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REVIEW

Time-resolved protein crystallography

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The Brookhaven Protein Data Bank now contains over 850 coordinate listings. Sixty-three new atomic coordinate sets were released in the January quarterly bulletin. With the obligation on authors to deposit coordinates, many entries represent a single amino acid mutant or a ligand binding study, and probably less than a third of these entries represent new structures. Nevertheless the figures indicate the explosion of successful structure determinations and the increased efficiency of both protein crystallographers and NMR spectroscopists. For how many of these structures do we understand the biological mechanism? The description of the constellation of atoms at the important sites almost always provides a unique insight into possible biological mechanisms, but to unravel the specific chemical and stereochemical details that are important in reaction requires a series of snapshots on the reaction pathway. In conventional protein crystallography, such data are usually obtained from studies on complexes with pseudosubstrates, inhibitors, and transition state analogues. The hope that these static pictures might be turned into dynamic pictures of transient structures whose lifetimes are too short for conventional X-ray methods has roused intense interest in the Laue diffraction methods. In the Laue method with bright synchrotron radiation sources, X-ray data may be obtained within seconds to milliseconds and there is one example of a picosecond recording time. Yet the method is not without its problems, and the full exploitation of the technology has required developments in physics and in the chemistry and biochemistry of certain well-defined proteins. A meeting organized at the Royal Society in London by D.W.J. Cruickshank, J.R. Helliwell, L.N. Johnson, K. Moffat, and D.C. Phillips in January 1992 provided a timely assessment of the field. The proceedings were to be published in August (*Philos. Trans. R. Soc. Lond. A* 340, 1992, and as a book by Oxford University Press). This review provides a summary of the meeting.

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Monochromatic and Laue methods

The achievements of conventional methods of protein crystallography with monochromatic radiation provide a solid basis for understanding. The advantages of monochromatic data are their reliability and precision. D.M. Blow showed how a series of conventional experiments with data from various complexes (including open and closed sugars) representing structures along the reaction pathway had led to proposals for the mechanism of xylose isomerase. By exploiting the low activity of the enzyme in the presence of Al^{3+}, the xylose/xylulose equilibrium in the presence of Mg^{2+} and Al^{3+} had resulted in a structure that mimicked the transition state of the isomerization. A conformational change had placed the cation close to a position in which it could act to polarize the relevant C1-O and C2-O bonds (Collyer et al., 1990). The major disadvantage of conventional methods is the long time scale required for data collection, which limits the studies to those of stable structures, but new developments have shortened these times. Using the giant Weissenberg camera developed by N. Sakabe at the Photon Factory in Japan (Sakabe, 1983), J. Hajdu reported data collection to 1.5 Å resolution for ribulose-1,5-bisphosphate carboxylase/oxygenase with exposure times less than 20 min (total data collection time was 2 h) (Andersson et al., 1991). This is an extraordinary achievement for one of the largest proteins under crystallographic analysis (molecular weight of the hexadecamer 550,000). In these experiments and those reported from the EMBL facility on the Deutsche Elektronen Synchrotron (DESY) at Hamburg by K.S. Wilson, a key factor has been the introduction of image plates (based on storage phosphor technology) that can reduce exposure times by at least an order of magnitude. Image plates are more sensitive than X-ray film especially at short wavelengths where crystals are usually more resistant to radiation damage and the

plates exhibit wide dynamic range with reasonable spatial resolution. Image plates are now available at most protein crystallographic installations at synchrotron radiation facilities.

