# Developing a Protocol for Infrared Cryocrystallographic Analysis of Carbonmonoxymyoglobin

Dave A. Chokshi

under the direction of
Dr. J. Timothy Sage
Northeastern University

Research Science Institute
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**Abstract**

Investigating intermediate states of biomolecular processes requires the development of methods to probe extreme conditions. In this study, Fourier-transform infrared cryocrystallography was applied to studying an intermediate state of carbonmonoxymyoglobin, with unbound CO lying stationary in the heme pocket of myoglobin. An experimental protocol was designed to lower myoglobin crystals to cryogenic temperatures and subsequently photolyze the bonds in this intermediate state. Spectral data on crystals at these temperatures were collected and analyzed to determine the effectiveness of procedural innovations. Several components of the protocol worked successfully, while other aspects may require revision and further theoretical analysis. The protocol provides a foundation for the field of infrared cryocrystallography and offers suggestions for future refinement and application of the technique.

# 1 Introduction

There are over 300,000 types of structural and functional proteins in the human body. The functional proteins play a variety of biological roles including catalyzing reactions, transferring signals, and providing defense as immunoglobulins. To better understand these proteins' mechanisms, their three-dimensional structure must be analyzed. For example, the structure of myoglobin must be determined to clarify its role in the body—storing oxygen in muscles. Myoglobin ($Mb$) reversibly binds small ligands such as $O_2$ and CO and thus has long served as a model system for probing protein control of ligand binding [5]. Such analysis has typically been performed by x-ray diffraction and spectroscopic techniques on both protein crystals and proteins in solution. Studies using these techniques have provided both macroscopic and atomic-level resolution of the myoglobin molecule [11].

Myoglobin is a useful laboratory tool for studying ligand binding because of its photolytic capability. Photolyzing myoglobin involves the liberation of a molecule (in this case, CO) bound to its heme. This property allows the examination of myoglobin in different states, represented by the following diagram:

$$MbCO \rightleftharpoons Mb * CO \rightleftharpoons Mb + CO$$

The leftmost state describes CO bound to the heme moiety of myoglobin, the intermediate state describes CO stationary (but not bound) in the heme pocket, and the rightmost state describes the complete separation of CO from the heme into solution [8]. Although myoglobin is an extensively studied protein, there are several fundamental unanswered questions

concerning this binding pathway [4]. In particular, the behavior of CO after photodissociation and protein response to this process is unknown. [8]. Myoglobin is simple enough that these dynamic concepts can be studied in detail, and yet sufficiently complex for concepts discovered to be valid for all proteins [2].

Previous studies in this area have focused on structure determination using x-ray diffraction techniques [13, 14]. Measurements of the orientation of CO in the heme pocket (*Mb*CO*) relative to the heme plane normal vary by 24° [5]. The precision of another strategy, photoselection spectroscopy using picosecond time resolution at room temperature, is also limited [5]. The method pursued in this work, cryocrystallographic analysis, differs intrinsically from those mentioned above. Instead of cryogenic x-ray diffraction or room-temperature photoselection, quickly-cooled protein crystals are examined using polarized Fourier-transform infrared (FTIR) spectroscopy. FTIR spectroscopy yields precise measurements of the details of CO binding to heme proteins because of the strong transition dipole of the CO in the infrared [1]. Further rationale for use of this technique was drawn from studies that dramatically refined x-ray diffraction measurements of CO *bound* to the heme of myoglobin [12, 10, 5].

Therefore, the purpose of this work was to design, implement, and analyze an innovative protocol to study carbonmonoxymyoglobin. The data presented in this work provide information for two frontiers of study: (1) the use of polarized infrared (IR) cryocrystallography as an experimental technique to resolve similar problems in protein structure determination and (2) the investigation of ligand binding discrimination in myoglobin. Ligand binding discrimination gives scientists a microscopic view of how a protein's structure relates to its

function. There has been progress in explaining this phenomenon [9], but the details of this process are as yet undiscovered. A precise measurement of the orientation of unbound CO in *Mb*CO* is crucial for progress in this area [5]. The presented experimental methods involving polarized IR cryocrystallography provide a basis for the use of this technique in examining this and related topics, such as the effect of extended illumination on an *MbCO* crystal.

# 2 Materials and Methods

**Protein Crystallization** Sperm whale myoglobin (Sigma Chemical Co.) was obtained in lyophilized form. The protein was crystallized by the hanging-drop method (Appendix A) [9]. Monoclinic ($P2_1$) crystals were formed in concentrated $4M$ ammonium sulfate solutions titrated with $4M$ sodium phosphate and $4M$ potassium phosphate. Solutions were titrated to adjust pH to either 6.1-6.2 or 6.9-7.0 to obtain crystals flattened on the {001} or {100} faces, respectively. Crystals used in the experiments ranged from approximately 70 microns in thickness and 100 microns in lateral distance.

**Crystal Preparation** After forming crystals with the above method, those optically suitable for measurement were prepared for cryogenic FTIR analysis. Crystals were placed in 10 mL of "mother liquor", ammonium sulfate titrated with potassium phosphate to the crystals' native pH. After sealing, 16 mg/mL sodium dithionite was added to free ligands from myoglobin hemes. CO gas was then dissolved into the solution to bind to the heme groups. Crystals were incubated in this solution overnight. Some crystals were instead bound with azide by a similar procedure, substituting solid sodium azide for the sodium dithion-

ite/carbon monoxide combination. Directly before measurement, crystals were transferred to a 20:80 (v/v) glycerol/4$M$ ammonium sulfate solution titrated to 8.0 pH with potassium phosphate and sodium phosphate. CO gas was dissolved into this solution as well. Crystals were soaked for approximately 25 minutes prior to spectroscopic analysis.

**Cryogenic FTIR Spectroscopy, Cooling, and Photolysis** Crystal samples were placed on a sapphire plate mounted in an oxygen-free, high-conductivity copper cold finger of a closed-cycle helium refrigerator (model 22C, CTI Cryogenics). Samples were surrounded but not submerged by a small amount of the glycerol/ammonium sulfate solution. The cold finger was encased in a radiation shield which was in turn placed in an insulating chamber with sapphire windows allowing radiation transmittance. The refrigerator was doubly interfaced to a commercial IR microscope (Bio-Rad UMA-500) and an FTIR spectrometer (Bio-Rad FTS-60A). This apparatus enabled recording IR spectra of crystals selected with an adjustable aperture located in the image plane. Since quick cooling was necessary to eliminate lattice disorder in the crystal, modifications were made to this assembled commercial apparatus (Appendix B). In order to keep the crystal and its surroundings free from condensation and frost, cold, dry nitrogen gas was flowed across the crystal. In addition, a combination mechanical-pump and diffusion-pump vacuum was used to evacuate the chamber (necessary for sub-80 K cooling). Adapting cryocrystallography to IR measurements required the development of an original rapid-cooling procedure. After placement onto the inner sapphire plate, crystals were submerged in liquid nitrogen and the cold nitrogen gas flow was started. This combination reduced the temperature of the crystals to about 180 K (the glass transition point) in 10 minutes. At that point, the nitrogen gas flow was shut

