm} Y_{l'm'} Y_{l'-m'} d\Omega. \tag{IV-7}
\]
and $v$ is a vector
\[ v_{l'm'}(\vec{r}_j) = h^{(1)}_l(kR_j) Y_{l'm'}(\Omega(\vec{r}_j)). \]  

(IV-8)

The scattered wave $\psi_s^{(j)}(\vec{r})$ emanating from $R_j$ is obtained in terms of the transition matrix $T_{II}^{(4)}$
\[ \psi_s^{(j)}(\vec{r}) = \sum_{LM, m'} h^{(1)}_L(k|\vec{r}-\vec{r}_j|) Y_{LM}(\Omega(\vec{r}-\vec{r}_j)) T_{LM, l'm'} B_{l'm'}, \]  

(IV-9)

where $T$-matrix is given by the phase shifts
\[ T_{LM, l'm'} = \delta_{LM} \delta_{m'm} \frac{1}{2} (e^{2i\delta_L} - 1). \]  

(IV-10)

Now we can expand $\psi_s^{(j)}(\vec{r})$ about the origin by the same method used above
\[ \psi_s^{(j)}(\vec{r}) = \sum_{LM, m'} C_{LM, m'} 2 j_{l'}(kr) Y_{LM'}(\Omega(\vec{r})). \]  

(IV-11)

Note that a factor of 2 has been introduced in Eq.(IV-11), because it consists of an incoming and an outgoing part and $2j_L = h^{(1)}_L + h^{(2)}_L$. And the coefficient $C_{LM}$ can be written in terms of matrix product
\[ C_{LM, m'} = \sum_{lm} Z_{LM, m', lm} A_{lm}, \]  

(IV-12)

with
\[ Z_{LM, m', lm} = \sum_j v(-\vec{r}_j) S_{LM'LM} T_{LM, lm} v(\vec{r}_j), \]  

(IV-13)

and
\[ [S_{LM}]_{LM', LM} = 2\pi i L_{L'} (-1)^{M'+M} \int Y_{LM} Y_{LM'} Y_{L'M'} d\Omega. \]  

(IV-14)

Let's think of a core electron in the state $l_0 m_0$ making a transition to an
outgoing wave of quantum number \( l'm' \) via the dipole matrix element \( \langle l'm' | \hat{r} | l_o m_o \rangle \). The outgoing wave is scattered by the surrounding atoms resulting in an incoming wave with quantum number \( l'm \) about the original atom. The amplitude of this incoming wave is given by \( Z_{l'm, l'm} \). This incoming wave is coupled back to the core state by matrix element \( \langle l_o m_o | \hat{r} | l'm \rangle \). In addition, the photoelectron suffers a phase shift of \( e^{i\delta'} \) on its outgoing trip and a factor of \( e^{i\delta} \) on its incoming trip. This incoming wave is part of the final state of the excited electron.

We can now substitute all above expressions and their complex conjugates into the Golden Rule

\[
w = \frac{2\pi}{\hbar} \left[ \sum_{l_0 m_0} \langle l_0 m_0 | \varepsilon \cdot \hat{r} | l'm \rangle \right] \sum_{l_0 m_0} \{ \sum_{l_0 m_0} \langle l_0 m_0 | \varepsilon \cdot \hat{r} | l_o m_o \rangle \} e^{i \delta_{l', m'}} \frac{Z_{l'm, l'm} \sum_{l_0 m_0} \sum_{l_0 m_0} Z_{l_0 m_0, l'm} \sum_{l_0 m_0} \sum_{l_0 m_0} e^{i \delta_{l_0 m_0, l'm}}}{\left| \sum_{l_0 m_0} \sum_{l_0 m_0} P_{l_0 m_0, l'm} \right|^2} \delta(E_f + \hbar \omega - E_p).
\]

(IV-15)

The atomic absorption coefficient \( \mu \) is proportional to the transition probability \( w \).

Let \( \mu_o \) be the absorption coefficient for the isolated atom and \( \mu \) for the atom in a molecule. EXAFS \( \chi \) is defined as \( (\mu - \mu_o)/\mu_o \)

\[
\chi = \frac{2 \text{Re} \left[ \sum_{l_0 m_0} \sum_{l_0 m_0} P_{l_0 m_0, l'm} \sum_{l_0 m_0} \sum_{l_0 m_0} P_{l_0 m_0, l'm} \sum_{l_0 m_0} \sum_{l_0 m_0} e^{i \delta_{l_0 m_0, l'm}} \right]}{\left| \sum_{l_0 m_0} \sum_{l_0 m_0} P_{l_0 m_0, l'm} \right|^2},
\]

(IV-16)

where

\[
P_{l_0 m_0, l'm} = \langle l_0 m_0 | \varepsilon \cdot \hat{r} | l'm \rangle
\]

(IV-17)

is the dipole matrix element between the core state \( l_0 m_0 \) and the excited state \( l'm \).

For an s core level and unpolarized x-rays, it can be shown that averaging over the diagonal elements of \( Z_{1m, 1m} \) yields
\[ \chi = \frac{1}{3} \sum_m 2 \text{Re} \left[ Z_{1m,1m'} e^{i2\delta_{1}} \right]. \]  

Eq. IV-18 is the one we will use to work out the useful EXAFS formulation by using approximations.

IV-2. The Plane-Wave Approximation

First we assume that the outgoing wave is approximated as a plan wave \( \Phi_{k}(\vec{r}) \) with the same phase and amplitude at \( \vec{R}_{j} \)

\[ \Phi_{k}(\vec{r}) = h_{1}^{(1)}(kR_{j}) \ Y_{lm}(\vec{R}_{j}) \ e^{i \vec{k} \cdot (\vec{r} - \vec{R}_{j})}, \tag{IV-19} \]

where

\[ \vec{k} = k \vec{R}_{j} \tag{IV-20} \]

and \( \vec{R}_{j} \) is a unit vector in \( \vec{R}_{j} \) direction.

By using the expansion

\[ e^{i \vec{k} \cdot \vec{r}} = 4\pi \sum_{lm'} i^{l} j_{l'}(kr) \ Y_{lm'}^{*}(\vec{k}) \ Y_{lm}(\vec{r}), \tag{IV-21} \]

we obtain the scattered wave in the plan wave approximation

\[ \Phi_{s,j}(\vec{r}) = \sum_{lm} h_{l}^{(1)}(k|\vec{r} - \vec{R}_{j}|) \ Y_{lm}(\Omega(\vec{r} - \vec{R}_{j})) \frac{1}{2} (e^{2i\delta_{r}} - 1) \]

\[ \times 4\pi i^{l} Y_{lm}(\vec{R}_{j}) \ Y_{lm}^{*}(\vec{R}_{j}) \ h_{l}^{(1)}(kR_{j}) \]  

\[ \times 4\pi i^{l} Y_{lm}(\vec{R}_{j}) \ Y_{lm}^{*}(\vec{R}_{j}) \ h_{l}^{(1)}(kR_{j}) \]  

\[ \times 4\pi i^{l} Y_{lm}(\vec{R}_{j}) \ Y_{lm}^{*}(\vec{R}_{j}) \ h_{l}^{(1)}(kR_{j}) \]  

with the same phase and amplitude at the origin propagating in the direction \(-\vec{R}_{j}\)

\[ \Phi_{\vec{k}}(\vec{r}) = \Phi_{s,j}(\vec{r} = 0) \ e^{i \vec{k} \cdot \vec{r}} \]  

\[ \Phi_{\vec{k}}(\vec{r}) = \Phi_{s,j}(\vec{r} = 0) \ e^{i \vec{k} \cdot \vec{r}} \]  

Using Eq.(IV-21), \( \Phi_{\vec{k}}(\vec{r}) \) is expanded
\[ \alpha_{l-k}(\vec{r}) = \sum_{l'm'} Z_{l'rm',lm} 2j_{l'}(kr) \ Y_{l'm'}(\vec{r}), \quad (IV-24) \]

with
\[ Z_{l'rm',lm} = 2\pi i^l \ Y_{l'rm'}(-\vec{r}) \ Y_{lm}(\vec{r}) \ h_{l'}^{(1)}(kR_j) \]
\[ \times \sum_{l'} i^{l'} (2l'+1) P_{l'}(\cos\theta) \frac{1}{2}(e^{2i\delta_{l'}} - 1) \ h_{l'}^{(1)}(kR_j). \quad (IV-25) \]

**IV-3. Long Wavelength Approximation**

The second approximation we will make is that \( kR_j \gg 1 \) for all \( R_j \). This means that \( h_{l'}^{(1)}(kR_j) \) can be replaced by its asymptotic form
\[ h_{l'}^{(1)}(kR_j) = -i \frac{e^{i \left( kR_j - \pi/2 \right)}}{kR_j} \quad (IV-26) \]

and Eq. (IV-25) becomes
\[ Z_{l'rm',lm} = -i2\pi i^{l'-l} \ Y_{l'rm'}(-\vec{r}) \ Y_{lm}(\vec{r}) \frac{e^{i2kR_j}}{k^2R_j^2} \]
\[ \times \sum_{l'} (2l'+1) e^{i\delta_{l'}} \sin\delta_{l'} \ P_{l'}(\cos\theta). \quad (IV-27) \]