The Laue method uses a stationary crystal and a spectrum of polychromatic radiation (outer limits usually 0.25 and 2.5 Å in protein crystallography) so that all Bragg reflections generated by the different wavelengths are recorded on the film. In crystals that exhibit high symmetry (e.g., cubic crystals), data can be recorded with just a single exposure, but for lower symmetry space groups two to five exposures may be required. In a discussion on the early history of the Laue method, D.W.J. Cruickshank traced the line of communication from von Laue's original experiment in 1912 through P.P. Ewald in Munich and S. Nishikawa in Japan and then to R.W.G. Wyckoff, R.G. Dickinson, and L. Pauling in the USA. The Laue method was used effectively for structure determination throughout the twenties. In hexamethylenetetramine, the first X-ray structure determination of an organic compound, Dickinson and Raymond (1923) determined the atomic positions to a precision of 0.02 Å of a more recent structure determination. In these studies the X-rays were generated from a conventional tube and a rather short wavelength range of 0.24–0.48 Å was used partly to avoid the silver absorption edge of the X-ray film and partly to avoid confusion between first and second order reflections. The introduction of monochromatic recording methods gave more complete data, important for Fourier methods, and the data were simpler to measure because there was no correction for wavelength-dependent effects. The Laue method fell into disuse. The realization that the intense beam of the synchrotron radiation source provided a wide spectral range, and the demonstration of the spectacular number of spots recorded with protein crystals (Fig. 1) led to a revival of the method in 1984 (reviewed in Moffat, 1989; Hajdu & Johnson, 1990; Helliwell, 1992). With the availability of technology for fast diffraction methods, interest has now focused on what biochemical reactions can be studied on the appropriate time scale, how to ensure that the start of the reaction coincides with the start of the data collection, and how to detect reaction intermediates, their lifetimes, and population distribution. An ideal system is one where a defined intermediate accumulates over a period of time that is long compared with the time taken to record the diffraction pattern so that there is a homogeneous population of molecules in the crystal. Accordingly, recent efforts have concentrated on ways of initiating the reaction in the crystal and diagnostic observations of the interconversion of reactants.

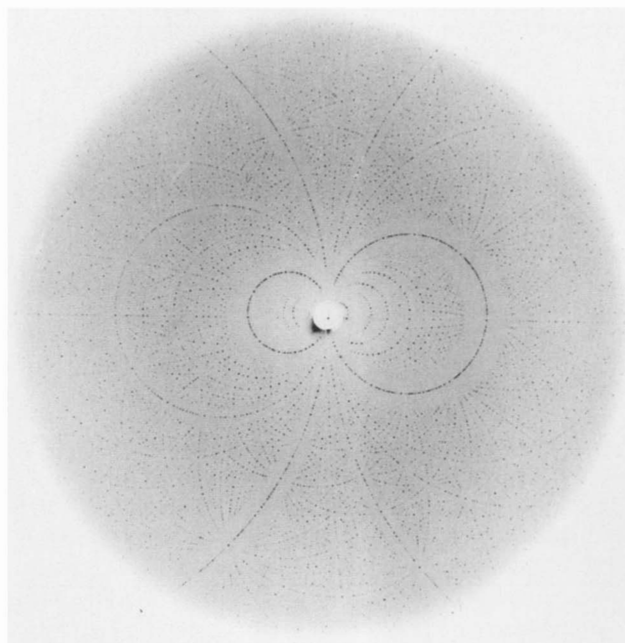

Fig. 1. Laue photograph of a glycogen phosphorylase *b* crystal recorded at the Synchrotron Radiation Source, Daresbury.

Initiation and monitoring of reactions

The method of choice for fast initiation of reaction is the use of photolabile "caged" compounds. The reactant is introduced into the protein or protein crystal in a blocked form and saturates the relevant binding sites. The reaction is then initiated by a flash of light that releases the photolabile blocking group. D.R. Trentham described the practical and theoretical considerations in the use of various caged compounds (McCray & Trentham, 1989). The major technical problem is to achieve sufficient conversion of caged compound in a single pulse of light so that all protein sites are occupied by the photoreleased ligand. Photosensitive compounds in which the functional groups are protected by a 1-(2-nitrophenyl)ethyl group have proved effective probes in physiological experiments and are fairly rapidly hydrolyzed with product quantum yield approximately 0.5 and photolysis rate of 100 s^{-1} for phosphate esters. 3,5-Dinitrophenyl and 3′,5′-dimethoxybenzoin esters are also potentially useful. 3,5-Dinitrophenyl phosphate has a quantum yield of 0.67 and releases P_i at $>10^4$ s^{-1}, but the dinitrophenyl group is not a general photosensitive blocking group with other compounds. The 3,5-dimethoxybenzoin ester of ATP photolyzes at $>10^5$ s^{-1} and is a promising compound for the future.

The most notable example in protein crystallography of the use of a caged compound to initiate the reaction has been the work with H-ras p21 to produce results on the active GTP complex. In this work reported by E.F. Pai, the protein was crystallized with stoichiometric amounts of the 1-(2-nitrophenyl)ethyl ester of GTP. The caged compound binds with a dissociation constant of 10^{10} M^{-1}, and this means that the reaction can be carried out without any excess caged compound. As described in work

that is already published (Schlichting et al., 1990), the reaction was triggered by a series of pulses from a xenon flash lamp to yield the GTP complex, which has a half life of 40 min. Data collection by Laue methods at DESY, Hamburg approximately 4 min after the photolytic removal of the protecting group allowed the p21–GTP structure to be determined. Subsequent data collections showed the conversion to GDP in the crystal. In new work, the structure of the p21 protein complexed with a single diastereomer of caged GTP was reported in which the caged compound, in contrast to previous studies with mixed diastereomers, bound in a similar way to that observed for GTP. Thus the caged compound is bound at the catalytic site, and there is no diffusion required.