off and a vacuum was established in the chamber. The refrigerator lowered the temperature of the crystals to approximately 9 K. The rapid-cooling "locked" the crystals in their room temperature conformations (*MbCO*). Photolysis of those conformations was achieved by illumination with red light (633 nm) from a 6 mW He-Ne laser. The cryogenic temperature was also necessary to ensure that ligand rebinding to *Mb* was slow on the experimental time scale. Variable polarization absorbance spectra of the photolyzed *Mb*CO* were taken by a rotatable wire-grid polarizer placed before the microscope condenser. Polarization angles of 45° and 135° relative to the microscope normal were analyzed because they correspond to the *b*- and *c*-axes, respectively. Due to the high degree of molecular orientation within a crystal, spectra are taken along these axes. 1024 scans were averaged at a spectral resolution of $2cm^{-1}$ before and after photolysis. Detailed procedures are included in Appendix B.

**Application of Polarization to CO Orientation** Polarized IR measurements can provide information on the photolyzed *Mb*CO* bond orientation in a manner similar to that of Sage and Jee [12, 9]. Spectra were analyzed by FTIR spectroscopy absorbance determinations using the following relation:

$$\tan^2 \Psi = \frac{\mathcal{A}_b}{\mathcal{A}_c} \tag{1}$$

where the absorbances $\mathcal{A}_b$ and $\mathcal{A}_c$ are measured with polarizations parallel to the crystallographic *b*- and *c*-axes, respectively. The angle $\Psi$ between the bond and the *a*-axis following projection onto the *ab*-plane is thereby determined. (See Figure 1.) The absorbance ratio of *MbCO* crystals at room temperature (whose $\Psi$ is known [9]) were compared to the absorbance ratio of *MbCO* at cryogenic temperatures prior to photolysis.

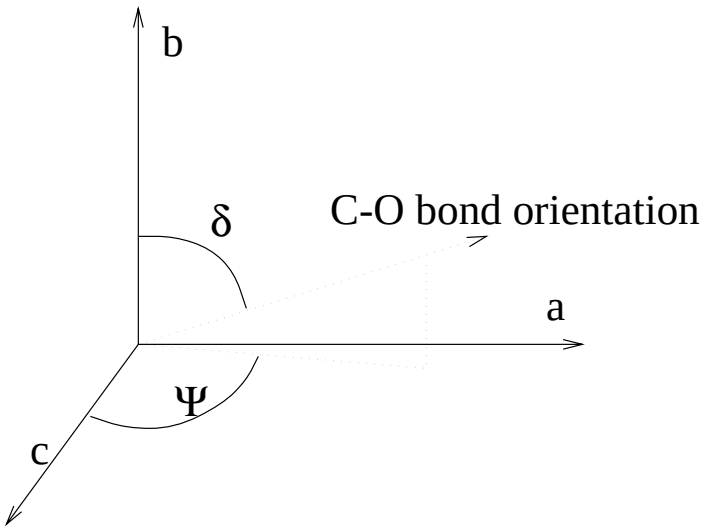


Figure 1: C-O bond orientation in crystallographic coordinate system

# 3 Results and Data

Figures 2 and 3 show the absorbance change (before and after photolysis) of a monoclinic {100} $MbCO$ crystal under cryogenic conditions recorded with IR polarization along the $b$- and $c$- axes, respectively.

The $c$- polarized spectrum (Figure 2) shows two significant features. The prominent band at 1945 $cm^{-1}$ corresponds to the known vibrational frequency of carbon monoxide bound to the heme prior to photolysis ($MbCO$). The less pronounced band at 2130 $cm^{-1}$ shows unbound carbon monoxide stationary in the heme pocket ($Mb * CO$).

The $c$-polarized spectrum (Figure 3) is similar to Figure 2. This polarization corresponds to the $b$-axis, generally the stronger axis of polarization in monoclinic {100} crystals. However, the absorbance peaks at 1945 $cm^{-1}$ are very similar. The $Mb * CO$ peak at this polarization is not apparent within the noise level achieved in this measurement.

The quantitative analysis of these two spectra are summarized and compared to room

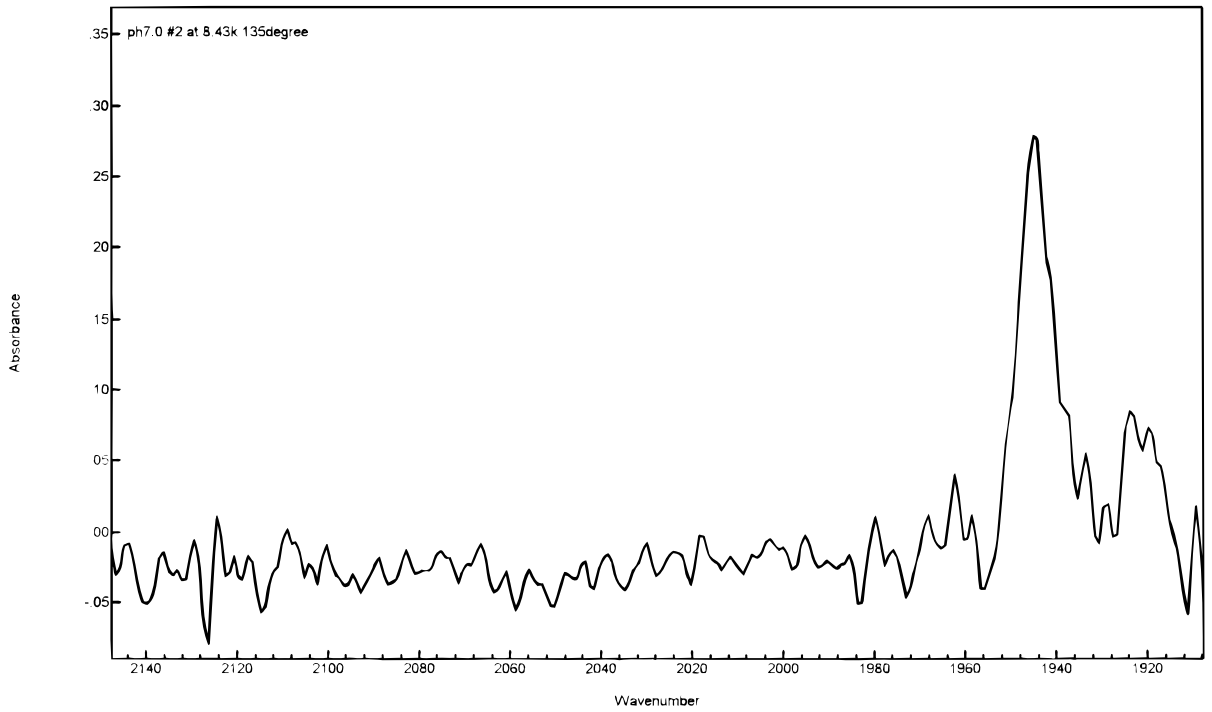


Figure 2: Absorbance change of monoclinic {100} $MbCO$ crystal before and after photolysis recorded with IR polarization parallel to the $c$-axis

temperature measurements in the table below (from [12]). Cryogenic absorbance measurements were used to derive $\Psi$ using equation (1), and the room temperature absorbance ratio was similarly derived using a known value of $\Psi$.