Since the scattering amplitude function \( f(\theta) \) is
\[ f(\theta) = \frac{1}{k} \sum_{l'} (2l'+1) e^{i\delta_l} \sin\delta_l \ P_l(\cos\theta), \quad (IV-28) \]

we have
\[ Z_{l'rm',lm} = -i2\pi i^{l'-l} \ Y_{l'rm'}(-\vec{r}) \ Y_{lm}(\vec{r}) \frac{e^{i2kR_j}}{k^2R_j^2} f(\pi). \quad (IV-29) \]
For s core states and unpolarized x-rays we use Eq. (IV-18) to obtain

$$\chi(k) = - \sum_j \frac{N_j}{kR_j^2} |f(k, \pi)| \sin[2kR_j + 2\delta'_1(k) + \psi(k)], \quad (IV-30)$$

where we let

$$f(k, \pi) = |f(\pi)| e^{i \psi(k)}. \quad (IV-31)$$

Here it is clear that $\chi$ is a function of $k$, the wave number of the excited electron. The final formula including an exponential damping term, $e^{-R_j/\lambda_j}$ due to the inelastic processes of the photoelectron, and $e^{-2\sigma_j^2 k^2}$, the Debye-Waller factor for the thermal smearing of the atomic positions, is given as

$$\chi(k) = - \sum_j \frac{N_j}{kR_j^2} |f(k, \pi)| e^{-2\sigma_j^2 k^2} e^{-R_j/\lambda_j(k)} \times \sin[2kR_j + 2\delta'_1(k) + \psi(k)], \quad (IV-32)$$

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4 see for example, Sakurai, J. J., 'Advanced Quantum Mechanics', p.39, Addison-Wesley, Reading, MA (1967)
5 see for example, Cohen-Tannoudji, C., Diu, B., and Lolee, F., 'Quantum Mechanics', Ch.VIII, John Wiley & Sons, New York (1977)
V. EXAFS DATA ANALYSIS

V-1. Introduction

The aim of EXAFS analysis is to determine the unknown quantities that appear in the Eq.(IV-32) derived in the previous section; namely,

$$\chi(k) = -\sum_j \frac{N_j}{kR_j^2} \left| f(k,\pi) \right| e^{-2\sigma_j^2k^2} e^{-R_j/\lambda_j(k)} \sin[2kR_j+\phi_j(k)], \quad (V-1)$$

where we have written

$$\phi_j(k) = 2\delta_j(k) + \psi(k) \quad (V-2)$$

as the total phase shift. The main quantities of interest are $R_j$, the average interatomic distance from the absorbing atom to atoms in the $j^{th}$ coordination shell, $N_j$, the number of such atoms and, $\sigma_j$, the root mean square variation in the distance. The other unknown quantities $f(k,\pi)$, $\lambda_j(k)$, and $\phi_j(k)$ are determined by comparison with standard compounds of known structure which have chemical properties similar to the unknown. $f(k,\pi)$, $\lambda_j(k)$, and $\phi_j(k)$ are assumed to be 'transferable' from the standard to the unknown system. Considerable evidence supports this procedure. Since Sayers et al. demonstrated that $R_j$ can be obtained by performing a Fourier transform in k-space of the EXAFS data, a method was developed which combines Fourier transforming and curve fitting. It consists of the determination of $\chi(k)$ from measured raw data, separation of the terms in Eq. (V-1) by Fourier filtering, elimination of $f(k,\pi)$, $\lambda_j(k)$, and
\( \phi_j(k) \) by comparison with the known (standard or theoretical values), and finally, extracting the information needed.

In this chapter, an overview of these steps is presented together with the analysis of our myoglobin data as an example.

V-2. Background Removal

Figure V-1 shows the iron K-edge absorption spectrum of a MbCO-PVA film sample. The modulations above the K-edge near 7 keV are EXAFS \( \chi(k) \) from several shells of neighboring atoms added together. The first step in the data analysis is to determine \( \chi(E) = (\mu - \mu_o)/\mu_o \) from the total absorption coefficient data \( \mu(E) \). Since the smooth absorption, \( \mu_o(E) \), of an isolated atom is usually unknown, in general practice\(^4\) one uses the smooth part of \( \mu(E) \) as \( \mu_o \). After removing the smooth part from \( \mu(E) \) itself, the remaining oscillatory part of \( \mu(E) \), \( \Delta \mu(E) = \mu(E) - \mu_o(E) \), is divided by the edge jump (height) to give \( \chi(E) \).

An alternative method is to use a smooth function fitted to the pre-edge part of the data then extrapolating it over the rest of the data. This second smooth fit accounts for background due to other absorption processes. The desired \( \chi(E) \) is then given by dividing \( \Delta \mu \) by the difference between the smooth part of \( \mu(E) \) and the extrapolated pre-edge fit\(^5\). I tried both methods whenever the pre-edge data were available. It seems that both methods yield almost identical EXAFS spectra.

Figure V-2 shows the procedure of background removal from the raw data presented in Fig. V-1. Since the basic equation (IV-32) was derived in the plane wave approximation, generally one fits the EXAFS formula to the portion of the spectrum whose energies are about 50 eV higher than the edge. Fig.
Figure V-1. The iron K-edge absorption spectrum of a MbCO-PVA film. Energy is plotted relative to the edge (~7113 eV). The x-ray absorption coefficient data was collected in the fluorescence mode at the liquid nitrogen temperature on the beam line VII-2, SSRL. The incident photon energy was tuned from about 7000 to 8000 eV with an intensity of 1 × 10^{11} photons/s·eV and a 2-3 eV resolution. The sample consisted of five pieces of MbCO embedded PVA dry films, each of them was about 40 μm in thickness and had 10 mM of heme concentration.
Figure V-2. Background removal. The oscillation part (EXAFS) of absorption coefficient above edge shown in Fig. V-1 was fitted by five piece cubic splines (solid line through it in the figure) with four fixed knots (denoted by small squares in the figure) where the fitted curves and their derivatives (dashed line) are forced to be continuous.
V-2 displays the EXAFS in this region along with its fitted smooth function. The latter is then subtracted from the former to yield the $\Delta \mu$, and finally it is divided by the edge jump to give $\chi(E)$. I have tried the least-square fitting with a (5 to 6th order) polynomial and the cubic spline with 3 to 4 fixed knots (all piecewise cubic polynomials are joined smoothly together by requiring their values and their first and second derivatives to be matched at the knots). I found that the latter was better than the polynomial fit.

V-3. Conversion to k-space

Since in Eq. (V-1) $\chi$ is a function of the photoelectron wave number $k$ instead of the photon energy $E$, the next step in the data analysis is to convert $E$ to $k$ according to the free particle dispersion relation

$$k = \left[ \frac{2m}{\hbar^2} (E - E_0) \right]^{1/2} \quad (V-3)$$

where $E_0$ is the threshold energy. Theoretically $E_0$ represents the threshold energy (or binding energy) for the photoexcitation. However, near the threshold the physical processes are complicated. The absorption spectra in general do not exhibit a clear edge.

Several workers have fixed $E_0$ arbitrarily at a special feature in the spectra (such as a point of inflection or the middle point of the edge)[6]. I followed the more generally accepted method of making $E_0$ an adjustable parameter[7]. The reason for doing this is not only because $E_0$ is experimentally unknown, but also because it can compensate for the inadequacies of the simplified EXAFS formula (such as plane-wave approximations).
Figure V-3a) EXAFS $\chi(k)$ vs. k. After converting $\chi(E)$ into k-space, the curve of $\chi(k)$ versus k is the experimental data which represents the theoretical equation Eq.(V-1).
Figure V-3b) \( \chi(k) \) has been multiplied by the weight factor \( k^3 \). This makes the amplitudes of EXAFS more uniform over the whole \( k \) range before Fourier transforming into \( r \)-space.
Figure V-3 shows the EXAFS spectrum derived from Fig. V-2 in the k-space. Fig. V-3a) is the direct transformation of Eq. (V-2). It is interpolated to an evenly spaced grid in k (about 0.03 Å⁻¹ in our case) for later Fourier filtering. Fig. V-3b) shows the resultant spectrum. Note that the curve has been multiplied by $k^3$. This factor cancels one power of k in Eq. (V-1) and roughly cancels the $1/k^2$ behavior of $|f_j(k,\pi)|$ at large values of k. Thus, the $k^3$ multiplication has the effect of weighing the EXAFS oscillations more uniformly over the range of the data. This step prevents the larger amplitude oscillations (in low k region) from dominating the smaller ones (in high k region) when determining the interatomic distances which depend only upon the frequency and not on the amplitude of the $\sin[2kR_j+\phi_j(k)]$ function in Eq.(V-1).

V-4. Fourier Transform Filtering

Now we have $k^3\chi(k)$ as a function in k-space which is a sum of damped sine waves $k^3\chi_j(k)$, $k^3\chi(k) = \sum k^3\chi_j(k)$. Each term corresponds to a shell of atoms. A coordination shell consists of a group of atoms that are approximately equidistant from the central photoabsorbing atom. One can isolate the contributions of the different shells and remove most of the noise by a Fourier transform filtering technique.