This is in contrast to the situation in glycogen phosphorylase where the low affinity for the phosphate substrate in the T state crystals presents problems. L.N. Johnson reported progress in the use of 3,5-dinitrophenylphosphate (DNPP) as a caged substrate. After diffusion of DNPP into the T state phosphorylase crystals at concentrations between 30 and 45 mM, the caged compound bound at the allosteric effector site and the nucleoside inhibitor site of the allosteric enzyme but not at the catalytic site. The concentration of phosphorylase in the crystal is about 7 mM, and hence there was unbound DNPP in the interstitial spaces of the crystal. Presumably on photolysis, the liberated phosphate diffuses to the catalytic site from these interstitial spaces in the crystal lattice. The distances are of the order of 10–50 Å and considerably shorter than distances of the order of 0.1 mm that would be required if the reaction were to be initiated by diffusion of ligand into the crystal. In trial experiments with the home source it had been shown that it was possible to release sufficient phosphate within 30 s from a 30 mM DNPP solution to generate catalysis and the conversion of heptenitol (a small molecular weight substrate) to heptulose-2-phosphate had been observed in the crystal (Duke et al., 1991). However in Laue diffraction experiments, where data had been collected at the Synchrotron Radiation Source (SRS), Daresbury within 3 min of initiation of the reaction with three exposures each of 800 ms, no phosphate was observed bound at the catalytic site. The low affinity for phosphate (K_m about 35 mM) requires that very high concentrations of the caged compound are needed. Under these conditions absorption of the illuminating light diminishes the effectiveness of release. A possible solution will be to work with the R state crystals where the K_m for substrate is about 3 mM but the R state crystals present a more difficult crystallographic problem because of the large unit cell and low symmetry.

In an alternative approach, D. Ringe described work in which the enzyme was caged. Building on preliminary experiments on caging γ-chymotrypsin with a mechanism-based photoreversible inhibitor developed in N.A. Porter's laboratory (Stoddard et al., 1991), Ringe and colleagues reacted a crystal of γ-chymotrypsin with trans-*p*-diethyl-amino-*o*-hydroxy-α-methylcinnamate to form a covalent adduct to the serine 195 (Fig. 2). The stability of this adduct appears to result from the fact that the carbonyl oxygen of the inhibitor is directed away from the oxyanion hole toward the solvent. Upon irradiation with UV light, the cinnamate isomerizes and deacylates, presumably by a nonenzymic reaction. Solution experiments indicated that the caged enzyme is less than 3% active and that the enzyme regains 90% of activity after irradiation (Stoddard et al., 1990). In the diffraction experiment the crystal soak solution also contained 3-benzyl-6-chloro-2-pyrone, another mechanism-based inhibitor. A series of single exposure Laue diffraction patterns were recorded at DESY, over a period ranging from $t = 0$ min (before irradiation) to $t = 24$ h to follow photorelease of the cinnamate followed by its replacement with the acyl-pyrone. The crystal was exposed to a 1-ms flash from a xenon flashlamp at $t = 5$ min simultaneously with an X-ray exposure lasting 5 s. The processing of these photographs led to a series of structures that have been interpreted as shown in Figure 2. In the initial (dark) experiment on the caged enzyme the cinnamoyl group is observed bound to the serine (Fig. 2A). The diffraction pattern recorded concurrently with photolysis showed streaking of spots, as observed with monochromatic studies and in studies on other systems reported by other workers. Consequently, the quality of the electron-density map was poorer than for others but showed the appearance of a weakly bound 10-membered bicyclic coumarin ring and the serine γ-oxygen free of covalently bound inhibitor (Fig. 2B). One minute after photolysis, free enzyme is observed (with the possibility of a small amount of peptide bound that is not displaced by the cinnamate) (Fig. 2C), and this structure persisted for more than 20 min after irradiation in a somewhat surprising reaction scheme. By 3 h after the flash, density corresponding to the acyl-pyrone complex was observed at the catalytic site and this structure remains stable (half life for inactivation by this inhibitor under similar conditions in solution is 65 min) for at least 24 h in the crystal (Fig. 2D). Thus the results give a series of snapshots documenting the course of the two reactions followed. The problem of triggering the reaction has been solved.

