CONF-921116--23

To be published Nucl. Instrum. Methods in Phys. Res.

BNL--48531

DE93 009537

LAUE DIFFRACTION PROTEIN CRYSTALLOGRAPHY AT THE NATIONAL SYNCHROTRON LIGHT SOURCE

E. D. Getzoff,[1] K. W. Jones,[2] D. McRee,[1] K. Moffat,[3] K. Ng,[3]

M. L. Rivers,[3] W. Schildkamp,[3] P. T. Singer,[4]

P. Spanne,[2] R. M. Sweet,[2] T.-Y. Teng,[3] E. M. Westbrook[4]

[1]The Scripps Research Institute, LaJolla, CA 92037 USA

[2]Brookhaven National Laboratory, Upton, NY 11973 USA

[3]The University of Chicago, Chicago, IL 60637 USA

[4]Argonne National Laboratory, Argonne, IL 60439 USA

Presented at

Twelfth International Conference on the

Application of Accelerators in Research and Industry

Denton, Texas

November 2-5, 1992

Laue Diffraction Protein Crystallography at The National Synchrotron Light Source*

E. D. Getzoff,[1] K. W. Jones,[2] D. McRee,[1] K. Moffat,[3] K. Ng,[3]
M. L. Rivers,[3] W. Schildkamp,[3] P. T. Singer,[4]
P. Spanne,[2] R. M. Sweet,[2] T.-Y. Teng,[3] E. M. Westbrook[4]

[1]The Scripps Research Institute, LaJolla, CA 92037 USA
[2]Brookhaven National Laboratory, Upton, NY 11973 USA
[3]The University of Chicago, Chicago, IL 60637 USA
[4]Argonne National Laboratory, Argonne, IL 60439 USA

ABSTRACT

A new facility for the study of protein crystal structure using Laue diffraction has been established at the X26 beam line of the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory. The characteristics of the beam line and diffraction apparatus are described. Selected results of some of the initial experiments are discussed briefly by beam line users to illustrate the scope of the experimental program. Because the Laue method permits the recording of large data sets in a single shot, one goal in establishing this facility has been to develop the means to study time-resolved structures within protein crystals. Systems being studied include: the reactions catalyzed by trypsin; photolysis of carbonmonoxy myoglobin; and the photocycle of photoactive yellow protein.

---

*Work supported in part by the US Department of Energy under Contracts W-31-109-ENG-38 (EMW,PTS) and DE-AC02-76CH00016 (RMS,KWJ,PS); by National Institutes of Health Grant GM44292 (EMW,PTS), GM36452 (KM), and GM37684 (EDG); and a grant from the Keck Foundation (KM).

1. Introduction

Laue diffraction crystallography using synchrotron radiation has become an important tool for studying the structure of proteins. In particular, the rapid data acquisition times and high resolution makes detailed studies of kinetic changes in the structure of proteins potentially achievable with the Laue method and may lead to a better understanding of the correlation between protein structure and function.

Despite the strengths of the Laue method, there are few locations where this type of work can be carried out. One facility recently put into operation is located at the X26C beam line of the Brookhaven National Synchrotron Light Source (NSLS). Here, the physical attributes of the facility are described, followed by discussion of the radiation damage problems and several examples of application to specific biological problems.

2. X26C beam line characteristics

Beam line X26C at the NSLS employs a bending magnet to produce synchrotron radiation. The experimental equipment is about 20 m from the electron orbit. A 1:1 cylindrical focussing mirror located 10 m from the orbit is used to increase the beam brilliance by focussing the 4 millirad horizontal divergence and the full vertical divergence onto a focal spot of less than 700 × 700 μm. The mirror is fabricated from Zerodur® [1] and coated with platinum. The experimental apparatus is isolated from the ring vacuum by beryllium windows. The absorption of these windows produces a low-energy cut-off in the photon spectrum at about 4 keV. The estimated photon flux integrated over the entire energy range of the beam is about 3×10^7 photons/($\mu m^2 \times s \times 100$ mA) or 10^4 photons/($\mu m^2 \times s \times eV \times 100$ mA) at a photon energy of 10 keV for the unfocussed beam. The energy spectrum of the beam has been measured by observing the energy of X-rays scattered at 45 degrees into a Si(Li) detector by air in the beam path. The results obtained for unfocussed and focussed white beam are shown in fig. 1. It can be seen that insertion

of the mirror increases the intensity by about two orders of magnitude in the range 4-11 keV photon energy.

3. Protein crystallography apparatus

The protein crystallography apparatus on beam line X26C falls into two classes: those components which are generally suitable for the acquisition of static Laue patterns on protein crystals and are available to all users; and those which are specific to particular time-resolved experiments and are added to the basic Laue camera by particular users according to their needs.