If we Fourier transform $k^3\chi(k)$ into r-space, we have

$$\zeta(r) = \frac{1}{\sqrt{2\pi}} \int k^3 \chi(k) e^{-i2kr} dk$$

(IV-4)

The modulus $|\zeta(r)|$ of the complex Fourier transform $\zeta(r)$ will exhibit peaks that correspond to the various coordination shells.
To show this clearly, consider the Fourier transform of the sinusoidal factor in the \( j \)th shell

\[
g_j(r) = \frac{1}{\sqrt{2\pi}} \int \sin[2kR_j + \phi_j(k)] e^{-i2kr} \, dk
\]

\[
= \frac{1}{2i\sqrt{2\pi}} \int [e^{-i(2k(r-R_j) + \phi_j(k))} - e^{-i(2k(r+R_j) + \phi_j(k))}] \, dk
\]

If \( \phi_j(k) \) is approximately a linear function in \( k \)

\[
\phi_j(k) = \phi_j(0) + k \phi_j'(0)
\]

\[
g_j(r) = e^{-\phi_j(0)} \left[ \delta(r-R_j - \phi_j'(0)) - \delta(r+R_j + \phi_j'(0)) \right]. \tag{V-5}
\]

As long as \( \phi_j(0) \) is known, we can obtain \( R_j \) from the peak position \( r = R_j + \phi_j' \).

However, since the extension of the wave number \( k \) is only within a finite range in actual data, the peaks in \( r \)-space will have finite widths. To achieve good resolution in \( r \)-space, one must expand the data points by adding zeros. The second effect of finite range in the Fourier transform is that the peak may also show considerable structure due to the sharp truncation at the data boundaries. It is desirable to taper \( k^3 \chi(k) \) so that it goes smoothly to zero at the data boundaries. This is usually done by multiplying \( k^3 \chi(k) \) by a 'window function', which is constant in the middle of the data range and falls smoothly to zero at the data boundaries. One must carefully choose the window function so that one can effectively eliminate the side ribs of the transformed peak without producing gross distortions in the data.

My program used the Hanning window\[8\]
\[ W(k) = \begin{cases} 
\frac{1}{2} \left[ 1 + \cos((k-k_{\min}^-\Delta)\pi/\Delta) \right], & k_{\min} \leq k \leq k_{\min} + \Delta \\
1, & k_{\min} + \Delta < k < k_{\max} - \Delta \\
\frac{1}{2} \left[ 1 + \cos((k-k_{\min}^-\Delta)\pi/\Delta) \right], & k_{\max} - \Delta \leq k \leq k_{\max} \\
0, & \text{otherwise} 
\end{cases} \] (V-6)

where \( \Delta \) is a parameter which can be adjusted to modify the data boundaries.

For a grid spacing of about 0.03 \( \text{\AA}^{-1} \), I transformed the \( 2^{15} \) (0 to about 1000 \( \text{\AA}^{-1} \)) k-space data points into r-space with a resolution of \( r \approx 0.003 \text{\AA} \) by a fast Fourier transform algorithm\[^9\]. The CPU time usage is less than 1 minute for double precision data on a VAX11/750 computer\[^*\]. Figure V-4 shows the Fourier transform from the data represented in Fig. V-3b. The large peak around 1.7 \( \text{\AA} \) is the contribution from the first shell (the six nearest neighbors of the heme iron) atoms. Other smaller peaks visible above the noise correspond to more distant neighbors.

One can isolate the \( j^{th} \) shell contribution by applying Fourier filtering techniques again. Inverse Fourier transformation of the transformed data from r-space to k-space with a window which excludes all peaks except the \( j^{th} \) one will separate \( \chi_j \) from other peaks

\[ \chi_j(k) = \frac{1}{\sqrt{2\pi}} \int W_j(r) \zeta(r) e^{ikr} \, dr. \] (V-7)

This is then the EXAFS signal of the \( j^{th} \) shell. However, one has to keep in mind that some distortion is unavoidable in the filtering process. Figure V-5 shows the first shell EXAFS \( k^2\chi_1(k) \) of MbCO-PVA film isolated from Fig. V-4.

\[^*\] I thank Dr. G. Bunker of ISFS for kindly providing the EXAFS analysis software developed at University of Washington so I could compare the results obtained from two programs and eliminate many possible errors in developing my own procedure.
Figure V-4. Fourier transform of Fig. V-3b). A Fourier transform translates χ(k) data from k-space to r-space, the large peaks above noises are contributed by the neighboring atoms of the center absorber iron atom. The distance numbered on the abscissa presents the interatomic distance R_j plus the total phase shifts φ_j(k) of the jth shell.
Figure V-5. First shell of MbCO iron K-edge EXAFS. It was separated out by inverse Fourier filtering of the first peak in Fig. V-4 where the window used in the filtering process is shown as the dashed line around the peak. The resultant sinusoidal curve and its envelope are the sine factor $\sin \Phi(k)$ and the amplitude factor $A(k)$ of the first term in Eq. (V-1).
Figure V-6. The total phase function $\Phi_1(k)$ extracted from the sine factor of the first shell shown on Fig. V-5. The interatomic distance can then be obtained.
In Eq. (V-7) if the window function $W_j(r)$ is chosen to select only the positive $r$ values, one obtains a complex $\hat{\chi}$

$$\hat{\chi}_j(k) = \frac{1}{2i} A_j(k) \, e^{i \Phi_j(k)}.$$  \hfill (V-8)

The $j^{th}$ shell EXAFS is then given by

$$\chi_j(k) = A_j(k) \, \sin \Phi_j(k).$$  \hfill (V-9)

Here the total phase $\Phi_j(k)$ is written in place of $2kR_j + \phi_j(k)$ and $A_j(k)$ is the amplitude function of the $j^{th}$ shell which is due all factors in the $j^{th}$ term of Eq. (V-1) except the sine factor.

It is then a simple matter to extract $A_j(k)$ and $\Phi_j(k)$ from $\hat{\chi}_j(k)$ as

$$A_j(k) = 2|\hat{\chi}_j(k)|,$$  \hfill (V-10)

$$\Phi_j(k) = \arg \left| \hat{\chi}_j(k) \right| + \frac{\pi}{2},$$  \hfill (V-11)

The amplitude function of the first shell of our example was already shown in Fig. V-5. The total phase extracted from Eq. (V-11) is plotted in Fig. V-6 and it looks like a straight line because $2kR_j$ is much larger than $\phi_1(k)$.

V-5. Amplitude and Phase Functions

After decomposing the contribution of the $j^{th}$ shell into its corresponding amplitude and phase function, it depends on the system as to what kind of structural information one can obtain from Eqs. (V-10), (V-11). For a simple system, a shell represents same kind of atoms with equal distance from the center absorbing atom. Then the coordination radius $R_j$ can be extracted without any
ambiguity as long as the phase shifts $\phi_j(k) = 2\delta_j(k) + \psi_j(k)$ are known. This can be done in two ways: either by using a structurally known sample which is similar to the one being investigated or using theoretical values$^{[10-12]}$ as the reference to obtain the total phase function $\Phi^S_j(k) = 2kR^S_j + \phi^S_j(k)$. The phase difference between the unknown and the reference samples is then given by

$$\Phi^X(k) - \Phi^S(k) = \phi^X(k) - \phi^S(k) + 2k(R^X - R^S), \quad (V-12)$$

(here the subscript $j$ has been omitted for simplicity). If the chemical transferability of the phase shifts holds exactly, then the intercept of $\Phi^X(k) - \Phi^S(k)$ vs $k$ equals zero

$$\Phi^X(k) - \Phi^S(k) = 2k(R^X - R^S) \quad (V-13)$$

and the interatomic distance $R^X$ can be extracted. In practice, one makes $E_0$ of the unknown sample as an adjustable parameter so that the linear fit straight line of the phase difference $\Phi^X(k) - \Phi^S(k)$ vs $k$ goes through the origin of the plot and extracts $R^X$ under this condition (Fig. V-7). For a complex system such as MbCO a coordination shell contains more than one atomic distance. Consequently extracting the interatomic distances is not an easy job. We will discuss it later.

The amplitude function also contains information. From Eq.(V-1), the amplitude function of the $j^{th}$ shell is

$$A_j(k) = \frac{N_j}{kR^2_j} \left| f(k,z) \right| e^{-2\sigma_j^2k^2} e^{-R_j/\lambda_j(k)}. \quad (V-14)$$

If one investigates it by the same strategy of comparing it with a known reference, the logarithm of their difference

$$\ln \left[ \frac{A_x(k)}{A_s(k)} \right] = \ln \left[ \frac{N_x}{N_s} \frac{R_x}{R_s} \right] + 2 \left[ \frac{R_s}{\lambda_s} - \frac{R_x}{\lambda_x} \right] + 2k^2(\sigma_s^2 - \sigma_x^2) \quad (V-15)$$
then is a linear function of $k^2$ with an intercept and slope, given by

$$a = \ln \left[ \frac{N_x R_s}{N_s R_x} \right] + 2 \left[ \frac{R_s}{\lambda_s} - \frac{R_x}{\lambda_x} \right],$$

$$b = 2(\sigma_s^2 + \sigma_x^2),$$ (V-16)

provided $\lambda(k)$'s are approximately constant in the energy range under consideration. Usually people extract the coordinate number $N_j$ and the Debye-Waller factor $\sigma_j$, which are of interesting in disordered system. In the case of myoglobin, the composition around the heme is known. Therefore my main objective was to obtain the the Debye-Waller factor, $\sigma_j$.