Other methods for initiating reaction have been explored. The method of initiation of reaction by diffusion of reactants into the crystal can still be effective when the ligand itself is small and the reaction intermediate lifetime is relatively long. J. Hajdu described experiments in which cytochrome *c* peroxidase was reacted with hydrogen peroxide in the flow cell and the structure of the resulting intermediate, compound I (half life about 20 min), was studied with Laue diffraction. Data were recorded within 10 s. The resulting difference maps provided positive features for the Fe^{4+}-oxy intermediate and indicated that the intermediate had been caught successfully

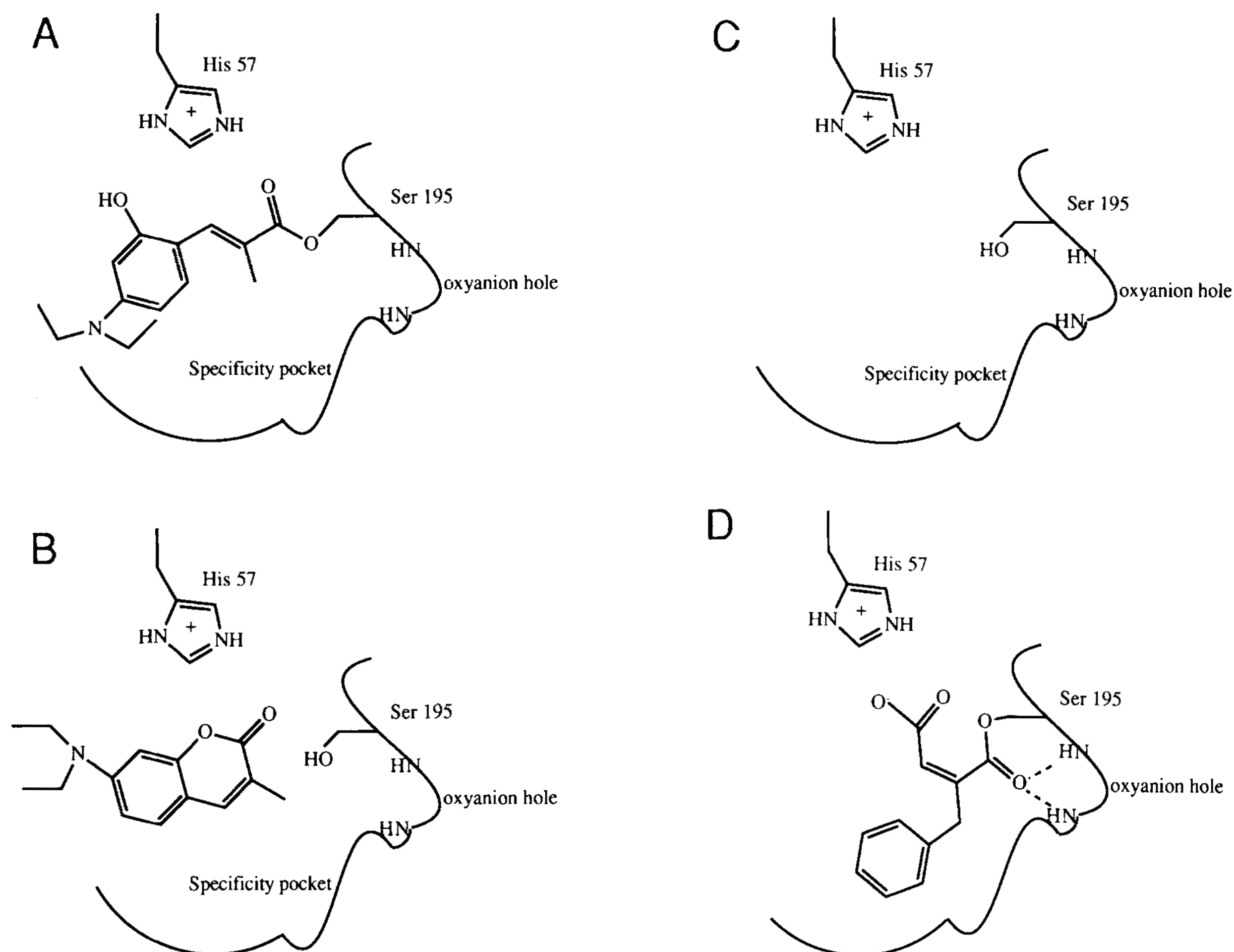

Fig. 2. Reaction sequence for the light induced deacylation of the enzyme-cinnamate γ-chymotrypsin complex followed by binding and reaction of pyrone to give the acyl-pyrone derivative complex. **A:** The caged enzyme at $t = 0$ with cinnamate covalently bound to Ser 195. **B:** The structure of the photolysis product at $t = 5$ min. The diffraction pattern was disordered but the electron density suggests a structure consistent with the coumarin ring. **C:** The structure of the free enzyme at $t = 6$ min. The photolysis products have diffused away. The pyrone has not yet reacted ($t_{1/2} = 65$ min). **D:** The structure of the pyrone-bound complex at $t = 3$ h. The inhibitor is covalently bound to Ser 195. (Redrawn from Stoddard et al. [1991].)

in the crystal. H.D. Bartunik described cryoenzymology studies in which a temperature jump was used to initiate the reaction. A crystal of elastase was cooled to 220 K in the presence of 70% methanol and then reacted with the peptide Boc-Pro-Ala-Ala-OMe to form an acyl enzyme intermediate. Scanning-Laue photographs (Bartunik et al., 1992) over a range of 1 Å, each taking 10 s, were recorded at temperatures increasing in steps by 5–10 K. At around 250 K there was a dramatic change in the diffraction pattern indicative of disorder that might have been caused by completion of the reaction in the crystal. These data have yet to be processed to produce electron-density maps. A conventional study with monochromatic data confirmed the presence of the acyl intermediate at 244 K. R.M. Sweet and colleagues have used pH jump to probe the steps in the deacylation of guanidinobenzoyl-Ser-195 trypsin. The acyl intermediate is stable for long periods at pH 5.5 (Mangel et al., 1990), but deacylation proceeds with a half life of about 1 h at pH 8.9. Laue diffraction data were recorded at the Brookhaven Laboratory's