X-rays are delivered to the camera through adjustable horizontal and vertical lead slits located about 19.5 m from the source. The basic camera is mounted on a computer-controlled table which permits translations and rotations of specified magnitude about any axis in space for alignment purposes. This design has been used successfully for several years at the Cornell High Energy Synchrotron Source (CHESS). On the table is rigidly mounted a Klinger X95 rail, to which the camera components can be attached in any desired configuration. A typical experiment would use the following components, listed in the order from upstream to downstream: X-ray beam position monitor based on a split beam ion chamber, with an accuracy horizontally and vertically of around 3 μm [2]; fast shutter with a single shot opening time from 1 ms to infinity; slow shutter and Supper collimator mount with tungsten collimators; Huber 410 goniometer to provide ϕ rotation capability for the crystal; helium beampath; X-ray backstop; and film or storage phosphor cassette and mount, either single or 6-cassette carousel. The crystal-to-detector distance is variable from ~ 50 mm to more than 500 mm. An air stream delivered from an FTS Systems crystal cooler through a nozzle with coaxial, temperature-controlled and warm gas streams [3] can maintain the temperature of the crystal in the range from roughly -50 to +50°C to within ± 1°C. The crystal can be visualized and centered in the X-ray beam by a CCD camera and monitor with electronic crosshairs. Exposed storage phosphors are scanned with a Fuji BAS2000

scanner using manufacturer-supplied software; files averaging 10 Mbytes can be written to an Exabyte tape for transport to the user's home laboratory. Computer control of the camera is provided by a VAX Station running under VMS and uses software written in part by Dr. Mark Rivers. User manuals describing the installation, alignment, and operation of the camera, as well as all other mechanical and electronic equipment, are being prepared.

Time-resolved experiments require that additional, user-supplied components be added. The (deliberately flexible) design enables these components to be attached either to the computer-controlled table or to the Klinger rail. Fig. 2 shows a block diagram of apparatus suitable for a representative time-resolved experiment [4], in which pulsed or CW laser stimulation of the crystal is possible, and the optical absorbance of the crystal can also be continuously monitored during the X-ray experiment via a single crystal microspectrophotometer. As an alternative to laser stimulation of the crystal, a flow cell may be used to introduce ligands or substrate, or to effect a change in pH. Certain experiments require lower crystal temperatures which can be obtained using cryostats based on boil-off of liquid nitrogen or liquid helium. The simple detector cassette mount can be replaced by translation stages which permit the detector to be moved in its plane during the experiment, so that individual time points for the X-ray reflections can be laid down in any desired array (see for example, fig. 5 of ref. 4). The critical coordination of the complex motor motions, shutter opening and closing, and laser and microspectrophotometer triggering, may be achieved via computer control of a programmable timing module resident in a CAMAC crate.

4. Experimental results from Laue diffraction measurements

4.1 Crystal damage

In recent experiments at the NSLS X26C and X25 beam lines, it was observed that the greater photon flux available at the 27-pole wiggler (beam line X25) resulted in greater damage to the crystals per usable film image than the spectrally similar beam from a

bending magnet (beam line X26C). This effect was observed by several groups using the two beam lines for Laue studies. A systematic effort to determine whether this effect was real was undertaken [5].

Crystals of bovine pancreatic trypsin (orthorhombic), hen egg-white lysozyme (tetragonal) and glutathione synthetase (hexagonal) were used to collect data at the two beam lines. In total, eight series of images were taken of each crystal, and the experimental parameters of beam line, temperature, and crystal mounting protocol ("dry" vs. "wet") were varied. The quantitation of damage was taken to be the number of frames of high quality data that could be measured at one location along the crystal. In brief, cooling the crystals always increased the longevity of the crystals in the X-ray beam, especially in the hotter beam at X25. More interestingly, one could consistently collect approximately twice as many usable images at X26C from putatively identical crystals. This effect was particularly pronounced for the radiation-sensitive glutathione synthetase, which deteriorated during a single 125 ms exposure at X25, but which gave at least two usable images at X26C (fig. 3).

We believe that this effect is due to a thermal gradient which is established by the beam within the crystal. Since it is primarily low-energy photons which are absorbed by protein crystals, we now routinely put a graphite filter in the beam path in order to reduce this problem.

4.2 Dynamic studies of catalysis

Crystals of bovine pancreatic trypsin were acylated at the active site residue Ser-195, resulting in the transiently stable p-guanidinobenzoyl ester. This complex mimics the covalent enzyme-substrate complex which lies along the mechanistic pathway of this enzyme. At pH 5.0, this bond is stable, with a lifetime measured in hundreds of hours, but at pH 8.9 the lifetime is approximately 1 hour. A crystal of p-guanidinobenzoyl-trypsin (GB-trypsin) measuring approximately 0.4 × 0.4 × 1.0 mm was mounted in a flow cell, and pH 5.0 buffer was flowed across it at approximately 10 ml/hour.

The deacylation reaction, which is analogous to the second step of hydrolysis by the serine proteases, was triggered by raising the pH of the eluent to pH 8.9. A Laue data set was collected just prior to the pH jump, two minutes thereafter, and then again after 90 minutes. The data, collected as packs of 5 sheets of Kodak DEF 5 X-ray film, were digitized using an Optronics P-1000 scanner and integrated using the Daresbury suite of programs [6]. The average intra-film-pack R-factors were 4.6% (t(0)), 3.9% (t(3)) and 4.8% (t(90)). After merging the data, the three sets contained 47, 68 and 60 percent of the data out to 1.8 Å, respectively. The data were used to refine the starting model of GB-trypsin (obtained in an earlier study using monochromatic data-collection techniques) via XPLOR, in both simulated annealing and conjugate gradient modes. After extensive refinement of atomic coordinates and temperature factors, the three models had final R values of 22.1%, 18.3% and 18.7%.