Figure 4 is an absorbance spectrum of azide-bound myoglobin ($metMbN_3^-$) at 8.84 K. This azide crystal was loaded and cooled according to protocol in order to test the feasibility of certain subprocedures within the protocol. This spectrum reveals a band corresponding to $metMbN_3^-$ located at 2032 $cm^{-1}$.

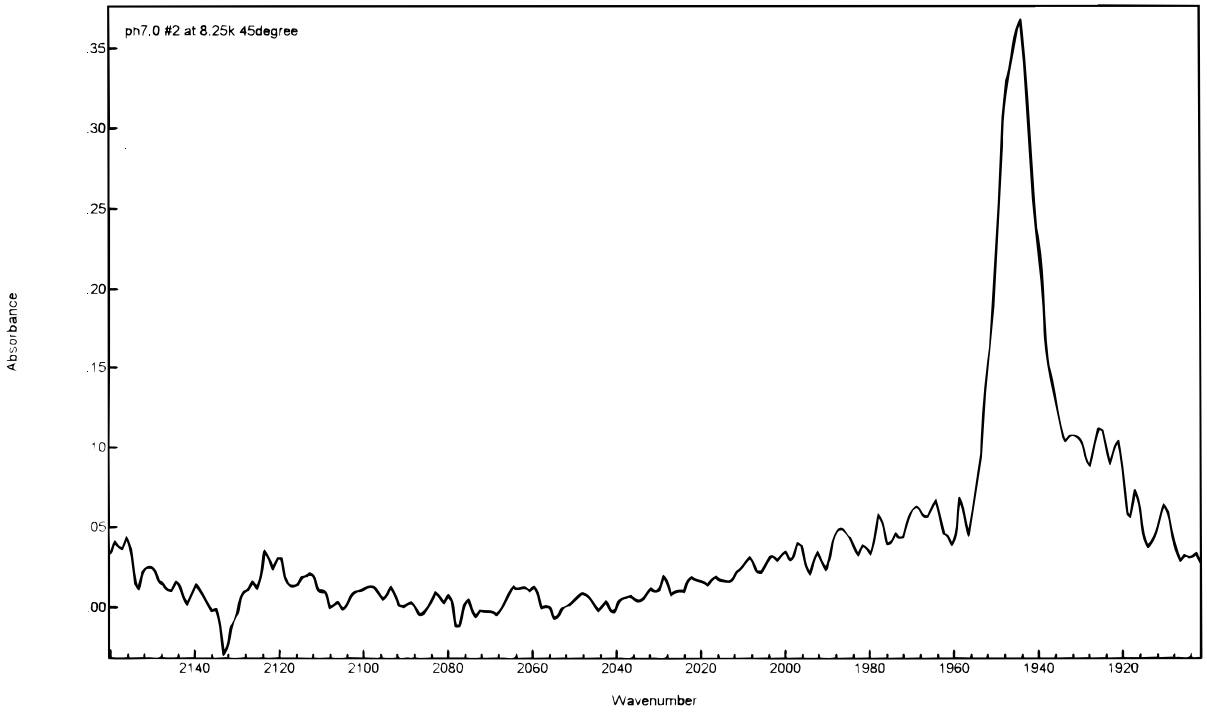


Figure 3: Absorbance change of monoclinic {100} $MbCO$ crystal before and after photolysis recorded with IR polarization parallel to the $b$-axis

| Temperature | $\mathcal{A}_b$ | $\mathcal{A}_c$ | $tan^2\Psi$ | $\Psi$ |
|---|---|---|---|---|
| 290 K | – | – | $6.25 \pm .20$ | $-111.8° \pm .3$ |
| 8-9 K | $.210 \pm .019$ | $.148 \pm .017$ | $1.42 \pm .160$ | $-128.7° \pm 2.92$ |

# 4 Discussion

This protocol required the management of multiple parameters for one purpose: to cool carbonmonoxymyoglobin crystals to 8-9 K without damaging the crystals or inducing carbon monoxide loss from the crystals. A way to test these parameters is to compare the spectra of protein crystals at 8-9 K with the spectra of crystals at room temperature. This challenge is formidable because of the sensitivity inherent to a protein. This difficulty must be confronted