National Synchrotron Light Source at low pH and 3 min and 90 min after the pH had been raised to 8.9 by use of a flow cell. The structures obtained from the Laue measurements had been refined by crystallographic restrained least-squares methods with data that were 60–70% complete from five photographs. Three minutes after the pH jump a shift in water molecules at the catalytic site was observed and a new water was bound at a position consistent with its role in deacylation. After 90 min the electron density for the guanidinobenzoyl group was substantially diminished, indicative that catalysis was in progress. A favorable system for reaction is represented by the photoactive yellow protein. This small (MW 14,000) photoreceptor protein undergoes a photocycle on illumination for which the kinetics reveal a series of spectrally distinct intermediates that decay via first order reactions

and whose lifetimes are quite different (Meyer et al., 1989). The quantum yield for photoinitiation is high 0.64. K. Moffat described spectroscopic experiments to examine conditions for bleaching, stabilization of intermediate states, and characterization of the diffraction pattern.

As is evident in these studies there is a need to know the stage of reaction in the crystal so that the relevant time window can be used to catch an appropriate intermediate. Many of the systems studied give rise to a distinct spectral signal and this allows reactions to be monitored. G.L. Rossi described experiments from his laboratory over the last 20 years with a sophisticated polarized absorption microspectrophotometer. Studies with γ-chymotrypsin, aspartate aminotransferase, hemoglobin, and tryptophan synthase had allowed detailed comparisons with the behavior of the protein in the crystal and in solution (e.g., Rossi & Bernhard, 1970). It was encouraging to note that when mixtures of several species accumulate in the crystal as a result of reaction it was possible to determine the concentration of the individual species from knowledge of their spectral characteristics (Mozzarelli et al., 1991). A number of microspectrophotometers have now been developed for similar crystal studies, some with time resolutions of 20 ms for a spectra, and several reports described prior monitoring of the events in the crystal. For example the liberation of phosphate from the caged phosphate compound, DNPP, in work with phosphorylase is accompanied by an increase in absorption at 400 nm from the released cage dinitrophenol. This diagnostic was used to determine the concentration of phosphate in the crystal.