Inspection of the three models leads to one initial conclusion--there was little or no change in the coordinates of the protein atoms (mean inter-model rms = ~0.25 Å), especially those comprising the backbone of trypsin (rms ~ 0.15 Å). There was however, an apparent shift in the water structure in and around the active site. One water molecule, which in the low pH structure (t(0)) is rigidly hydrogen bonded in such a manner so as to block attack on the carbonyl carbon of the serine ester bond, moves approximately 2 Å upon the rise of the pH to 8.9. Consequently, a new water site is now accessible, and is essentially fully occupied in the t3 and t90 structures. Unlike the first water, this water molecule is located close enough to, and directly above the plane of, the scissile bond to allow it to act as the hydrolytic nucleophile.

A model of the active site of the digestive enzyme trypsin, determined by time-resolved Laue crystallography, is shown in fig. 4 [7]. The enzyme had been acylated by guanidinobenzoate. The three suspended spheres in the foreground represent water molecules, the central one of which is poised to attack the carbonyl carbon atom of the acyl group.

The Laue method proved effective in collecting sufficiently high-quality diffraction data to confidently assign the atomic coordinates of catalytically relevant solvent molecules. More importantly, the ability to collect the relatively complete data set within 3 minutes allowed conclusions to be drawn about the movement of these water molecules over the same time period. This shows conclusively the promise of Laue method for dynamic experiments on protein crystals.

4.3 Study of carbonomonoxy myoglobin

Berendzen and Schlichting [8] are carrying on an investigation of the oxygen-storage protein myoglobin (Mb), using it as a model system for physical studies of the relation between molecular structure and biological function. Mb reversibly binds O_2, CO, and other small ligands at its heme iron. Absorption of a photon causes the ligand to dissociate, and the time course of the subsequent rebinding reaction may be followed by Laue diffraction at liquid-helium temperatures.

Some of the important points about the relation between structure and function to be addressed in the experiment are: differences in the structures of the various conformational substates; structural changes corresponding to conformational relaxation; the structural nature of the solvent process; and the role of the protein in the control of the binding of small molecules.

The result of a number of initial experiments at X26C is that high-resolution data may be obtained at 5°K on a time scale of the process to be studied. A room-temperature diffraction pattern of a mutant met-myoglobin that crystallizes in a hexagonal space group is shown in fig. 5. Analysis of this pattern indicates that the crystal diffracts to better than 1.9 Å, which is comparable to results obtained with monochromatic methods.

4.4 Photoactive yellow protein

Almost all successful time-resolved crystallographic studies to date have concentrated on slow reactions, where intermediate formation and breakdown is measured in minutes or longer [9,10, see also the papers in ref. 11]. Slower reactions allow longer X-ray exposure

times and lower incident X-ray intensities. Reaction initiation can proceed smoothly via diffusion of reactants into the crystal or via slow, repeated photoinitiation with a flash lamp. If, however, the system under study exhibits much faster reactions with short-lived intermediates lasting for seconds or much less, then reaction initiation must be much more rapid via, for example, laser photoactivation of a caged biochemically inert precursor, or pulsed laser stimulation of a naturally-occurring chromophore. Brief laser pulses may introduce experimental artefacts, as discussed in more detail elsewhere [12,13,4,14].

One such system which exhibits rapid, light-stimulatable reactions is photoactive yellow protein, PYP, a small, soluble, 14 KDa photoreceptor protein with a fully-reversible photocycle resembling that of sensory rhodopsin. The crystal structure of PYP isolated from the halophilic bacterium *Ectothiorhodospira halophila* [15] has been determined at 2.4 Å resolution [16] and refinement to a resolution of less than 2 Å is under way. The chromophore is believed to be retinal-like, but its identity has not been established. Immediate bleaching of its yellow color by a laser pulse is followed by further bleaching on a much slower time scale, and finally by recovery of the initial spectrum over several seconds.

The PYP system is nearly ideal for rapid time-resolved studies. The crystals are of known structure, of low mosaic spread, scatter X-rays strongly to around 1.4 Å resolution, and are relatively resistant to radiation damage, both from synchrotron X-rays and from pulsed and CW lasers. PYP crystallizes in a high symmetry, hexagonal space group of moderate cell dimensions [17] that allows the unique data to be collected on only a small number of Laue images, and avoids Laue spot overlap problems [18]. The kinetics of the photocycle [19,20] reveal a series of spectrally distinct intermediates which decay via first order reactions and whose lifetimes are quite different, so that there are time periods when the crystal is essentially homogeneous (see figs. 3 and 4 of ref. 4).