V-6. Decomposition of a Mixed Shell

The iron atom in myoglobin has six coordinated atoms in the first shell. Four porphyrin nitrogen atoms are approximately equidistant from Fe. The fifth nitrogen on the proximal imidazole has a longer distance from iron atom. These distances may change during photoexcitation. The sixth distance is to the carbon of CO; this distance is of course a variable in photolysis. My goal is to determine the average Fe-$N_{\text{porphyrin}}$ distance by using the Fe-C and Fe-$N_{\text{imidazole}}$ distances from the crystallographical data. First, I used an iron (III) derivative of tetraphenylporphine, $\alpha,\beta,\gamma,\delta$-tetraphenylporphine iron chloride (ClFeTPP), as a reference sample. The average distance of the iron atom to its porphyrin nitrogen neighbors was known from x-ray crystallography to be 2.049 Å$^{[13]}$. Its x-ray absorption spectrum was taken and treated exactly as described above so its first shell phase $\Phi_1^S(k)$ was obtained.
Figure V-7. Extract $R_1$ from the total phase function. The phase differences between the MbCO sample and the known model C1FeTPP were plotted against $k$ (solid line) which intercepts the ordinate at 0.21 rad (Fig. V-7a). It would be zero only if $E_0$ is lowered by 1.8 eV (Fig. V-7b). In this condition, the interatomic distance $R_1$ is determined by the slope of the fitted line.
In analyzing the EXAFS data of a biological system in which an absorber atom usually has two or three very closely positioned neighbors and all of which contribute to the first peak of the Fourier transform in the r-space, one usually faces a problem in distinguishing the distances to each of the different atoms. Different methods have been proposed to solve this problem. Martens et al.\textsuperscript{[14]} suggested that if two R's are very close together in the first shell then the beats of the amplitude function can be decomposed. I found this to be a difficult method. I could not even properly determine the two distance in the first shell of an Fe\textsubscript{2}O\textsubscript{3} sample by this method. Perutz et al.\textsuperscript{[15]} have investigated deoxyhemoglobin by fitting models to the EXAFS by varying the contributions of all shells; thereby, they obtained interatomic distances from the best fit, to both EXAFS and its Fourier transform. This is a good method; however, it requires the precise knowledge of theoretical amplitude, phase shifts, and the Debye-Waller factor. Furthermore, considering that there are more than ten parameters that have to be varied in the fitting process, I wonder if the fits were unique. In the following treatment, I used a phase correction method proposed by Eisenberger et al.\textsuperscript{[16]}.

From general equation (V-1), one can write the EXAFS specifically for the first shell atoms as

\[
\begin{align*}
  k^3 \chi(k) &= -\frac{4}{kR_1^2} k^3 |f^N(k,\pi)| \ e^{R_1/\lambda} e^{-2\sigma_1^2 k^2} \sin[2R_1 k + \phi^N(k)] \\
  &\quad - \frac{1}{kR_2^2} k^3 |f^N(k,\pi)| \ e^{R_2/\lambda} e^{-2\sigma_2^2 k^2} \sin[2R_2 k + \phi^N(k)] \\
  &\quad - \frac{1}{kR_3^2} k^3 |f^C(k,\pi)| \ e^{R_3/\lambda} e^{-2\sigma_3^2 k^2} \sin[2R_3 k + \phi^C(k)] \\
  &= \sum_j a_j(k) \sin\phi_j(k)
\end{align*}

\text{(V-17)}
More conveniently, one can write Eq. (V-17) in exponential expression which physically means that one includes the negative frequencies

\[ k^3 \chi(k) = \sum_j a_j(k) e^{i \phi_j(k)} \]
\[ = A(k) e^{i \phi(k)} \]

or explicitly,

\[ k^3 \chi(k) = a_1 e^{i \phi_1} + a_2 e^{i \phi_2} + a_3 e^{i \phi_3} \]
\[ = a_1 e^{i \phi_1} \left[ 1 + \frac{a_2}{a_1} e^{i (\phi_2 - \phi_1)} + \frac{a_3}{a_1} e^{i (\phi_3 - \phi_1)} \right] \]
\[ \equiv a_1 a_c e^{i (\phi_1 + \phi_c)} \quad (V-18) \]

Where \( a_c e^{i \phi_c} \) is defined as,

\[ a_c e^{i \phi_c} \equiv 1 + \frac{a_2}{a_1} e^{i (\phi_2 - \phi_1)} + \frac{a_3}{a_1} e^{i (\phi_3 - \phi_1)} \]

Thus,

\[ \phi_c = \arctan \left\{ \frac{\frac{a_2}{a_1} \sin[2k(\phi_2 - \phi_1)] + \frac{a_3}{a_1} \sin(\phi_3 - \phi_1)}{1 + \frac{a_2}{a_1} \cos[2k(\phi_2 - \phi_1)] + \frac{a_3}{a_1} \cos(\phi_3 - \phi_1)} \right\}. \quad (V-19) \]

If one assumes that \( \lambda_1 = \lambda_2 = \lambda_3, \sigma_1 = \sigma_2 = \sigma_3, \) and \( f(k, \pi) = Z/k \) (for \( k > 4 \).
\( \lambda^{-1} \)), then

\[ \frac{R_1^2}{4R_2^2} \sin[2k(R_2 - R_1)] + \frac{6R_1^2}{28R_3^2} \sin[2k(R_3 - R_1) + \phi_c - \phi_N] \]
\[ \phi_c = \arctan \left\{ \frac{\frac{R_1^2}{4R_2^2} \cos[2k(R_2 - R_1)] + \frac{6R_1^2}{28R_3^2} \cos[2k(R_3 - R_1) + \phi_c - \phi_N]}{1 + \frac{R_1^2}{4R_2^2} \cos[2k(R_2 - R_1)] + \frac{6R_1^2}{28R_3^2} \cos[2k(R_3 - R_1) + \phi_c - \phi_N]} \right\}. \quad (V-20) \]
Using axial distances $R_2$ and $R_3$ from x-ray diffraction data (Table V-1) and theoretical values for $\Phi^C(k)$, $\Phi^N(k)$, one can generate $\Phi_c(k)$ for each measurement after assuming a value for $R_1$. This $\Phi_c(k)$ is subtracted from the total phase $\Phi_1(k)$ which was previously determined by Fourier filtering of the first shell (Eq. V-11). One then obtains the real total phase contributed by $N_{\mathrm{porphyrin}}$ only

$$\Phi_1^N(k) = \Phi_1(k) - \Phi_c(k)$$

$$= 2kR_1 + \phi_1^N(k) + \frac{\pi}{2}, \quad (V-21)$$

from which $R_1$, the Fe-$N_{\mathrm{porphyrin}}$ distance, can be determined since now $\phi_1^N(k)$ is the phase shifts in Eq. (V-1) due to Fe and N atoms only. The mixed shell problem has been reduced to the determination of a single distance. One can then apply the previously described analysis to $\Phi_1^N(k)$. This was done for various values of $R_1$ until self-consistency was reached. Figure V-8 shows the flow chart of this procedure.

V-7 Results and Discussion

Following the method described above, I analyzed the Fe K-edge EXAFS data of deoxymyoglobin and carboxymyoglobin aqueous solution and film samples taken at various times on different synchrotron beam lines. Figures V-9 to V-12 are the iron K-edge EXAFS of buffered solution MbCO sample taken by the conventional counting method. It is interesting to compare the measuring time per data point shown in Fig. V-1 and V-9. Fig. V-1, measured with our own
Figure V-8. Flow chart of data analysis
spectrometer at SSRL, with a 100 μs measuring interval and $10^4$ iterations resulted in 1 s per point data throughout the whole spectrum. In Fig.V-9, measured at X21A NSLS with a four NaI detector array, the spectrum was divided into five regions, having 1, 0.5, 1, 2, 3 seconds per data point which translated into 1 to 3 s per data point for the various EXAFS regions. Furthermore, the spectra presented in Fig.V-9 was the result of the average of nine scans. One can see clearly from Figs. V-3b and V-10 that the quality of data between two measurement are about the same. Two factors are different between the SSRL and the NSLS measurements. One is that the beam intensity at SSRL is about a factor of 5 higher than at NSLS. The second and more important factor is that we used our own integration method at SSRL but used a conventional counting method at NSLS. Considering all these facts, we can conclude that an equal or better quality x-ray absorption spectrum can be obtained by our spectrometer with about an order of magnitude higher time efficiency than by the conventional method.

A summary of measured interatomic distances is listed in Table V-1 along with data from other authors for comparison.