The quality of Laue data

In many of the experiments reported, exposure times ranged from milliseconds to a few seconds, and total data collection times were usually in the range between 1 and 30 s. With such rapid accumulation of data, what is the quality of the resulting electron-density maps and refined structures? The results reported for the small proteins (chymotrypsin, trypsin, elastase, p21, cytochrome *c* peroxidase, and carbonic anhydrase) gave cause for considerable optimism that high quality structures could result. A. Liljas described results on carbonic anhydrase complexed with HSO^{3-} obtained by Laue data recorded at SRS, Daresbury and by monochromatic measurements (Lindahl et al., 1992). The Laue maps showed the bisulfite ion bound with one of its oxygens at the position of the zinc water, and this clear result was confirmed by the monochromatic maps. In a study at pH 6, the Laue data showed the zinc still bound to a water molecule as at higher pH and thus demonstrated the ability to distinguish between a tetra and a penta coordinated zinc atom by Laue crystallography. In work of Sweet and colleagues a comparison of refined trypsin structures based on Laue and monochromatic data at 2 Å resolution showed closely identical structures with root mean square (rms) differences in coordinates of all atoms of about 0.2 Å, close to the limit imposed by the quality of the data and refinement protocols. Even the constellations of water molecules were similar in the maps derived from the two different data collection strategies. These results give encouragement that subtle structural features may be discerned with Laue data. In a small molecule structure refinement, Laue data were of sufficient precision to detect positions of hydrogen atoms (Helliwell et al., 1989).

Calculations of reflections predicted to be recorded on the films with a few Laue photographs indicate that data sets nominally about 85% complete can be recorded (in work with trypsin and carbonic anhydrase where time resolution was not important, data sets 60–70% complete were obtained). For larger proteins or when fine time resolution is required, Laue data sets may be only 30–40% complete. There are a number of limitations that give rise to incomplete data sets. A substantial proportion of reflections are rejected in the data processing because intensities are judged unreliable if their intensity is less than a few standard deviations, because reflections are judged too close in space to deconvolute if their spot separation is close (<0.1 mm), because of wavelength cutoffs introduced in the data processing to optimize precise measurements, and because harmonic reflections generated by different wavelengths that superimpose on the photograph are not unscrambled (e.g., the reflections 110 generated at λ and 220 generated at $\lambda/2$ will superimpose on the film). Does the partial character of Laue data sets matter? The above examples with small proteins suggest that the answer is no; yet a more detailed analysis of these effects with the larger protein phosphorylase (MW 97,000) suggested that systematic loss of the low resolution terms severely distorts difference electron-density maps. Many of the low resolution terms that are recorded are not measured because wavelength multiplets have not been unscrambled. Methods for unscrambling these multiplets are available that depend on estimates of the wavelength dependence of the absorption of the X-ray film. Inclusion of these terms greatly improves the quality of the difference maps. Once a suitable interpretation of an electron-density map has been obtained, then the effects of partial data sets in crystallographic least-squares refinement appear to be less severe. For example, K. Moffat reported that with a small organic molecule of known structure Laue data gave a data set that was 52% complete to 1.1 Å resolution, and although the structure could not be determined ab initio from these data, it was possible to refine it from a starting model.

Prospects

The third generation of synchrotron sources is about to come on line, such as the European Synchrotron Radiation Facility (ESRF) at Grenoble and the Advanced Pho-

ton Source (APS) at Argonne, Chicago, and there is a need to meet the increased brightness with increased detector efficiency. Film has been the method of choice in Laue work with the facility to unscramble wavelength overlaps, but image plates offer greater sensitivity and precision. J.R. Helliwell described the comparative time responses of various detectors and recording systems that includes practical considerations of film change times and readout facilities. Charge coupled devices (CCD) may be of interest for the future. At present the active area in these devices is too small to record the whole of a diffraction pattern, but Helliwell described an application in which a CCD device area 6×8 mm^2 was used to record part of a Laue diffraction pattern from concanavalin A. A series of 120-ms exposures of 35 shots recorded over a total time of 190 s were used to demonstrate radiation damage. Such an approach could be used as a diagnostic in preliminary experiments to determine the appropriate times to record complete data. K. Moffat and his colleagues have pioneered the very shortest exposures for crystals with their report of picosecond recording times for lysozyme that exploited the pulsed nature of the synchrotron radiation when operated in single bunch mode (Szebenyi et al., 1992). In a new experiment with the CHESS/Argonne undulator at the Cornell Electron-Positron Storage Ring, 120-ps Laue data were collected from crystals of a diterpenoid, briarane B, using a Kodak storage phosphor detector, an ultrafast shutter, and single bunch currents up to 49 mA. The accomplishment of this technically difficult experiment emphasizes more than ever the need for advances in the chemistry to understand the time processes in the crystal and to provide rapid initiation of reactions.