Since all reactions are unimolecular, the high protein concentration in the lattice, 63 mM, is of no (kinetic) consequence and diffusion problems are absent. However, the

closely-packed lattice may restrict the conformational changes which indirect evidence suggests [20] accompany photocycling. The quantum yield for photoinitiation in solution is high, 0.64 [20], although polycrystalline slurries of microcrystals and single crystals of X-ray dimensions appear somewhat harder to bleach. The photocycle is fully reversible, which permits X-ray data to be accumulated over many cycles if necessary. Finally, PYP may be a particularly simple photoreceptor and as such, is of considerable biophysical interest.

Since PYP has a long-lived intermediate which decays with a first-order rate constant of around 3 s^{-1} at room temperature [20], a bleached photostationary state can be readily established via continuous illumination with a relatively low power CW laser. Using a single crystal microspectrophotometer either separately or as an integral part of a Laue camera [4], it was possible to obtain clear optical evidence for bleaching of small single crystals by illumination with a CW Ar laser at 488 or 496.5 nm, but not at 514 nm where the crystals are completely transparent. The spectral changes saturated as the laser power was increased and were reversible. On rapidly shutting off the laser the time course of the spectral decay to the dark, yellow state appeared to be biphasic.

In initial X-ray experiments conducted without the use of the microspectrophotometer, an increase in mosaic spread was seen whenever the laser beam struck the crystals. This increase rapidly and completely disappeared when the laser was shut off (see fig. 5 of ref. 4). Later experiments using the microspectrophotometer have indicated that there is only a narrow range of laser energies within which the spectral changes saturated, but essentially no increase in mosaic spread occurred. Lower energies did not produce full bleaching, and higher energies produced an increase in mosaic spread sufficiently large to make extraction of precise structure amplitudes more difficult. A typical Laue exposure time of 30 ms was obtained on the rather small crystals (say 50 × 50 × 250 μm) necessary to ensure suitable optical properties.

The change in X-ray integrated intensities which occur when the photostationary state has been established, and when decay from that state to the dark state is initiated by shutting of the laser, are being evaluated.

5. Conclusions

The characteristics of the X26C Laue diffraction facility have been discussed. Examples of results from several investigations are given to illustrate limitations to the work caused by radiation damage as well as to show the type of data that can be obtained for several different biological investigations. It is expected that this facility will become of increasing importance to a diverse community of biologists.

6. Acknowledgements

We are greatly indebted to Dr. Joel Berendzen, Los Alamos National Laboratory, and Dr. Ilme Schlichting, Max Planck Institute, Heidelberg, for making available the material on the Mb experiment and especially for permitting use of the Laue diffraction pattern of Mb shown in fig. 5.

References

1. Zerodur - Schott Glass Technologies Inc., 400 York Avenue, Duryea, PA 18642.
2. Schildkamp, W., unpublished.
3. Teng, T.-Y., Schildkamp, W., Dolmer, P., and Moffat, K., to be submitted.
4. Moffat, K., Chen, Y., Ng, K., McRee, D., and Getzoff, E. D. Phil. Trans. Roy. Soc. Lond. A340 (1992) 175-190.
5. Singer, P. T., Carty, R. C., Berman, L. E., Schlichting, I., Stock, A., Smalås, A., Cai, Z., Mangel, W. F., Jones, K. W., and Sweet, R. M. Phil. Trans. Roy. Soc. Lond. A 340 (1992) 285-300.
6. Helliwell, J. R., Habash, J., Cruickshank, D. W. J., Harding, M. M., Greenhough, T. J., Campbell, J. W., Clifton, I. J., Elder, M., Machin, P. A., Papiz, M. Z., and Zurek, S. J. Appl. Crystallogr. 22 (1989) 483-497
7. Singer, P. T., Smalås, A., Carty, R. P., Mangel, W. F., and Sweet, R. M. Science 1992, in press.
8. Berendzen, J. and Schlichting, I., private communication, October 1992.
9. Hajdu, J., Machin, P. A., Campbell, J. W., Greenhough, T. J., Clifton, I. J., Zurek, S., Gover, S., Johnson, L. N., and Elder, M. Nature, Lond. 329 (1987) 178-181.
10. Schlichting, I., Almo, S. C., Rapp, G., Wilson, K., Petratos, K., Lentfer, A., Wittinghofer, A., Kabsch, W., Pai, E. E., Petsko, G. A., and Goody, R. S. Nature, Lond. 345 (1990) 309-315.
11. Cruickshank, D. W. J., Helliwell, J. R., and Johnson, L. N., eds. Time-resolved macromolecular crystallography. Phil. Trans. Roy. Soc., Lond. A340 (1992) 169-334.
12. Moffat, K. Ann. Rev. Biophys. Biophys. Chem 18 (1989) 309-332.
13. Hajdu, J. and Johnson, L. N. Biochem. 29 (1990) 1669-1678.
14. Petsko, G. A. Phil. Trans. Roy. Soc., Lond. A 340 (1992) 323-334.
15. Meyer, T. E. Biochim. Biophys. Acta 806 (1985) 175-183.

16. McRee, D. E., Tainer, J. A., Meyer, T. E., Van Beeumen, J., Cusanovich, M. A., and Getzoff, E. D. Proc. Nat. Acad. Sci. USA 86 (1989) 6533-6537.