In order to assess the accuracy of our analyses, systematic errors must be included in the final error estimate. This includes the effects of the different data analysis procedures used, as well as non-EXAFS background modulation due to multiple excitations and the presence of other absorption edges, etc. All these led to a total error estimate of 0.01 Å and 0.03 Å for the first and second shell respectively in our results. Artificial data of MbCO with 4N, 1N, 1C, 8C neighboring atoms including reasonable damping factors, random noise and glitches were used to test our analysis program. This test confirmed the validity of the total error estimate given above. For this reason I will only discuss the first-shell
Figure V-9. The iron K-edge absorption spectrum of a MbCO solution sample.

The x-ray absorption coefficient data was collected in the fluorescence mode at room temperature at beam line X-21A, NSLS. The incident photon intensity was about $2 \times 10^{10}$ photons / s-eV. The sample was confined in mylar films with about 2 mm in thickness and had 10 mM of heme concentration. The spectrometer was an array of 4 NaI scintillation detectors operating in the counting mode. Although the counting rates were controlled to be less than $5 \times 10^4$ s$^{-1}$ the spectrum already appeared saturated.
Figure V-10. EXAFS $k^3 \chi(k)$ vs k of Fig. V-9 after background removal and converting to k-space. Compared with Fig. V-3b), one can see that the EXAFS spectra of the MbCO solution and the film are essentially the same.
Figure V-11. Fourier transform of Fig. V-10.
Figure V-12. First shell of MbCO iron K-edge EXAFS obtained from Fig. V-11 by inverse Fourier filtering. The resultant $\Phi_1(k)$ has been used as an example to extract $R_1$ by comparing it with a model compound (see Fig. V-7).
### TABLE V-1. INTERATOMIC DISTANCE OF Mb AND Hb\textsuperscript{a}

<table>
<thead>
<tr>
<th>Sample</th>
<th>$R_1$</th>
<th>$R_2$</th>
<th>$R_3$</th>
<th>$\Delta \sigma^2 \times 10^3$</th>
<th>Method, Material &amp; Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>MbCO</td>
<td>2.00(1)</td>
<td></td>
<td>3.01(3)</td>
<td>0.6</td>
<td>E, solution, 300K</td>
</tr>
<tr>
<td>MbCO</td>
<td>2.00(1)</td>
<td></td>
<td>3.00(3)</td>
<td>-1.0</td>
<td>E, film, 300K</td>
</tr>
<tr>
<td>MbCO</td>
<td>2.00(1)</td>
<td></td>
<td>2.98(3)</td>
<td>-3.7</td>
<td>E, film, 4K</td>
</tr>
<tr>
<td>deoxyMb</td>
<td>2.06(1)</td>
<td></td>
<td></td>
<td>2.3</td>
<td>E, solution, 300K</td>
</tr>
<tr>
<td>MbCO</td>
<td>1.97</td>
<td>2.2</td>
<td>1.9</td>
<td></td>
<td>D, Kuriyan\textsuperscript{[17]}</td>
</tr>
<tr>
<td>MbCO</td>
<td>2.01</td>
<td>2.20</td>
<td>1.93</td>
<td></td>
<td>E, Powers\textsuperscript{[18]}</td>
</tr>
<tr>
<td>MbO\textsubscript{2}</td>
<td>1.95(6)</td>
<td>2.07(6)</td>
<td>1.83(6)</td>
<td></td>
<td>D, Philips\textsuperscript{[19]}</td>
</tr>
<tr>
<td>Met-Mb</td>
<td>2.04</td>
<td>2.13</td>
<td>2.00</td>
<td></td>
<td>D, Takano\textsuperscript{[20]}</td>
</tr>
<tr>
<td>deoxy-Mb</td>
<td>2.06</td>
<td>2.1</td>
<td></td>
<td></td>
<td>D, Takano\textsuperscript{[21]}</td>
</tr>
<tr>
<td>HbO\textsubscript{2}</td>
<td>1.986(10)</td>
<td></td>
<td></td>
<td></td>
<td>E, Eisenberger\textsuperscript{[16]}</td>
</tr>
<tr>
<td>deoxy-Hb</td>
<td>2.055(2)</td>
<td></td>
<td></td>
<td></td>
<td>E, Eisenberger\textsuperscript{[16]}</td>
</tr>
<tr>
<td>deoxy-Hb</td>
<td>2.06(1)</td>
<td></td>
<td></td>
<td>3.01</td>
<td>E, Perutz\textsuperscript{[15]}</td>
</tr>
<tr>
<td>deoxy-Hb</td>
<td>2.06(2)</td>
<td>2.12(4)</td>
<td></td>
<td></td>
<td>D, Fermi\textsuperscript{[22]}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} All myoglobins are from sperm whale, hemoglobins from human blood.

\textsuperscript{b} Bond lengths are in the units of Å, the digits in parentheses are experiment errors.

\textsuperscript{c} Debye-Waller factor is given in difference from the reference ClFeTPP sample.

**Method:**

D-- diffraction; E -- EXAFS
distance in the following.

Comparing our results with the x-ray diffraction results, we find that they are in good agreement with deoxyMb but not with MbCO. The discrepancy was probably due to the Fe-N_{imidazole} and Fe-C distances I used\textsuperscript{[17]} for phase correction, (R_{2} and R_{3} of diffraction data), which have only two digits, and the final error estimation of x-ray diffraction data were not given. As for the EXAFS results of other authors\textsuperscript{[18]}, we will discuss them later (see Ch. VII).

EXAFS are best used for comparing similar samples. The variation of the Fe-N_{porphyrin} distance for different binding states of iron verified the well known hypothesis of the iron movement in the heme. The resemblance of EXAFS spectra and the agreement of Fe-N_{porphyrin} distance between aqueous samples and film samples proves that the local structure around the iron atom in the heme is not affected by embedding the myoglobin molecule into the poly(vinyl alcohol) matrix. This gives us the confidence for using the film samples in time resolved EXAFS experiments.

\begin{itemize}
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\end{itemize}
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16 Eisenberger, P., Shulman, R. G., Kincaid, B. M., Brown, G. S., and Ogawa, S.,
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VI. TIME RESOLVED EXAFS OF PHOTODISSOCIATED MbCO

As mentioned in the Introduction the goal of this thesis was to determine the heme structures of photo-excited MbCO. To this end our laboratory constructed a time-resolved EXAFS spectrometer which was described in detail in two papers\textsuperscript{[1,2]} and in my Masters' degree thesis\textsuperscript{[3]}. The instrumental time-resolution of this spectrometer is about 100 \(\mu\)s. However the absorption spectra of dynamic systems have to be measured point by point in energy. In other words, the sample has to be excited for each energy point. Since an absorption spectrum typically consists of 100 points or more and each energy point has to be repeated in order to obtain good photon statistics, this is a time-consuming procedure. Nevertheless this is the only workable method of measuring time-resolved EXAFS of dilute biological samples. The often-mentioned (but so far unsuccessful) energy dispersion method, which measures the entire spectrum simultaneously, can only be performed in the transmission mode and therefore is not applicable to most biological samples. How much beamtime is required of course depends on the beam intensity. At the time we planned this experiment (1982) SSRL proclaimed a beam intensity of \(10^{12}\) photons/s-eV and was projecting an intensity increase to \(10^{14}\) photons/s-eV in a couple of years. At \(10^{12}\) photons/s-eV, we figured that a 100-\(\mu\)s-resolved EXAFS of photolyzed MbCO at 80 K (recombination time \(\sim\) 1 s) would take 7 to 10 days to accomplish. Unfortunately, it turned out that \(10^{12}\) photons/s-eV was the optimal condition. Most of the time the beam intensity available to us was about \(10^{10}\) photons/s-eV. To make the matter worse the completion of NSLS was repeatedly
delayed. That meant that even the low intensity beamtime was hard to get for the last several years. For this reason we were forced to scale down our original goal and measured only at two temperatures, one at 4 K where the recombination time of photolyzed MbCO is infinite and one at 40 K where a 10-s-resolved measurement is meaningful. The 40 K measurement is the first time-resolved EXAFS of any kind.

VI-1. Set-up of Time Resolved EXAFS Experiment

The basic idea of time resolved EXAFS is to measure an EXAFS point (at a given x-ray energy) over a short time interval \( \tau \) (time resolution) at a time \( t \) measured from the instant when a process began in the sample. In order to do this, one needs a standard EXAFS facility, a time-resolved data acquisition system, and most importantly an excitable sample. Figure VI-1 shows a schematic diagram of our experimental set-up. The synchrotron radiation is filtered by a monochromator before it is incident on the sample. The incident monochromatic beam \( I_0 \) and the transmitted \( I_t \) or fluorescence \( I_f \) beam are measured as a function of incident photon energy \( E \) to yield the absorption coefficient \( \mu(E) \). The data acquisition system controls the time \( t \) and the time interval \( \tau \). A specially designed cryostat serving as the sample holder provides temperature control (4 - 300 K), flash (for photolysis) as well as on line optical absorption monitoring (see Sec.III-2).