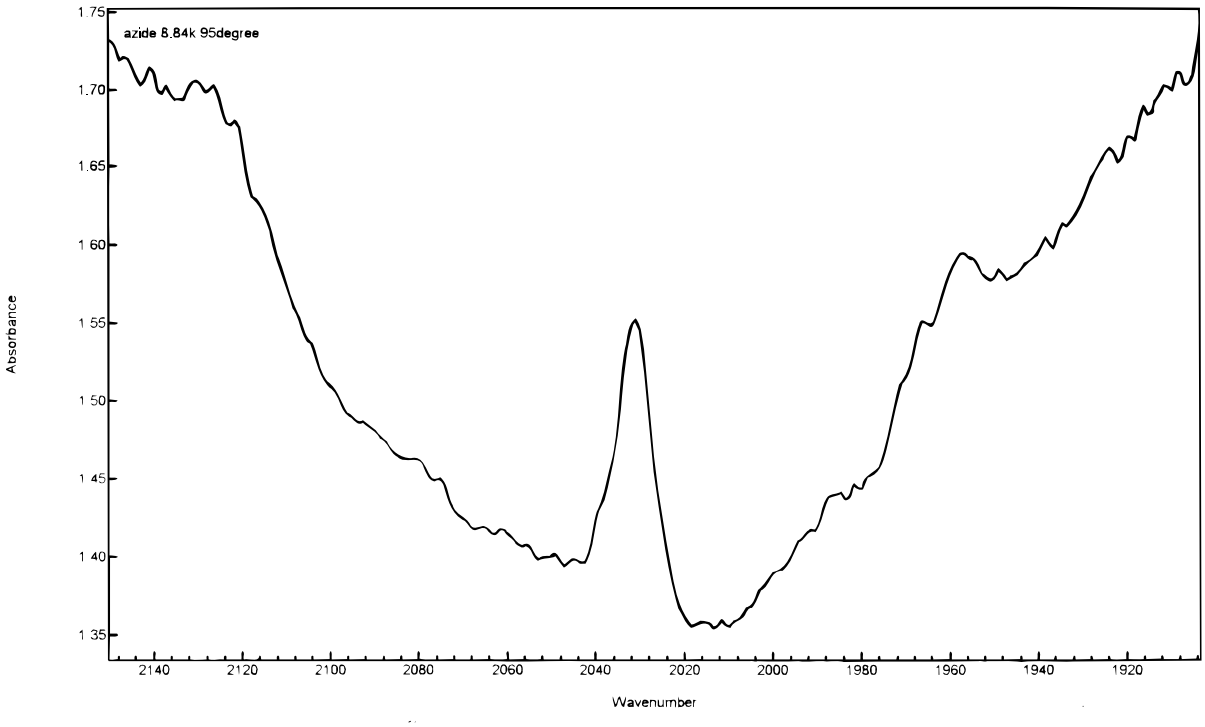


Figure 4: Unpolarized bsorbance of monoclinic $\{001\}$ $metMbN_3^-$ crystal at 8.84 K referenced to clear region of sapphire plate

if intermediate states are to be studied in detail. Promising results, obstacles, and suggestions for confronting the difficulties evident from data are outlined and analyzed in the following sections.

**Polarized Infrared Cryocrystallography of Carbonoxymyoglobin** The bands at $1945cm^{-1}$ in Figures 2 and 3 correspond to carbon monoxide bound to the heme of myoglobin. This reference frequency was obtained from and roughly corresponds to previous measurements of photolysis at room temperature [5]. However, the signal-to-noise ratio is greater than that of previous room-temperature measurements of $MbCO$ [11].

The polarization data elicited from FTIR crystallography of $MbCO$ is useful in deter-

mining ligand binding orientation [12]. This same principle applies to the $Mb*CO$ state in determining the behavior of CO in the heme pocket. However, the 1945 $cm^{-1}$ band in figures 2 and 3 is less strongly polarized than at room temperature, resulting in an absorbance ratio ($\frac{\mathcal{A}_b}{\mathcal{A}_c}$) close to 1 (see Table 1). Comparing this ratio to the polarization ratio of $MbCO$ at room temperature, it can clearly be seen that the polarization has been skewed. A possible cause of this is lattice stress induced by the cooling process. The fact that an accurate polarization ratio is not obtained hinders the pursuit of the precise quantitative angular measurement of CO in the heme pocket; indeed, it invalidates the value given for $\Psi$ in Table 1. Nevertheless, this piece of data provides valuable information: because the polarization ratio is close to 1, it can be inferred that there is a random orientation of myoglobin within the crystals instead of the regular pattern usually found in a lattice. This implies lattice stress or disorder.

Several factors could be affecting the lattice pattern. The most obvious is the cooling procedure. The procedure involves pouring liquid nitrogen onto the crystals and immediately establishing a cold, dry nitrogen gas flow in order to rapidly cool the crystals to 180 K. This may place the crystals' lattice under excessive stress. Future revisions to this protocol should vary these methods. Another possible factor is the effect of the cryoprotectant/buffer solution on the crystals. The cryoprotectant is a cholesterol that protects the crystal at very low temperatures. However, excess exposure to cryoprotectant disrupts the lattice [3]. Although crystals were only soaked for 20 minutes in a 20% glycerol solution, varying the time and concentration could clarify the effects of the buffer.

Photolysis of the crystal proved that a significant part of the protocol was successfully developed. A stronly polarized photoproduct bands can be seen at 2130 $cm^{-1}$, and the single-

beamspectra (see Appendix C) demonstrate a direct relationship between the $MbCO$ and $Mb*CO$ states. This relationship indicates that the laser setup under cryogenic conditions was successful and that enough CO was bound to the $Mb$ at the start of the experiment. This accomplishment counters the relatively weak absorbance.

The absorbance of the $Mb*CO$ states displayed after photolysis in this experiment were quantified as follows:

| $\mathcal{A}_b$ | $\mathcal{A}_c$ | $\tan^2\Psi$ | $\Psi$ |
|---|---|---|---|
| $.061 \pm .006$ | $.0197 \pm .004$ | $3.10 \pm .309$ | $60.41^\circ \pm 1.673$ |

The strongly $b$-polarized 2130 $cm^{-1}$ band qualitatively agrees with predictions for $\Psi$ made by X-ray crystallography [8, 6, 13, 14]. The measurement given here is not acceptably accurate because of the weak absorbance and high signal-to-noise ratios present in the 2120-2130 $cm^{-1}$ band. In principle, FTIR crystallography resolves bond orientations more precisely than X-ray crystallography; however, this assumes a highly oriented lattice structure. Therefore, FTIR is the first step toward determining the first accurate value for $\Psi$. Since little is known about the orientation of CO in the heme, it is possible that there is no regular orientation. If that is the case, then X-ray crystallography records spurious measurements that the method presented in this work can recognize.

**Cryocrystallography of $metMbN_3^-$** Working with this innovative method at cryogenic temperatures produced some unexpected results. This was the case with a $metMbN_3^-$ crystal whose spectrum is displayed in Figure 4. The frequency of the azide band seems to be shifted from the normal frequency. This could be the result of cooling effects and would provide a cross-reference with $MbCO$ and $Mb*CO$ for further study on the effects of cooling on

protein crystals. It supports the notion that the cooling procedure somehow does alter the crystal structure, thereby altering the absorbance readings.

**Relevance of cryocrystallography to proteins in biological conditions** Cryocrystallography shows promise as a powerful method for studying proteins. However, the necessity of taking the proteins out of their natural environment raises questions about the interpretability of data gathered under such conditions. It is conceivable that there are real differences in MbCO's structure in the two environments. However, it has been proven that crystallography itself is consistent with the presence in the crystal of the same range of conformations as in solution[10, 3]. Futher evidence is drawn from the fact that frequencies corresponding to $MbCO$ at room temperature and cryogenic temperatures are equal. Therefore, if revisions made to the protocol proposed here could ensure stable lattice structure, then information derived from infrared cryocrystallographic measurements would provide an appropriate basis for understanding the function of myoglobin in the body.

# 5 Conclusion

This work takes a large step toward developing methods relating to infrared cryocrystallography. The potential application to $Mb*CO$ adapts the presented protocol to this particular protein. The method for photolyzing the crystal was successful, as was the method for inducing CO-binding. The innovative cooling procedure differs from those currently used to "lock" CO in its intermediate state. Further studies to evaluate the relative contributions of lattice disorder and temperature-dependent structural changes are necessary. Understand-

ing these structural requirements for protein functionality provides a rational basis both for treating associated pathologies (e.g., sickle cell anemia) and ultimately for engineering new proteins [12].