In a perceptive summary of the meeting, G.A. Petsko provided a critique of some problems that still remain. First is the requirement for highly ordered crystals. The Laue method is most sensitive for detection of disorder in crystals. In the work with wild-type H-ras p21, only 1 out of 11 crystals was found to be sufficiently well ordered (but crystals of a mutant protein [Pro 12] were very much better and crystals of another mutant [Val 120] were not usable at all). In addition, diffusion of ligands or triggering reactions with light also leads to a transient disorder of the lattice in many of the systems described, and although the crystals heal within minutes, the diffraction pattern is lost at the very time the reaction process may be of greatest interest. Radiation damage continues to be a severe problem, and as synchrotrons become brighter the difficulties in recording more than one exposure from a portion of the crystal will become more severe. Petsko commented on the need to develop chemical protection schemes and described the advantages in use of the flow cell and the presence of high concentrations of reducing agents such as dithiothreitol.

Cooling offers a possible relief in some cases, and interesting insights into why enzymes may be inactive at very low temperatures are beginning to emerge. Many systems show a broad transition around 220 K both in experimental and in molecular dynamics simulations. The transition is thought to be associated with the change in vibration mode of the atoms. In recent work, Rasmussen et al. (1992) showed that ribonuclease is unable to bind substrate at 212 K but will bind at 228 K, with the strong implications that flexibility and mobility are required for protein functions of recognition and catalysis.

Summary

Advances in synchrotron radiation technology have allowed exposure times from protein crystals of the order of milliseconds to be used routinely, and in exceptional circumstances exposure times of 100 ps have been obtained. However, many data sets take seconds to record because of the slow time scale of film change or crystal reorientation or translation when more than one exposure is required. This problem has been addressed by Amemiya et al. (1989).

There has been considerable progress in methods to initiate reactions in protein crystals, especially the development of photolabile caged compounds but also temperature jump, pH jump, and diffusion. Although flash lamps deliver pulses of 100 mJ/ms, often several pulses are required to release sufficient product, and reaction initiation can take several seconds. Laser illumination can provide more powerful input, but the laser must be accommodated within the restricted space at the synchrotron station.

The requirement to maintain synchrony among the molecules in the crystal lattice as the reaction proceeds and to ensure that the lifetime of intermediates is longer than data collection rates emphasizes the need for chemical characterization of the reaction under study. As Ringe advocated in the studies with chymotrypsin, it may be more profitable to devise conditions under which certain intermediates along the reaction pathway accumulate in the crystal and to record these in a series of discrete steps rather than continuous monitoring of the reaction.

The Laue method is limited to those proteins that give well-ordered crystals and problems of transient disorder on initiation of reaction and problems of radiation damage need to be overcome or avoided by suitable experimental protocols.

The Laue method gives reliable structural information for small protein molecules. For larger proteins or for ab initio structure determination, the low resolution terms are important. Methods applicable for data recorded on multiple film packs allow unscrambling of those spots that occur as wavelength overlaps of multiple reflections. These data comprise a substantial proportion of the low resolution terms, and their inclusion leads to improved electron-density maps.

Time-resolved macromolecular crystallography is at

present applicable to a small number of systems for which the reaction intermediates have lifetimes of the order of seconds. Several reactions might be brought into this time domain through a combination of moderately low temperature (e.g., $-10\,^{\circ}C$), slow substrates, or mutant enzymes. The method is also limited to those proteins that can undergo their biological activity in the crystal, including complete conformational response, without restrictions from the crystal lattice. Nevertheless the opportunity to produce dynamic descriptions of protein molecules and to extend our understanding of the structural basis of biological function in the fourth domain of time opens up fascinating research and the promise of new insights.

References

Amemiya, Y., Kishimoto, S., Matsushita, T., Satow, Y., & Ando, M. (1989). Imaging plate for time resolved X-ray measurements. *Rev. Sci. Instr. 60*, 1552-1556.

Andersson, I., Clifton, I.J., Fulop, V., & Hajdu, J. (1991). High speed high resolution data collection on spinach Rubisco using a Weisenberg camera at the Photon Factory. In *Crystallographic Computing 5: From Chemistry to Biology* (Mora, D., Podjarny, A.D., & Thierry, J.C., Eds.), pp. 20-28. Oxford University Press, Oxford.

Bartunik, H.D., Bartsch, H.H., & Qichen, H. (1992). Accuracy in Laue X-ray diffraction analysis of protein structures. *Acta Crystallogr. A48*, 180-188.

Collyer, C.A., Henrick, K., & Blow, D.M. (1990). Mechanism for aldose/ketose conversion by D-xylose isomerase involving ring-opening by a 1,2-hydride shift. *J. Mol. Biol. 212*, 211-235.