In our experiment ionization chamber detectors were used to monitor \( I_0 \) and \( I_t \), and a NaI scintillation detector was used to monitor \( I_f \). Figure VI-2 shows the setup with the cryostat in the experimental hutch along with the detectors.
Figure VI-1. Block diagram of time-resolved EXAFS experiment. A standard x-ray monochromator filters incident synchrotron radiation. Specially designed cryostat holds the photolyzed sample at the desired temperature. The data acquisition system controls measuring time.
Figure VI-2. Cryostat in a synchrotron radiation experimental hutch along with detectors. The picture was taken at NSLS beamline X-23B.
Figure VI-3. Schematic diagram of sample holder. The incident and fluorescent x-rays are 45° with respect to film samples. Xenon flash light is focused at the sample by a 'cold mirror' (not shown). The monitoring light guided by optical fibers is perpendicular to the sample and through two small quartz windows.
VI-2. Time Resolution and Sample Temperature

The choice of time resolution depended on the nature of the dynamic process of interest. In our case, we were interested in the relaxation process of the photolyzed MbCO; the relaxation time can be controlled by temperature. It varies from infinite at 4 K to microseconds at room temperature. Therefore the metastable photolyzed state Mb* can be studied with stationary (time-independent) EXAFS at 4 K. The time-resolved experiment is much more difficult. Because the sample has to be photolyzed repeatedly, each process of photolysis has to be identical; not only must the photolysis be 100%, but the sample must be 100% relaxed before each photolysis also.

It was learned from the extensive study of photolysis kinetics of MbCO that in practice a photolyzed sample has to be raised above 120 K in order for it to completely return to the ground state. Since above 120 K the relaxation time (< 1 s) is too short for time-resolved EXAFS measurement, we are forced to chose T below, say, 80 K. However, raising the cryostat to above 120 K and cooling it down again is a time-consuming process. We decided that we could not afford to make repeated measurements at each energy point. Examining the recombination curves of photolyzed MbCO at various temperature, we found that at 40 K photolyzed MbCO remains 60% unbound for about 10 s. With a 10 s integration time, we could obtain a good quality spectrum with a beam of $10^{10}$ photons/s-ev without iterations. Thus 40 K was chosen for our time-resolved EXAFS experiment.
VI-3. Flash and Temperature Control

Although we have shown that our film samples of MbCO have the desirable quality of being 100% photolyzable, it is quite another matter to maintain the sample control in the cryostat. In other words, we have to prove that under the condition of the x-ray experiment the sample is indeed photolyzed and the temperature of the sample is not changed significantly by flash.

This problem was handled by carefully designing and, after months of painstaking testings, redesigning the cryostat. To appreciate the complexity of this problem, one has to realize that, to reach a low temperature, a cryostat is basically a small vacuum chamber with an interior radiation shield. In the meantime, it is connected to a liquid helium transfer line (which extends into a cold finger) and provides three x-ray windows (for incidence, transmission and fluorescence), one flash window and two optical windows for light absorption monitor. The geometry of this arrangement is shown in Fig. VI-3. The major components of the finished product are:

1. SuperTran (liquid helium transfer line) by Janis Research Company, (Wilmington, MA), with a temperature range from 4 K to 300 K at the cold finger. (Refrigeration capacity > 2 watts at 4 K, cool down time ~ 15 min from 300 K to 4 K, liquid consumption ~ 0.7 l/hr at 4 K.) The temperature is controlled by a DRC-81C controller with a DT-500DRC-D silicon diode sensor (Lake Shore Cryotronics, Inc., Westerville, OH).

2. A xenon flash tube (FXQ-260-3, EG&E Electro-Optics, Salem, MA) capable of delivering 200 J with 85 μs pulse width. I built the power supply for
the tube. Its characteristics are \( V \leq 2 \text{ kV}, \ C = 126 \ \mu\text{f} \) with a 12 kV flash trigger pulse (initiated by a TTL signal from the data acquisition system with a delay time less than 10 \( \mu\text{s} \)).

3. The flash light was focused toward the sample by a 'cold mirror' (#61.4200, Rolyн Optics Company, Covina, CA), which transmits red and longer wavelength light but reflects light of wavelength shorter than 650 nm. The flash window further filters this light to a narrow band between 450 nm and 650 nm. This is achieved by a short path filter ('Hot mirror' #60.5200, Rolyн Optics Company) on the vacuum shroud and a long path filter (yellow glass filter #5149, Oriel Corporation, Straford, CT) on the radiation shroud. This combination of two filters allow 80% of 550 nm light to pass through. This optical arrangement was designed to utilize the absorption peaks around 550 nm (Fig. III-2) for efficient photolysis but block out the other wavelengths to avoid unnecessary heating of the sample. The effect of a single flash on the sample temperature was tested. It was found that the temperature would rise from a few degrees for \( T < 20 \text{ K} \) to less than one degree for \( T > 100 \text{ K} \) and last for a few seconds after a flash of 200 J (pulse width 85 \( \mu\text{s} \)). If the flash was repeated less than one flash per second, the temperature change would not exceed 10 degrees.

4. The monitoring light for detecting the degree of recombination occurring in the photolyzed sample is introduced through a small optical window with a fiber optical illuminator. The incident light was directed perpendicular to the film sample. The transmitted light guided by another fiber optical cable was filtered and delivered to a photomultiplier. The photodetector was recorded with a waveform digitizer for time-resolved recording (also see Sec.III-2 and Fig.III-3).
VI-4. EXAFS Measurement

VI-4-1. EXAFS of MbCO and Mb

Static EXAFS spectra of carboxymyoglobin and deoxymyoglobin samples in buffered solutions and in PVA films (10 mM for liquid samples and 12-15 mM for film samples) at different temperatures (from room temperature to 4 K) were taken at several synchrotron beamlines (SSRL VII-2, NSLS X-21A and X-23B). The data acquisition systems used for static EXAFS were either our own spectrometer converted for static runs or the standard equipment supplied with the beamlines. Iron K-edge EXAFS of a high spin ferric iron derivative of tetraphenylporphine[^4], α,β,γ,δ-tetraphenylporphine iron chloride (ClFeTPP) (Aldrich Chemical Company, Milwaukee, WI), was taken for the purpose of computing the phase shift function ϕ (see Sec. V-5). The static EXAFS of Mb and MbCO are important references for the photolyzed states.

VI-4-2. X-ray Absorption Spectrum of Mb* at 4 K

The x-ray absorption experiment of photodissociated carboxymyoglobin was performed by using a combination of our cryostat-photolysis equipment and the standard EXAFS facility. Two 12 mM MbCO-PVA films facing both the incident x-ray and the fluorescence detector at 45° angle were mounted on the cold finger of the cryostat. The sample temperature was kept at 4 K during the entire process. The EXAFS spectrum was taken after flash. The optical absorption monitor indicated that the photolyzed state, Mb*, had the same optical spectrum as Mb between 400 nm and 700 nm and this condition remained stationary indefinitely as long as the
sample was kept at 4 K (see Sec. III-2). The x-ray absorption measuring time per point was adjusted according to energy regions. A complete scan from 100 eV below the Fe K-edge to 800 eV above the edge was usually divided into 4 regions. They were 1, 2, 2, and 4 s/poin for pre-edge (-100 eV to -10 eV), edge (-10 eV to 50 eV), 50 eV to 250 eV, and 250 eV to 800 eV regions, respectively. A single complete scan took about 20 min. With an incident beam intensity of about $5 \times 10^{10}$ photon/s-eV, a good quality spectrum could be obtained by an accumulation of 5 scans averaged together. This is consistent with the estimate of the photon statistics stated in Sec. I-2 when the absorption of the mylar windows was taken into account. There were three x-ray windows on the cryostat. Their thicknesses were 2 mils for the incident and fluorescence x-rays, and 4 mils for the transmission.

To test the completeness of photolysis of MbCO, two kinds of scans were taken: scans right after one flash and, scans after multiple flashes. A set of short (near edge) scans with different numbers of flashes were also taken with long sampling time thus eliminating the need of iteration.

Although we knew (from the transient optical study and the x-ray data taken) that the frozen excited state can be kept for a long time, we did not keep taking data on a photolyzed system very long. For the one flash data, we always brought the sample temperature up to 120 K and then lowered it to 4 K in complete darkness before photolysis.

**VI-4-3. 10 s-Resolved EXAFS at 40 K**

The 10 s-resolved EXAFS of photolyzed MbCO at 40 K was performed in September 1986 at NSLS-X-23B. The experimental setup was almost identical to the setup used for the static Mb+ spectrum described in §VI-4-2 except the data
acquisition system was switched to our time resolved spectrometer. The data taking process was as follows: i) the monochromator was moved to the desired incident photon energy; ii) the sample temperature was lowered from 120 K to 40 K in complete darkness; iii) two 10 s-measurements were taken, one before photolysis (ground state), one after flash with 1 ms delay to avoid the effect of flash on electronics; iv) the sample temperature was raised to 120 K in order to let the sample relax before the next point. In this procedure two spectra at 40 K (the ground and the excited states) were taken back to back for the purpose of comparison. Also, raising the temperature to 120 K each time before data taking avoided any possible accumulation of unbound species in the sample. Each up-down temperature cycle required 12 to 15 minutes. The complete scan from 100 eV below the K-edge to 800 eV above the edge took approximately 24 working hours to accomplish.