# 6 Acknowledgments

I wish to thank my mentor, Dr. J. Timothy Sage of Northeastern University, whose guidance and assistance in suggesting the problem and providing materials for its solution were pivotal towards the completion of this project. Also from this organization are Mr. William Jee and Dr. M. Mylrajan, whose aid was greatly appreciated. I would also like to thank the Center for Excellence in Education for creating and sponsoring the Research Science Institute, and the members of the staff of the RSI 1998 for their assistance in the development of this paper.

# References


[1] D. P. Braunstein , et al.: Ligand Binding to Heme Proteins: III. FTIR Studies of His-E7 and Val-E11 Mutants of Carbonmonoxymyoglobin. *Biophysical Journal* (1993). **65**, 2447.

[2] H. Frauenfelder, S.G. Sligar, and P.G. Wolynes: The Energy Landscapes and Motions of Proteins. *Science* (1991). **254**, 1598.

[3] E. F. Garman and T. R. Schneider: Macromolecular Crystallography. *Journal of Applied Crystallography* (1997). **30**, 211.

[4] M.K. Hong, et al.: Conformational Substates and Motions in Myoglobin. *Biophysical Journal* (1990). **58**, 429.

[5] M. Lim, T. A. Jackson, and P. A. Anfinrud: Binding of CO to Myoglobin from a Heme Pocket Docking Site to Form Nearly Linear Fe-C-O. *Science* (1995). **269**, 962.

[6] K. Moffat: X-ray Crystallography at Extremely Low Temperatures. *Bio/Technology* (1995). **13**, 133.

[7] G. U. Nienhaus, K. Chu, and K. Jesse: Structural Homogeneity and Ligand Binding in Carbonmonoxy Myoglobin Crystals at Cryogenic Temperatures. *Biochemistry* (1998). **37**, 6819.

[8] G. A. Petsko: The Little Big Bang. *Nature* (1994). **371**, 740.

[9] J. T. Sage: Infrared Crystallography: Structural Refinement through Spectroscopy. *Journal of Applied Spectroscopy* (1997). **51**, 568.

[10] J. T. Sage: Myoglobin and CO: Structure, Energetics, and Disorder. *Journal of Biochemistry* (1997). **2**, 537.

[11] J. T. Sage: Personal communication (1998).

[12] J. T. Sage and William Jee: Structural Characterization of the Myoglobin Active Site Using Infrared Crystallography. *Journal of Molecular Biology* (1997). **274**, 21.

[13] I. Schlichting, et al.: Crystal-structure of photolyzed carbonmonoxy-myoglobin. *Nature* (1994) **371**, 808.

[14] T. Teng, V. Srajer, and K. Moffat: Initial trajectory of carbon monoxide after photodissociation from myoglobin at cryogenic temperatures. *Nature Structural Biology* (1994). **1**, 701.

# A Hanging Drop Method of Protein Crystallization

This section details the method by which crystals were grown for analysis. It is a step-by-step procedure that can be used to reproduce the type of crystals used during experimentation (monoclinic $P2_1$).

- Prepare 4 solutions: (1) 10% lyophilized sperm whale myoglobin (50 mg Mb in 500 $\mu$ L deionized water), (2) $4M$ ammonium sulfate (250 mL), (3) $4M$ potassium phosphate (100 mL), (4) $4M$ sodium phosphate (100 mL).
- Centrifuge solution (1) for 15 minutes.
- Filter solutions (1)-(4): use a syringe filter for solution (1) and filter paper for (2)-(4).
- Titrate solution (2) using solutions (3) and (4) to either 6.20 pH or 7.00 pH (for 001 and 100 crystals, respectively). This solution will now be referred to as solution (5).
- Prepare a well plate with dilute solution (5). The well plate used in this experiment was 6 wells across by 4 wells down (denoted columns 1-6 and rows A-D). Place .25 mL of water in each well. Place titrated solution (5) in columns 1-6 as follows (in mL): .80, .82, .84, .86, .88, .90.
- Prepare cover slips corresponding to each well. Place 10 $\mu$ L of solution (5) from each well onto the middle of a cover slip.
- Place 1 $\mu$ L (rows A and B) or 2 $\mu$ L (rows C and D) of solution (1) onto the top of droplet created on cover slip.
- Grease top rims of well plate. Invert each cover slip and place it on the corresponding well.

Some crystals were observed within 24 hours of sealing.

# B Detailed Procedures for IR Cryocrystallography

The specific procedures used in this work are outlined in this appendix. These step-by-step methods can be used to reproduce the results of this work and also as a guideline for infrared

cryocrystallography protocol that can be revised and added to in the development of more refined methods.

## B.1 Crystal Preparation

This section describes how crystals were prepared for infrared cryocrystallographic measurement.

- Prepare two vials. In vial (1), place 1 mL of mother liquor (at native pH). In vial (2), place 9 mL of mother liquor and 160 mg of sodium dithionite.
- Remove crystals from cover slips. Invert cover slip and use pipette to saturate droplet on slip with mother liquor while holding over vial (1). Repeat until all crystals have been removed from cover slip and placed in vial (1).
- Perform same procedure on multiple cover slips until desired number of crystals is obtained.
- Seal both vials with rubber stopper. Flush out both vials with ultra-high purity argon gas for ten minutes.
- Place contents of vial (2) into vial (1) using syringe.
- Flush out vial (1) with ultra-high purity carbon monoxide for 30 minutes.
- Seal with Parafilm and allow binding to occur for 24 hours.
- Just before loading, soak crystals in a cryoprotectant/buffer (c/b) solution for 15 minutes. The c/b solution used in this work consisted of 20% glycerol/80% native pH mother liquor titrated to a pH of 8.0 using potassium phosphate.
- Flush out solution using UHP carbon monoxide for thirty minutes and seal.
- An observation made in formulating this procedure was the deleterious effect of extended soaking on protein crystals. Special care should be taken not to exceed the 15 minute soaking duration.

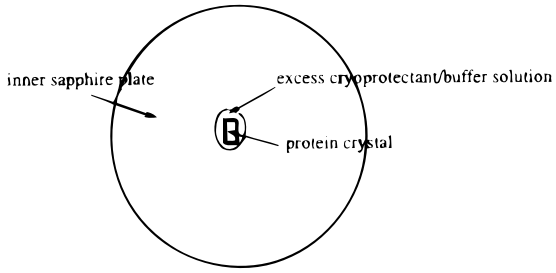


Figure 5: Protein crystal on inner sapphire plate

## B.2 Crystal Loading

This section discusses the logistics of loading protein crystals onto the inner sapphire window (see Figure 5 below).