17. McRee, D. E., Meyer, T. E., Cusanovich, M. A., Parge, H. E., and Getzoff, E. D. J. Biol. Chem. 261 (1986) 13850-13851.

18. Cruickshank, D. W. J., Helliwell, J. R., and Moffat, K. Acta Crystallogr. A47 (1991) 352-373.

19. Meyer, T. E., Yakali, E., Cusanovich, M. A., and Tollin, G. Biochem. 26 (1987) 418-423.

20. Meyer, T. E., Tollin, G., Hazzard, J. H., and Cusanovich, M. A. Biophys. J. 56 (1989) 559-564.

Figure Captions

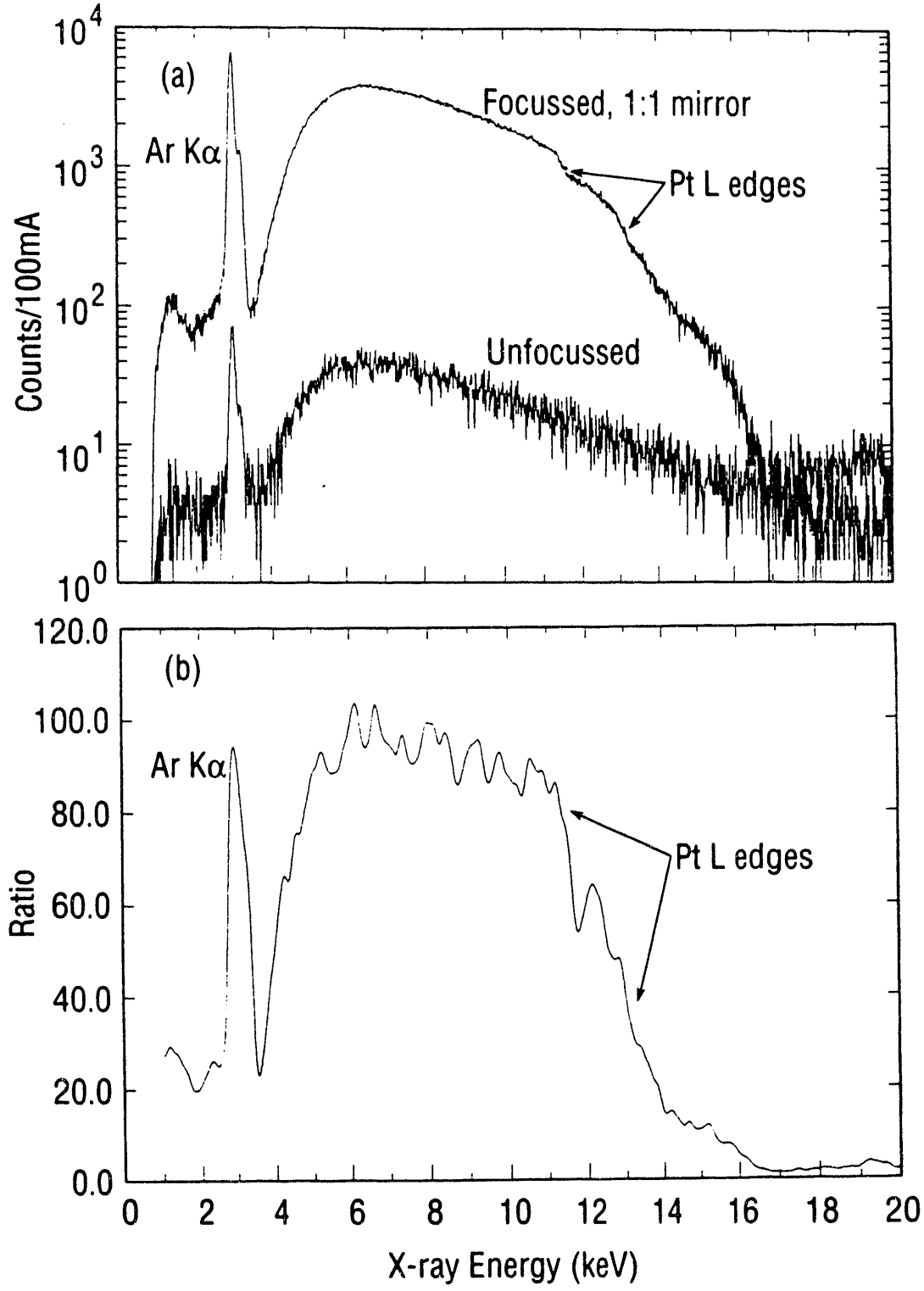
Figure 1. Energy spectrum of X-rays for focussed and unfocussed beams. This shows the enhancement of intensity produced by the mirror and the alteration of the energy distribution. (Figure 7 from B. M. Johnson, Atomic Physics at the Advanced Photon Source: Workshop Report. Argonne National Laboratory, March 29-30, 1990, ANL/APS/TM-8 pp. 200-226, with permission).