VI-5. Radiation Damage and Sample Replacement

Although we showed that our film samples had better resistance to radiation damage (§III-1c) compare to solution samples, it was still a good practice to change the sample often. At room temperature we replaced the sample after two or three scans; at low temperature the risk of radiation damage is lower; therefore a film sample might be allowed to run for more than six scans. One common problem of many synchrotron radiation beamlines is that the operating computer often runs out of phase with the monochromator. Therefore, an iron foil was positioned behind the MbCO sample and the transmission EXAFS was used as an edge reference for later use during data reduction.
Replacing film samples in the cryostat was easier than replacing solution samples. We simply raised the temperature, dismounted the old film samples and replaced them with the new ones. We did not have to worry about degassing, bubble formation, and so on as, as in the case of solution samples.

VII. RESULTS AND DISCUSSIONS

VII-1. The Completeness of Photolysis

The first question concerning the experiment of the excited state at 4 K is whether all the MbCO molecules have been photodissociated. The transient optical absorption studies (Sec. III-2) performed in our lab showed that our film sample can indeed be 100% photolyzed by a single shot of a 100 joule (with a 60 µs pulse width) xenon flash. Neither increasing the energy up to 200 J with a 85 µs pulse width nor repeating the flashes altered the absorbance of the excited sample. A simple calculation (Eq. III-6) indicated that N(t) = 1, i.e., 100% photodissociation was achieved.

The same situation can be seen in the x-ray absorption studies. Figure VII-1 shows that photolysis alters the near edge spectrum of MbCO. Figure VII-2 shows the near edge spectra taken after single flash and after multi-flashes. They are essentially the same, proving that one flash is sufficient for the maximum effect of photolysis. This gives us the confidence in performing 40 K 10-s-resolved EXAFS study with one flash. It will be impossible to do time resolved EXAFS if more than one flash was needed to guarantee complete photolysis. Chance et al[1] reported that they needed more than 40 flashes (200 J/flash) to reach a complete photolysis of a 10 mM (1mm thick) solution sample at 4 K. This is an important difference between their technique (setup, sample, etc) and ours. The kinetic studies by optical absorption indicated[2] (Sec. III-2) that it is difficult to achieve a
Figure VII-1 Near edge spectra of MbCO (dashed), Mb* (solid), and Mb (dotted) species. The data were taken at beam line X-23B, NSLS, in Dec. '86. MbCO and Mb* were film samples as described in Fig. VII-1. The Mb experiment was performed on a fresh chemically deoxygenated myoglobin solution (10 mM) at room temperature. The spectra were normalized.
Figure VII-2. Near edge portion of x-ray absorption spectra show that film samples can be 100% photolyzed by a single flash. The data was taken at beam line X-23B, NSLS, in Sept. '86 with two MbCO film samples at 4 K (each of them was 12 mM in heme concentration and ~40 μm in thickness). The xenon flash light used was a 200 J/pulse with a 85 μs pulse width. The solid line was the spectrum taken after one flash, and the dotted line was taken after multiple flashes. They were identical.
complete photolysis of a sample if its heme concentration times thickness \( C \cdot l \)
is greater than 0.4 mM-cm. Our samples and theirs were about the same in
congestion ( \(-10 \text{ mM}\) ) but different from each other in thickness (theirs were
more than 20 times thicker than ours). The value of \( C \cdot l \) for film sample was about
0.2 mM-cm, whereas the value for their solution sample was about 1.0 mM-cm.

VII-2. Existence of Metastable State \( \text{Mb}^* \)

As mentioned earlier (Sec. I-1) the existence of a metastable photolyzed
myoglobin had been studied with several spectroscopies, including optical
(infrared) absorptions, time resolved Raman, and Mossbauer. These studies
indicated that spectral properties of a photolyzed sample is somewhat different from
chemically deoxygenated myoglobin. Chance \textit{et al.}\(^1\) were the first to report a
change in the x-ray absorption near edge spectrum of \( \text{MbCO} \) upon photolysis. Our
investigations supported their claims.

VII-2-1. Edge Shifts

There were earlier reports\(^3,5\) on hemoglobin studies indicating a shift of a
few eV of the x-ray absorption edge to lower energy upon deoxygenation. This kind
of shift seems to be universal for heme proteins. For a simple argument, consider
the peak at the absorption edge as an excited state in the potential well formed by the
first shell atoms. Absence of one ligand is equivalent to an expansion of the potential
well, which lowers the energy levels of the eigenstates. Figure VII-1 shows the near
edge spectra of the carboxymyoglobin \( \text{MbCO} \), its photolyzed product \( \text{Mb}^* \), and the
chemically deoxygenated myoglobin Mb. The red shift of a chemically deoxygenated
myoglobin is more than 2 eV from the edge position of carboxymyoglobin; on
the other hand, the edge shift of Mb* is only about 1 eV (Fig. VII-1).

VII-2-2. Change in the First Peak height

Besides the edge shift, there are significant differences in their general
shapes. It seems that the first peak height (and also the second peak or bump) of the
x-ray absorption spectra increases while the degree of deligation increases. I
measured the peak heights from all available data and obtained the ratios of 1.15 and
1.31 for Mb* to MbCO and Mb to MbCO, respectively. These values differ slightly
from the result of Chance et al.\textsuperscript{[1]}, (They took the ratio from a set of short scans
only), but there is agreement in the ratio of Mb* to Mb (ours: 0.88; Chance's: 0.87).

VII-3. EXAFS of Photodissociated MbCO at 4 K

The optical absorption spectrum of photolyzed MbCO at 4 K is identical to Mb
from 400 nm to 700 nm. There has been a controversy as to whether Mb* is
different from Mb. The near edge x-ray absorption spectra showed a distinct
difference between the two states. However there is as yet no structural
interpretation for the spectra in the near edge region. Therefore the EXAFS
investigation of this problem is particularly important.

I analyzed the EXAFS of Mb* in two ways. First I assumed that Mb*CO is like
Mb without the sixth ligand. I then fixed the Fe-N\textsubscript{imidazole} distance between
Figure VII-3. The entire x-ray absorption spectra of MbCO (lower curve), Mb* (middle curve), and Mb (upper curve) species. See the figure caption of Fig. VII-2 for samples and experimental conditions.
Figure VII-4. EXAFS spectra of MbCO (lower curve), Mb* (middle curve), and Mb (upper curve). The data reduction procedure (background removal and converting to k-space) was applied to the spectra shown in Fig. VII-3. In order to enhance the difference between the spectra, the low k ($< 4 \text{ Å}^{-1}$) spectral portion was also displayed.
Figure VII-5. Fourier transforms of EXAFS spectra of MbCO (bottom curve), Mb* (middle curve), and Mb (top curve). A noticeable first peak shift (~ 0.1 Å) to smaller R from Mb to Mb* and MbCO can be seen.
Figure VII-6. The filtered first shell EXAFS of MbCO (dashed curve), Mb* (solid curve), and Mb (dotted curve). Again, the Mb EXAFS is seen to have a shorter \( k \) periodicity. Also, the interatomic distance in the heme of the excited species (Mb*) did not change very much from the ground state at very low temperatures. See text for discussions.
2.1 Å (as in Mb) and 2.2 Å (as in MbCO) and calculated the average Fe-N\textsubscript{porphyrin} distance from the first shell EXAFS. (Note that this crystallographical analysis gave distances only to two digits, and it is not clear what are the magnitudes of uncertainty, see Table V-1.) In all cases the values I obtained were 1.97 Å or less. This is to be compared with 1.97 Å for MbCO and 2.06 Å for Mb. In particular for Fe-N\textsubscript{imidazole} = 2.1 Å, i.e. the Mb value, the obtained Fe-N\textsubscript{porphyrin} distance was 1.96 Å, less than in MbCO where Fe is in the plane of heme. This of course does not make sense. Therefore it is clear that Mb\textsuperscript{*} is structurally different from Mb.

Next I include the sixth ligand in the analysis. In this case I fixed the Fe-N\textsubscript{imidazole} distance in the range from 2.12 Å to 2.20 Å and Fe-C distance from 1.90 Å to 2.50 Å and used the method described in Sec. V-6 to find the average Fe-N\textsubscript{porphyrin} distance. Recall that the edge energy \(E_0\) is an adjustable parameter. However its value should be consistent in comparison with MbCO. From the observed edge shift (§ VII-2-1) we know that \(E_0\) of Mb\textsuperscript{*} should be about 1 or 2 eV below that of MbCO. The solution consistent with this condition gave

\[
\begin{align*}
\text{Fe-C} &= 2.17 \text{ Å}, \\
\text{Fe-N}_{\text{imidazole}} &= 2.18 \text{ Å}, \\
\text{Fe-N}_{\text{porphyrin}} &= 1.98 \text{ Å}.
\end{align*}
\]

This is rather different from the result of Powers et al\textsuperscript{[3]}

\[
\begin{align*}
\text{Fe-C} &= 1.97 \text{ Å}, \\
\text{Fe-N}_{\text{imidazole}} &= 2.22 \text{ Å}, \\
\text{Fe-N}_{\text{porphyrin}} &= 2.03 \text{ Å}.
\end{align*}
\]

Clearly further study on this important problem is necessary.
VII-4. Results from Time Resolved EXAFS at 40 K

As mentioned in the Introduction, the beam conditions of the synchrotron radiation are unpredictable. In general they are changeable and unstable. When the beam quantity changed, it often affected the quality of data so much that comparison of different scans could be a problem. One great advantage of time-resolved scan is that one can measure the spectral point before and after flash. This allows us to compare the excited state with the ground state without worrying about the effect of changing beam conditions.