Dickinson, R.G. & Raymond, A.L. (1923). The crystal structure of hexamethylenetetramine. *J. Am. Chem. Soc. 45*, 22-29.

Duke, E.M.H., Hadfield, A., Martin, J.L., Clifton, I.C., Hajdu, J., Johnson, L.N., Reid, G.P., Trentham, D.R., Bruce, I., & Fleet, G.W.R. (1991). Towards time resolved diffraction studies with glycogen phosphorylase. In *Protein Conformation. Ciba Foundation Symposium*, Vol. 161, pp. 75-90. Wiley, Chichester, UK.

Hajdu, J. & Johnson, L.N. (1990). Progress with Laue diffraction on protein and virus crystals. *Biochemistry 29*, 1669-1678.

Helliwell, J.R. (1992). *Macromolecular Crystallography with Synchrotron Radiation*. Cambridge University Press, Cambridge, UK.

Helliwell, M., Gomez de Anderez, D., Habash, J., Helliwell, J.R., & Vernon, J. (1989). A comparison of Laue and monochromatic X-ray analyses: The determination of the hydrogen atom positions of an organic small molecule. *Acta Crystallogr. B45*, 591-596.

Lindahl, M., Habash, J., Harrop, S., Helliwell, J.R., & Liljas, A. (1992). The sensitivity of the Laue method to small structural changes: Binding studies of human carbonic anhydrase II. *Acta Crystallogr. B48*, 281-285.

Mangel, W.F., Singer, P.T., Cyr, D.M., Umland, T.C., Toledo, D.L., Stroud, R.M., Pflugrath, J.W., & Sweet, R.M. (1990). Structure of an acyl-enzyme intermediate during catalysis: Guanidinobenzoyl trypsin. *Biochemistry 29*, 8351-8357.

McCray, J.A. & Trentham, D.R. (1989). Properties and uses of photoreactive caged compounds. *Annu. Rev. Biophys. Biophys. Chem. 18*, 239-270.

Meyer, T.E., Tollin, G., Hazzard, J.H., & Cusanovich, M.A. (1989). Photoactive yellow protein from the purple phototrophic bacterium, *Ectothiorhodospira halophila*. *Biophys. J. 56*, 559-564.

Moffat, K. (1989). Time resolved macromolecular crystallography. *Annu. Rev. Biophys. Biophys. Chem. 18*, 309-332.

Mozzarelli, A., Rivetti, C., Rossi, G.L., Henry, E.R., & Eaton, W.A. (1991). Crystals of haemoglobin with the T quaternary structure bind oxygen non-cooperatively with no Bohr effect. *Nature 351*, 416-419.

Rasmussen, B.F., Stock, A.M., Ringe, D., & Petsko, G.A. (1992). Crystalline ribonuclease A loses function below the dynamical transition at 220 K. *Nature 357*, 423-424.

Rossi, G.L. & Bernhard, S.A. (1970). Are the structure and function of an enzyme the same in aqueous solution and in the wet crystal? *J. Mol. Biol. 49*, 85-91.

Sakabe, N. (1983). A focussing Weisenberg camera with multilayer line screens for macromolecular crystallography. *J. Appl. Crystallogr. 16*, 542-547.

Schlichting, I., Almo, S.C., Rapp, G., Wilson, K.S., Petratos, K., Lentfer, A., Wittinghofer, A., Kabsch, W., Pai, E.F., Petsko, G.A., & Goody, R.S. (1990). Time resolved X-ray structural studies on H-ras p21: Identification of the conformational change induced by GTP hydrolysis. *Nature 345*, 309-315.

Stoddard, B.L., Bruhnke, P., Koenigs, P., Porter, N., Ringe, D., & Petsko, G.A. (1990). Photolysis and deacylation of inhibited chymotrypsin. *Biochemistry 29*, 8042-8051.

Stoddard, B.L., Bruhnke, P., Porter, N., Petratos, K., Petsko, G.A., & Ringe, D. (1991). Observation of the light triggered binding of pyrone to chymotrypsin by Laue X-ray crystallography. *Proc. Natl. Acad. Sci. USA 88*, 5503-5507.

Szebenyi, D.M.E., Bilderback, D.H., Legrand, A., Moffat, K., Schildkamp, W., Smith Temple, B., & Teng, T.-Y. (1992). Quantitative analysis of Laue diffraction patterns recorded with a 120 picosecond exposure from X-ray undulator. *J. Appl. Crystallogr. 25*, 414-423.