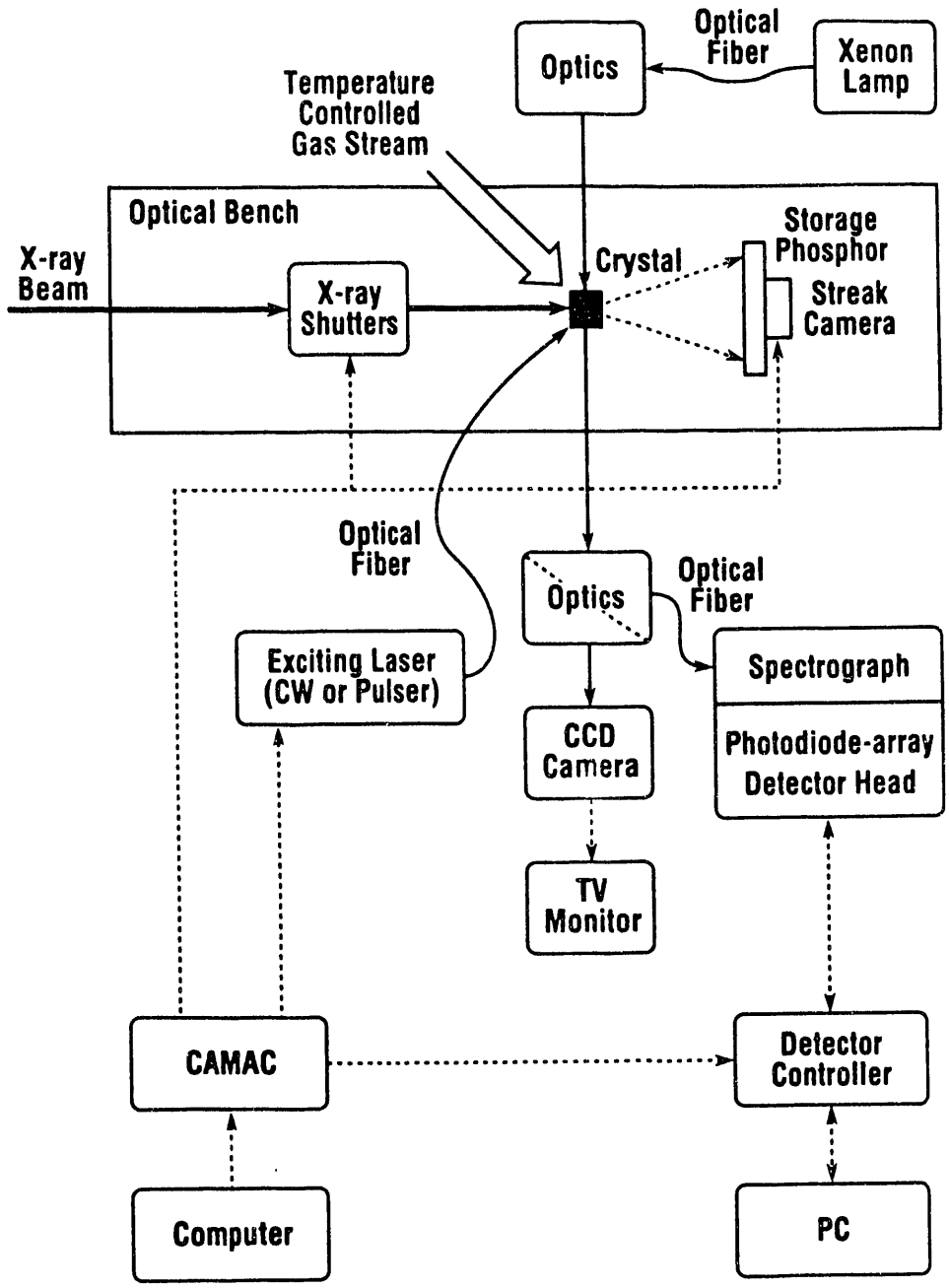
Figure 2. Block diagram of X-ray and optical apparatus for time-resolved crystallography (currently used by the University of Chicago group at beam line X26C).

Figure 3. Relative crystal damage at X26C and X25. Composite photograph of Laue diffraction patterns generated by crystals of glutathione synthetase. Upper left--first exposure at X26C (second was essentially similar); upper right--third exposure at X26C; lower--only exposure X25. (Fig. 5 from ref. 3.)