- Throughout the loading of the crystal onto the sapphire plate, the duration of crystal exposure to air should be minimized.
- Extract crystals from c/b solution using 100 $\mu$L pipette.
- Deposit crystal within droplet of c/b solution on sapphire plate.
- Repeat procedure for desired number of crystals.
- Immediately before cooling procedure begins, remove excess c/b solution.

## B.3 Cooling Procedures

This section outlines the specific means by which cryogenic temperatures were achieved as a guide for modification, reference, and for reproducibility. The apparatus described is a modified form of a closed-cycle helium refrigerator (see A.4 for illustrations).

- Load crystal onto inner sapphire plate and remove excess c/b solution.
- Replace doughnut-shaped copper plate, and radiation shield.
- Replace outer shield except for outer sapphire window and doughnut-shaped ring for outer sapphire window.
- Turn on refrigerator.
- Pour liquid nitrogen onto inner sapphire plate and crystals.
- While the liquid nitrogen is boiling, position outer sapphire window and doughnut-shaped ring. As soon as pressure allows placement of both, partially screw them in. There should be an opening for nitrogen gas to escape.
- Begin cold, dry nitrogen gas stream at 25 psi.
- Refill dewar containing copper coil with nitrogen as needed.
- Allow vacuum pumps to warm up, but keep valve connecting to cryostat closed.
- Allow nitrogen gas to flow until a temperature of about 180K is reached (approximately 12 minutes).
- Stop nitrogen gas flow and simultaneously open vacuum valve.
- Completely screw on plate which holds top outer sapphire window in place.
- Wipe condensation from outer shield until vacuum is established.
- Allow refrigerator to cool to 8-9K.

## B.4 Apparatus

Figures 6, 7, 8, 9, 10, and 11 depict the apparatus modified and used for experimentation. The cold nitrogen gas flow was obtained by pumping nitrogen gas through a copper coil submerged in liquid nitrogen (see figures 6 and 7). The laser was interfaced to the microscope as shown in figure 9 and figure 11.

Figure 6: Overhead view of liquid nitrogen-filled dewar containing copper coil

Figure 7: Dewar and nitrogen gas tube attachments

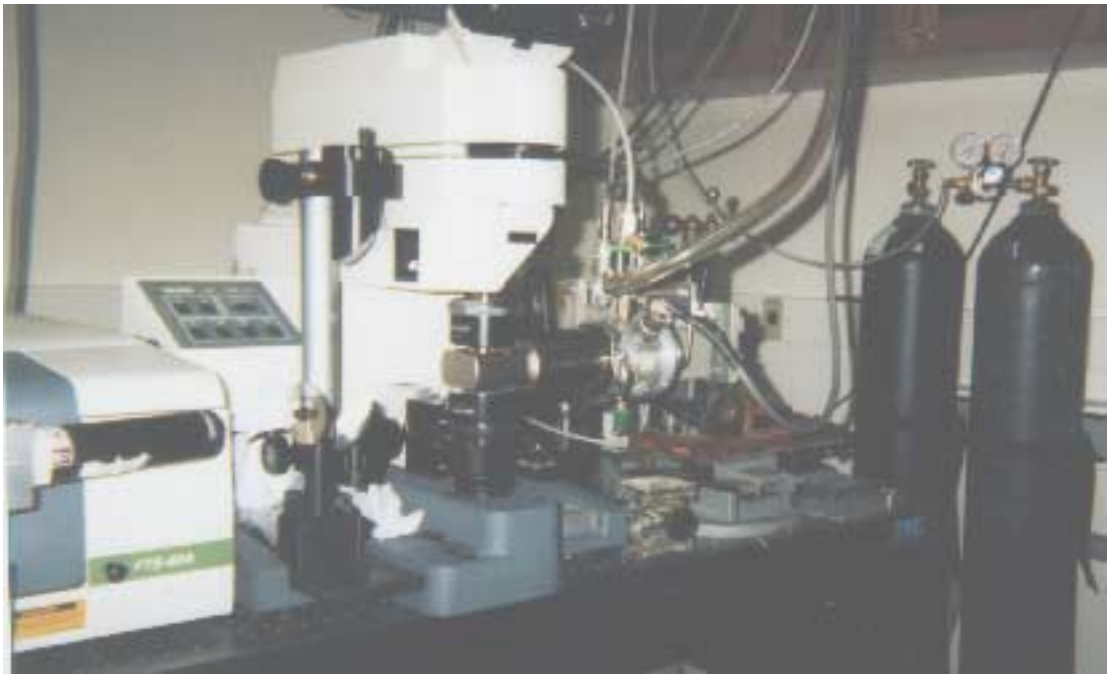

Figure 8: Apparatus interface: laser, FTIR spectrometer, microscope, and cooling mechanism

# C Intensity Spectra

Intensity spectra of a monoclinic {100} protein crystal at cryogenic temperatures and varying polarization are displayed here. These spectra, taken before and after photolysis, compose the absorbance scans shown in the Results and Data section. Future experiments following the protocol outlined here can use these spectra as a guide and reference.