VII-4-1. The 40 K 10 s-Resolved Data

The time resolved EXAFS of photolyzed MbCO at 40 K consists of two sets of data, one was taken before photolysis, another one right after photolysis, both with a 10 s/point measuring time. The first step of data reduction was to reconstruct the spectra. Because the experiment lasted more than two days, there were many unavoidable interferences which could affect the data such as sample changing, beam damping, computer problems, etc. The raw data were formed from several segments with amplitude jumps due to different amplifications of the electronics used in the data taking process under different beam conditions. The first data set was normalized according to known MbCO static EXAFS spectrum taken at 4 K. This is simply assuming that the first data set, which was taken before flash, represents the relaxed MbCO state. The second set of data which represents the state of photolyzed MbCO in the first 10 seconds after photolysis at 40 K was then recovered by the ratio of its readings to the corresponding points of the first set of data.
Figure VII-7. Near edge portion of the 40 K 10-s-resolved x-ray absorption spectrum of photolyzed carboxymyoglobin. Dashed curve: data taken before the triggering of the flash; solid curve: after photolysis (with 1 ms delay). A spectrum of Mb⁺ at 4 K is also plotted for comparison (dotted line).
Figure VII-8. The Fourier filtered first shell of the 40 K 10 s-resolved EXAFS (dashed curve: before photolysis; solid curve: after photolysis). The dotted curve is for Mb*. A quantitative analysis shows that the solid curve is a linear combination of MbCO (42%) and Mb* (58%).
VII-4-2. Fraction of Excited States

Figure VII-7 shows the 40 K 10-s-resolved EXAFS of photolyzed MbCO. The pure excited state (4 K static EXAFS of Mb*) is also plotted for comparison. It is clear that the spectrum taken after flash is somewhat in between the two extreme states. I treated the 40 K data by the regular procedure of Fourier filtering used in analyzing the static EXAFS data. Figure VII-8 shows the filtered first shells. Again, it is in between the MbCO and Mb* states. An attempt was made to see if the spectrum of 40 K (the second data set) can be formed by a linear combination of MbCO and Mb*. The answer was positive. The best fit was a linear combination of 42% MbCO plus 58% Mb* with a resultant residual square of 2.1. From the optical absorption studies (Sec. III-2) we knew that there is about 60% molecules remaining unbound within 1 to 10 seconds after the photolysis at 40 K. This agreement between the x-ray and optical results is very encouraging.

VII-4-3. The Comparison of Excited States

The portion of the excited state was extracted by subtracting the 42% ground state (MbCO) out of the time resolved spectrum. The resultant spectrum represents the photodissociated carboxymyoglobin at 40 K. I found that within the experimental errors I could not distinguish this spectrum from 4 K Mb*.

VII-5. Conclusions

For more than twenty years there has been the controversy as to whether there is a low temperature metastable photolyzed state Mb*. And what is its
structural difference from MbCO and Mb if it does exist? Innumerable investigations using all sorts of spectroscopic techniques have been attempted to answer this question without success. Based on our studies we conclude that there is a metastable state Mb* in the transition between relaxed MbCO and tense Mb. In this low temperature metastable state, CO is displaced from the bound position but the heme structure changes are minimal. The first shell atomic distance in the heme from the iron atom changes less than 0.02 Å from that of MbCO. Previous 4 K EXAFS investigation by Chance et al. and and Powers et al.\textsuperscript{[1,3]} also concluded the existence of Mb*. But our structure of Mb* is different from theirs.

The procedure of measurement used by Chance et al. was quite different from ours, in particular in the sample preparation. They used a solution sample whereas we used PVA film samples. We have tried to use solution samples but found it to be very difficult, particularly in the attempt to achieve 100% photolysis. We believe that the film samples as described in this thesis are superior to solution samples in performing x-ray absorption experiments of photolyzed heme protein. One aspect of the experiment which will definitely improve in the future is the synchrotron radiation facilities, in particular the stability and intensity of the radiation beams. Since it is clear now that structural dynamics is an important aspect of protein functions, and there is as yet no other way of directly measuring this structural dynamics, this experiment is definitely worth repeating with a better beam condition in the future.

APPENDIX Fluctuations in the Synchrotron Radiation

The noise in synchrotron radiation has been measured as a by-product by our experiment[1]. Figure A-1 shows a 1ms-resolved measurement of a synchrotron beam intensity. Also displayed is the background measurement (i.e. the electronic noise measured when the beam was shut off) in the same scale for comparison. It is evident that the variation is not due to the noise of our data acquisition system. Figures A-2 and A-3 show the synchrotron beam fluctuations in 100 μs and 1 ms resolution. It is clear that fluctuations are of the beam, not of the detecting system.

TABLE A-1 NOISE FREQUENCY SPECTRA
OF SYNCHROTRON RADIATION

FREQUENCIES (Hz)

3.2 17 29 49 53 54 58 60 63 70 73 75 85 117

BEAMLINES

<table>
<thead>
<tr>
<th></th>
<th>SSRL VII-2</th>
<th>NSLS X-21A</th>
<th>NSLS X-23B</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOISE AMPLITUDES (% of total noise)</td>
<td>1.3 1.9 3.5 5.1 1.0</td>
<td>1.5 2.0 8.6 1.3</td>
<td>1.8 1.3 2.3 1.4 3.7 1.0</td>
</tr>
</tbody>
</table>

A frequency analysis[2] using the same fast Fourier transform technique described in Chapter V gives more details. Figures A-4 to A-6 show the frequency spectra of the synchrotron beam noises as measured on three different occasions (corresponding to Fig. A-1 to A-3). Table A-1 lists all noise
components where the contribution exceeds more than 1%. The main frequency components are characteristic of the electron storage rings, that is, a 60 c/s variation at SPEAR (Stanford Positron Electron Annihilation Ring) and a 75 c/s modulation for the X-Ray Ring of NSLS (National Synchrotron Light Source). The smaller peaks depend on the beam situations. For example, the beam condition was worst when we were on the beamline X-23B. Its noise structure (Fig. A-3) yielded a more "colorful" frequency spectrum (Fig. A-6) than X-21A (Figs. A-1, A-4) even though both beam lines are from the same NSLS ring. One usually could determine the origins of some of the small peaks. For instance, a low frequency (~6Hz) beat in Fig. A-2 resulted from the interference of a 54 Hz modulation and the main 60 Hz power noise (Fig. A-5). Also, the noticeable 3 Hz peak in Fig. A-6 was most likely due to mechanical vibration when the "phase two" building expansion in NSLS was going on. Higher frequency components possibly were from the high harmonics of the lower frequency components. No noticeable peak was found in the frequency range higher than 250 Hz.

Chance et al.[3] have investigated the beam fluctuations in SSRL. They found a dominant 5.2 Hz noise in the low frequency region which, I think, was really the 6 Hz beat mentioned above. Because of our faster sampling rate and better Fourier transform resolution, our frequency analysis can go much higher than 250 Hz (compared with 25 Hz in their report)

Figure A-1. Fluctuations of synchrotron beam intensity. Incident x-ray intensity (upper curve) was measured by our data acquisition system with 1 ms time resolution in July '85 at beamline X-21A of NSLS (beam condition: 2.4 GeV, 40 mA). The lower curve is the background (electronic noise of the system) plotted on the same scale.
Figure A-2. Fluctuations of synchrotron radiation expressed as percentage of the average intensity (DC component). Both $I_o$ (upper curve, incident x-ray) and $I$ (lower curve, transmitted x-ray) were measured simultaneously. The measurements were performed with 100 µs time-resolution in Feb. ’83 at beam line VII-2 of SSRL (beam conditions: 3.0 GeV, 65 mA, wiggler field 14 kG).
Figure A-3. Fluctuations of synchrotron radiation expressed as percentage of the average intensity (DC component). Both $I_o$ (upper curve, incident x-ray) and $I$ (lower curve, transmitted x-ray) were measured simultaneously. The measurements were performed with 1 ms time resolution in Sept. '86 at beam line X-23B of NSLS (beam conditions: 2.5 GeV, 90 mA).
Figure A-4. The frequency spectrum of synchrotron radiation noise. The Fourier transform was performed on the data shown in Fig. A-1. The noise amplitude of a certain frequency is expressed as the percentage of its contribution to the total noise spectrum.
Figure A-5. The frequency spectrum of synchrotron radiation noise. The Fourier transform was performed on the data shown in Fig. A-2. The noise amplitude of a certain frequency is expressed as the percentage of its contribution to the total noise spectrum.
Figure A-6. The frequency spectrum of synchrotron radiation noise. The Fourier transform was performed on the data shown in Fig. A-3. The noise amplitude of a certain frequency is expressed as the percentage of its contribution to the total noise spectrum.
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