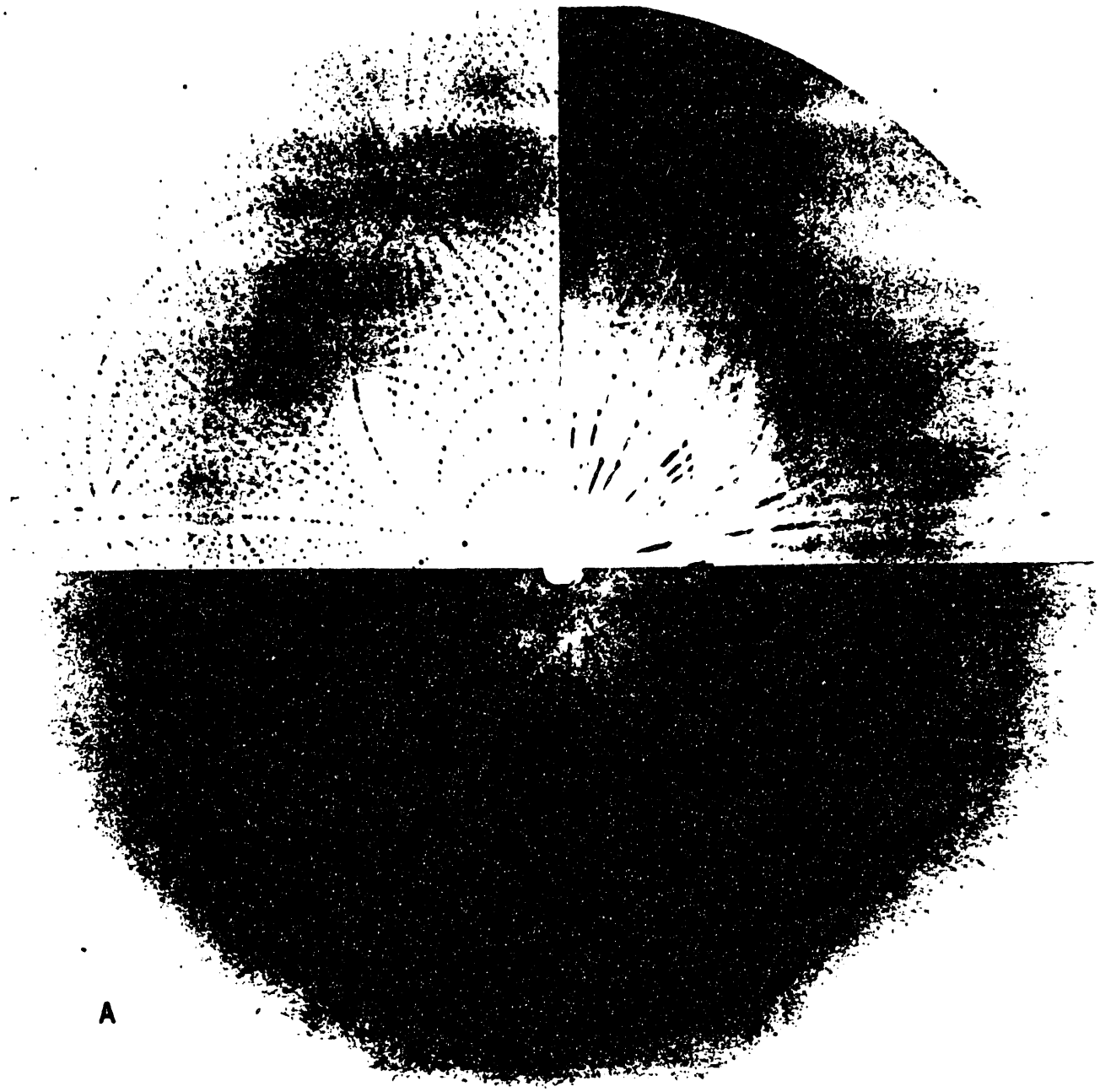
Figure 4. Electron density maps of guanidinobenzoyl-trypsin at pH 5.0 (A) and three minutes later, after raising the pH to 8.9 (B). Note the appearance of water 1082 at the higher pH. (Fig. 1 from ref. 7.)

Figure 5. Laue diffraction pattern of sperm whale myoglobin. Exposure time was 500 ms, crystal to film distance 100 mm.