Figure 9: Laser interfaced to microcope

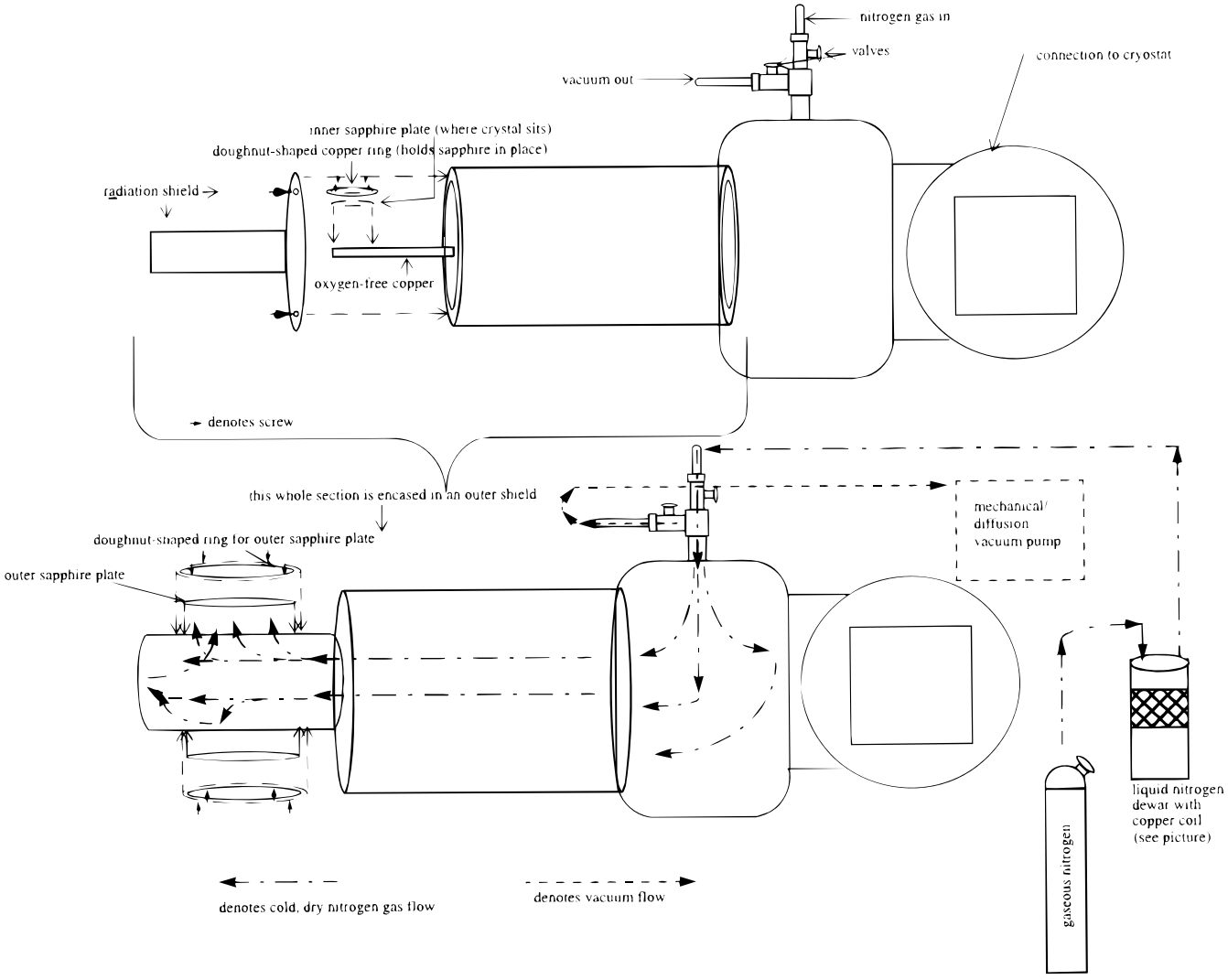


Figure 10: Interaction diagram of apparatus

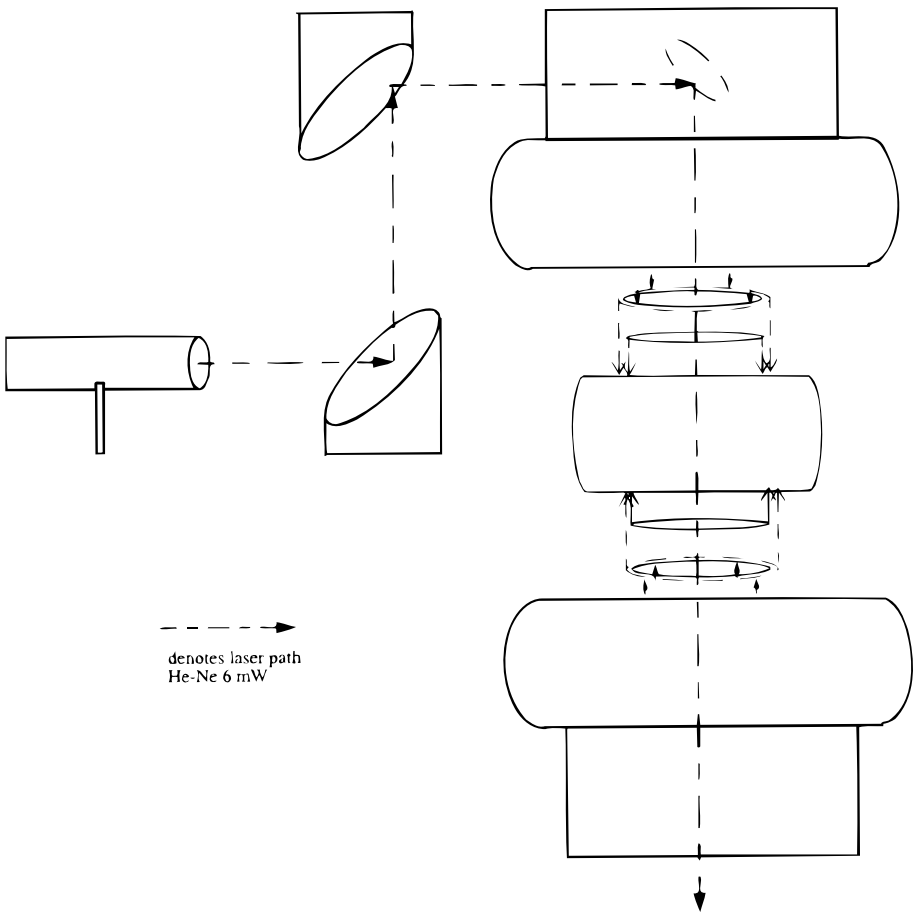


Figure 11: Laser setup

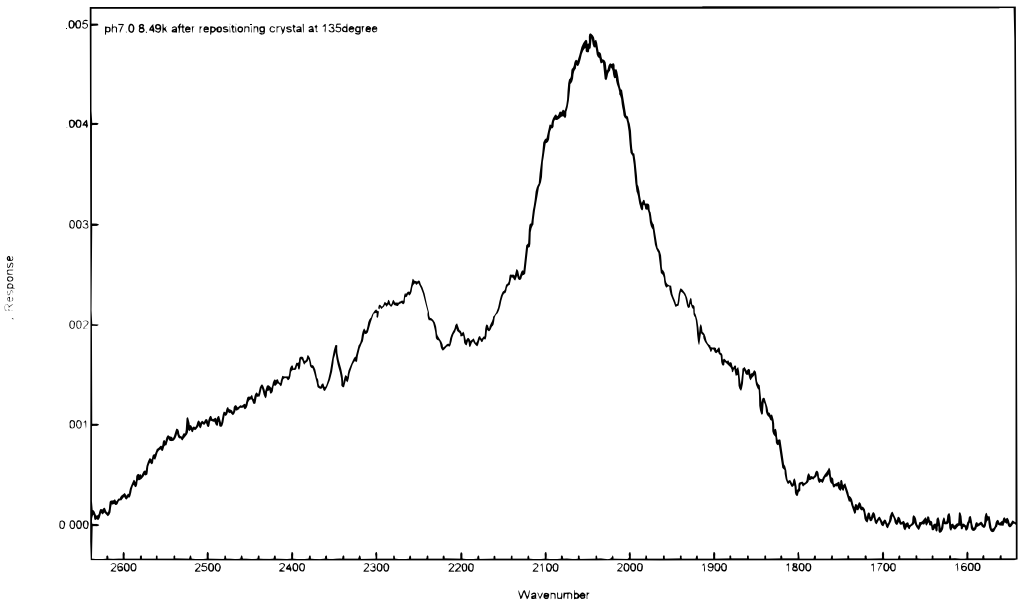


Figure 12: Response of monoclinic {100} *MbCO* crystal at 8.49 K and 135° polarization after photolysis