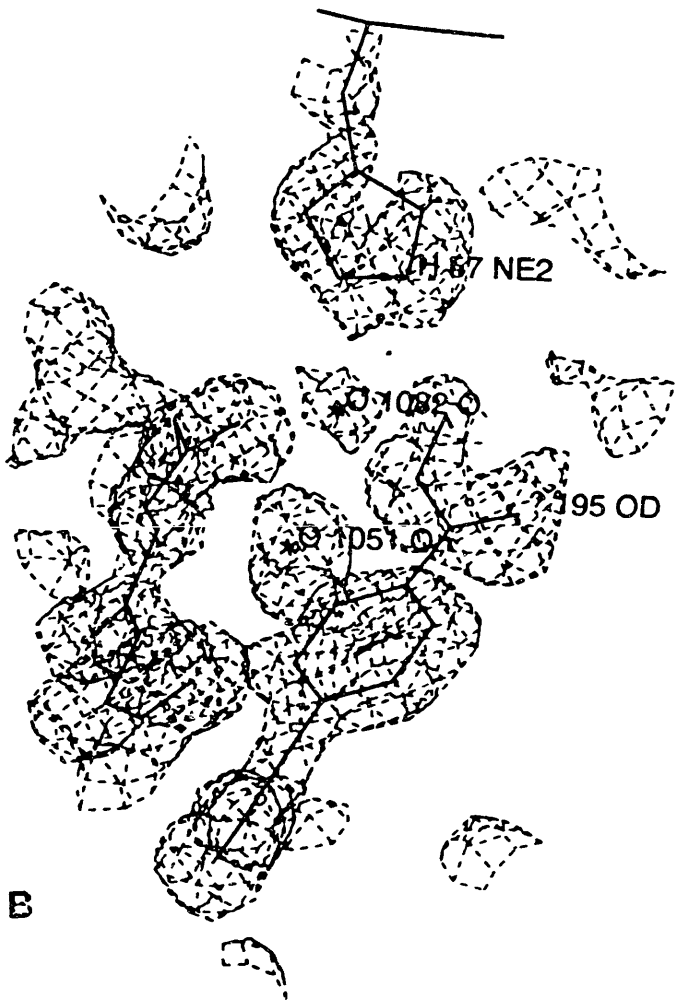

(a)
Ar Kα
Focussed, 1:1 mirror
Pt L edges
Unfocussed
Counts/100mA
10^4
10^3
10^2
10^1
10^0
(b)
Ar Kα
Pt L edges
Ratio
120.0
100.0
80.0
60.0
40.0
20.0
0.0
0
2
4
6
8
10
12
14
16
18
20
X-ray Energy (keV)

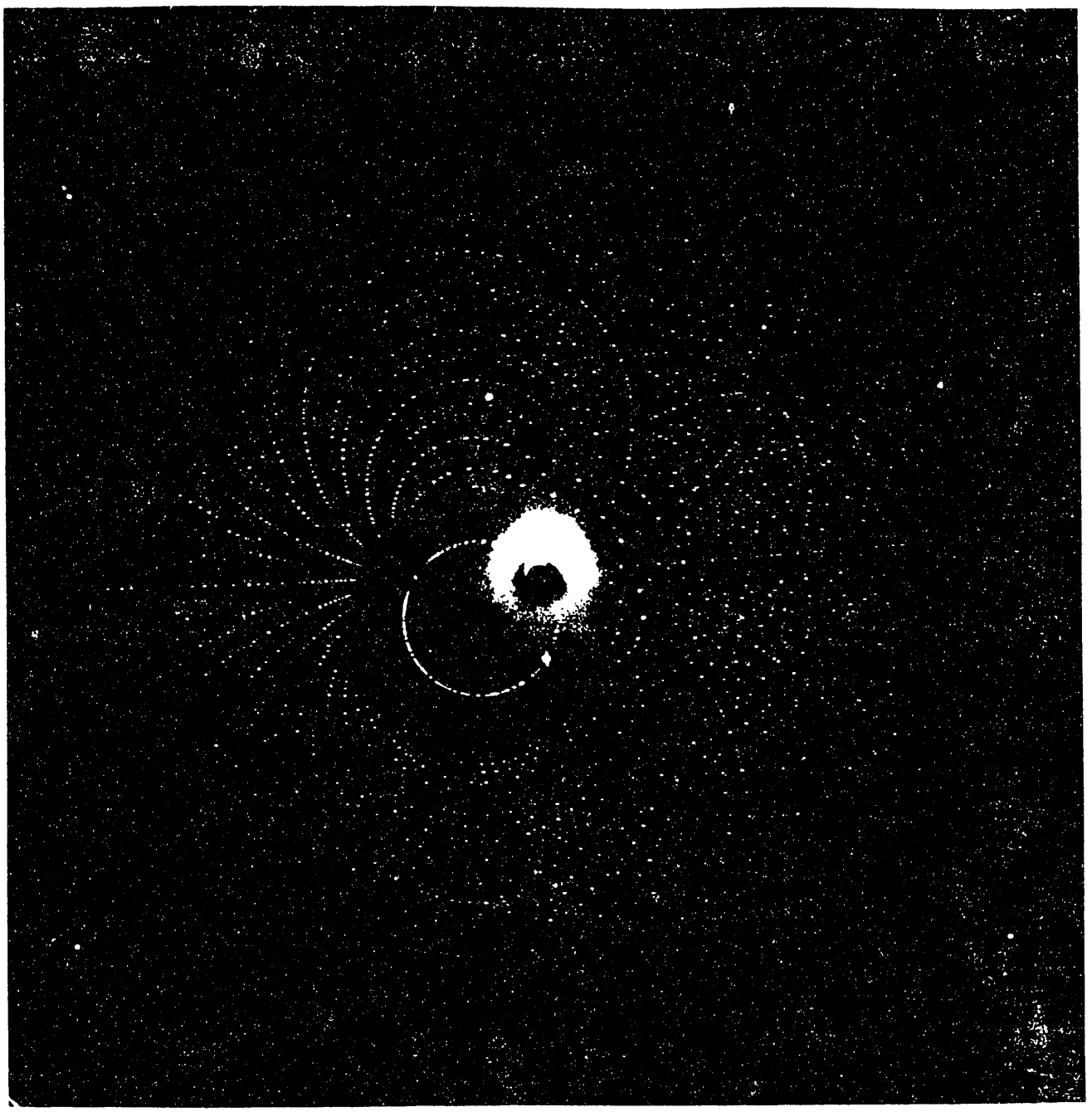


Block Diagram of X-ray and Optical Apparatus For Time Resolved Crystallography


A


A


B



END

DATE
FILMED
5/20/93