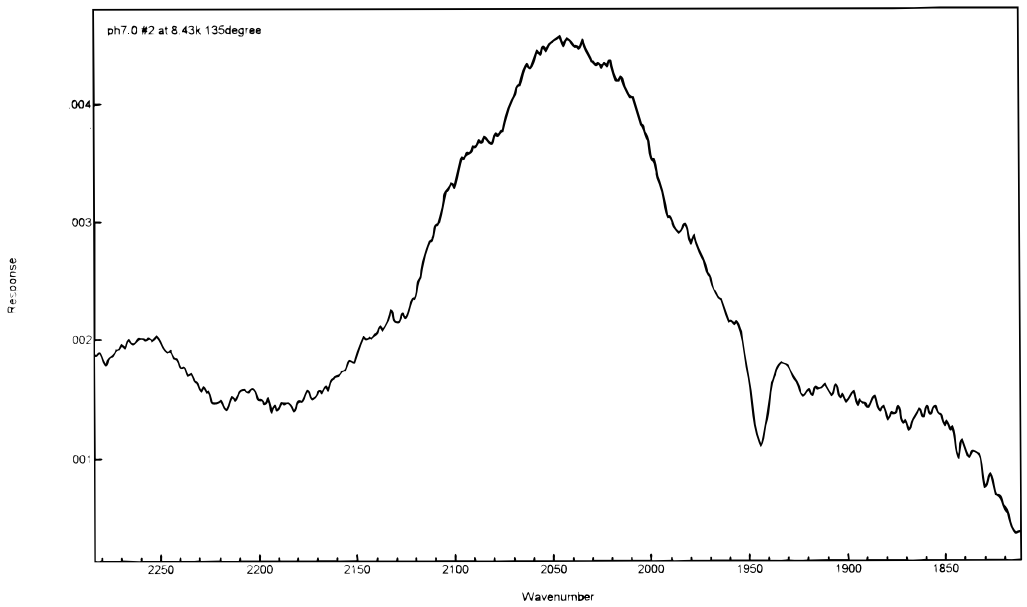


Figure 13: Response of monoclinic {100} *MbCO* crystal at 8.43 K and 135° polarization before photolysis

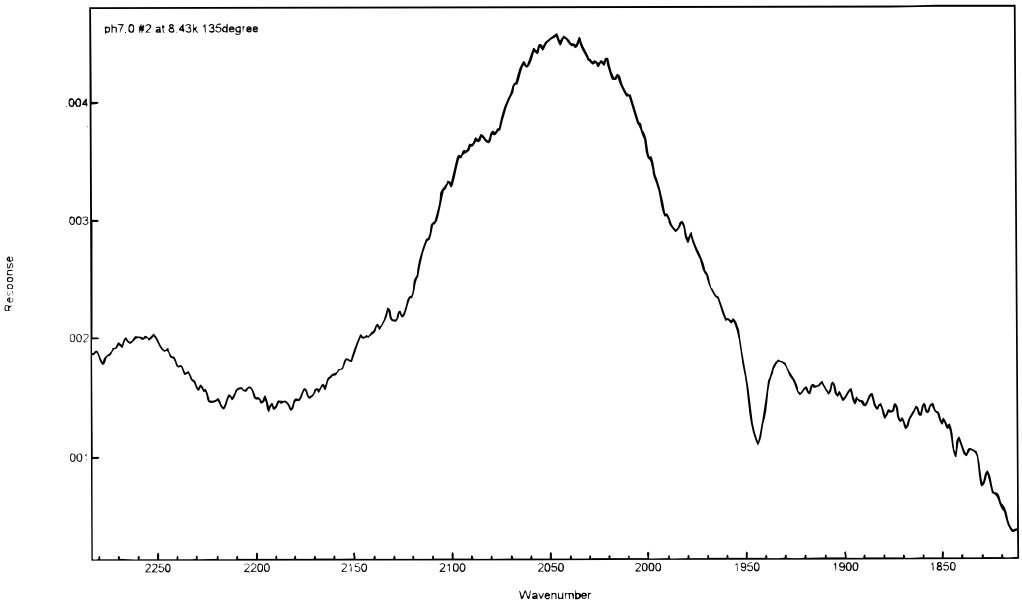


Figure 14: Absorbance comparison of monoclinic {100} *MbCO* crystal at 45° polarization before and after photolysis

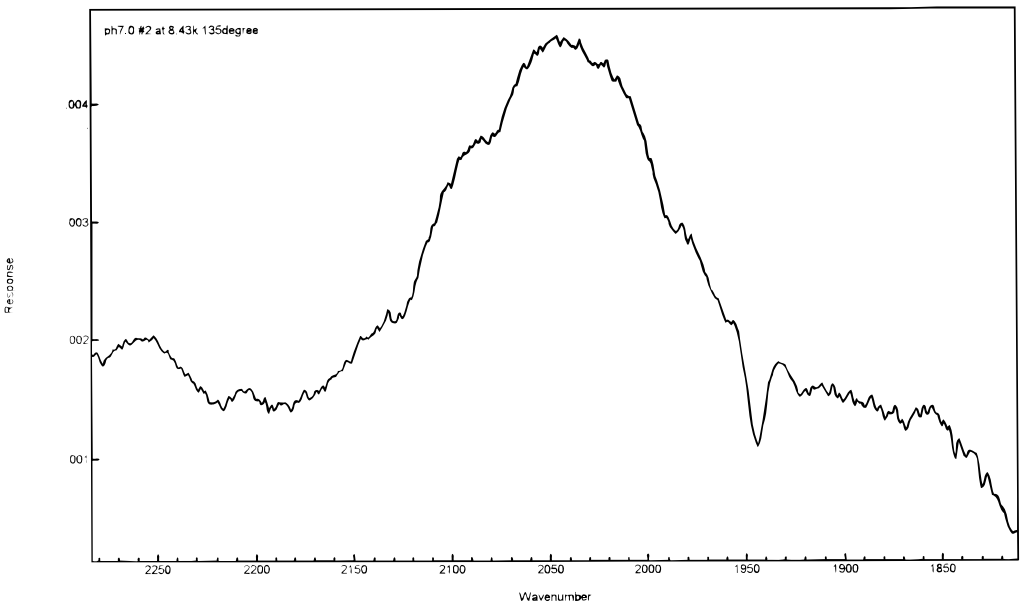


Figure 15: Response of monoclinic {100} *MbCO* crystal at 8.48 K and 45° polarization after photolysis