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Time-Resolved X-Ray Diffraction Studies of Enzymes under Cryoconditions [and Discussion]

Hans D. Bartunik, Lesley J. Bartunik, Hans Viehmann, J. Hajdu and G. A. Petsko

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Time-resolved X-ray diffraction studies of enzymes under cryoconditions

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Cryoenzymological techniques provide a means of initiating enzymatic reactions in crystals homogeneously and of prolonging the lifetimes of intermediate reaction steps. Catalytic intermediates may accumulate to sufficiently high population for X-ray crystal structure analysis by time-resolved monochromatic or Laue diffraction data collection using synchrotron radiation. Due to short exposure times, intermediates may be studied at moderately low temperatures. A combination of cryoenzymology and time-resolved crystallography has been applied to crystal structure analysis at high resolution of an acyl-enzyme intermediate of a productive reaction catalysed by porcine pancreatic elastase.

1. Introduction

Eigen (1967) suggested stopping enzymatic reaction pathways temporarily in order to study intermediates under defined conditions. The development of cryo-enzymological techniques provided a means of maintaining the activities of certain enzyme systems in cryosolvents at subzero temperatures, but with considerably slower reaction speeds (Douzou 1971, 1977). The conditions for accumulating specific intermediates were investigated for a number of enzymatic reactions including, in particular, serine protease catalysis (Douzou 1971; Fink & Ahmed 1976; Fink 1976; Fink & Cartwright 1981). Petsko was the first to combine cryoenzymological and X-ray diffraction techniques (for a review see Douzou & Petsko 1984), and to attempt an X-ray crystal structure analysis of an enzymatic reaction intermediate trapped at low temperatures (Alber *et al.* 1976). The structural analysis was limited to medium resolution; it was therefore not possible to derive detailed information on interactions between enzyme and substrate.

The availability of intense X-radiation from storage rings and of area detectors reduced the time required for diffraction data collection by monochromatic techniques by 3–4 orders of magnitude. The possibility of applying Laue diffraction techniques to protein structures (Moffat *et al.* 1984; Helliwell 1985; Cruickshank *et al.* 1987) in theory even provides the basis for one-shot time-resolved studies on very short timescales. At presently operated (second generation) synchrotron radiation sources, white-beam Laue diffraction exposures extending to high resolution may be recorded within a few milliseconds, at least in the case of well-diffracting crystals of medium-sized structures. Such a time resolution is comparable with typical lifetimes of enzymatic intermediates at room temperature.

Broad-bandpass Laue methods are only applicable if the crystal lattice is highly

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ordered. In studies of enzymatic reactions external initiation of the reaction, and conformational changes involved in structural transitions, may cause substantial broadening in the crystal mosaicity. Even under such conditions, scanning Laue methods or reduced-bandwidth Laue methods (Bartunik & Borchert 1989) may be used; they involve significantly shorter timescales than conventional monochromatic crystal rotation techniques. However, crystal cooling to moderately low subzero temperatures is required. A combination of cryo and time-resolved diffraction techniques has been applied at 244–264 K to crystal structure analysis at 2.0 Å† resolution of an acyl-enzyme intermediate in the proteolytic degradation of Boc–Pro–Ala–Ala–OMe by porcine pancreatic elastase. This application illustrates the potential, but also some of the limitations, of crystallographic investigations of unstable states.

2. Cryoenzymological techniques

Conventional methods of cryoenzymology involve mixed organic/aqueous solvents as cryosolvents. Stepwise increase in the concentration of such cryosolvents under simultaneous reduction in the temperature preserves the dielectric constant. Suitable buffers stabilize the protonic activity. These techniques were reviewed by Douzou and Petsko (Douzou 1977; Douzou & Petsko 1984). In crystallographic applications, aqueous methanol has frequently been used as a cryosolvent, because of its low viscosity; the freezing point of 70% aqueous methanol is near 150 K (Douzou 1977).

Organic cryosolvents may raise the K_m of the reaction (Fink & Cartwright 1981) and inactivate certain enzymes during turnover (Cartwright & Waley 1987; Malthouse 1986). As possible alternatives without such adverse effects, undercooled water (Douzou *et al.* 1978; Franks 1985) or salt solutions have been suggested. For temperatures above 215 K, it is feasible to use ammonium acetate solutions as cryosolvents (Cartwright & Waley 1987).

High population of an intermediate may be reached, if the decay of the intermediate has a smaller rate constant and larger activation energy than its formation. At room temperature, turnover occurs in solution on timescales ranging from 10 ns to 100 ms. The rate constant varies with $T \exp(-AE/T)$, where AE is the activation energy and T the ambient temperature; this corresponds to an Arrhenius behaviour. In the example given by Douzou (1971) of a reaction with an activation energy of 18 kcal mol^{-1}‡, cooling to 200 K reduces its speed by six orders of magnitude. An intermediate with a lifetime of 1 ms at room temperature may thus be stable for a few hours at 200 K. Even lower temperatures will generally not be of interest, since the increase in the viscosity of the solvent with decreasing temperature appears to damp fluctuations in the protein molecule (Singh *et al.* 1981); transitions to a glass-like state have been observed near 200 K for myoglobin by different spectroscopic techniques (Singh *et al.* 1981; Doster *et al.* 1989). In diffraction experiments using synchrotron radiation, exposure times may be so short that only cooling to moderately low temperatures around 250 K may be needed; this is further discussed below. A combination of low temperatures and low pH values may possibly favour the accumulation of intermediates (Fink 1976; Malthouse & Scott 1983).

† 1 Å = 10^{-10} m = 10^{-1} nm ‡ 1 cal ≈ 4.184 J

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3. Initiation and monitoring of reactions

Reaction initiation

Rapid initiation – as compared with the rate of turnover – of an enzymatic reaction within the entire crystal volume may be achieved by external activation of the substrate. Inactive caged metabolites may be introduced into the enzyme crystal by diffusion or co-crystallization and activated *in situ*, e.g. by laser or flash-lamp pulse photolysis (McCray *et al.* 1980). Such activation processes have lifetimes of about 100 μs to a few milliseconds.

Alternatively, the active substrate may be diffused into the crystal at a pH outside the activity range of the enzyme or at low temperatures; the enzymatic reaction may then be triggered by pH jumps and by rapid reheating respectively. Use of a flow cell is essential; therefore, low-viscosity cryosolvents have to be used. An example of such a cooling/reheating protocol is described below. A suitable cooling system with a flow cell was previously described (Bartunik & Schubert 1982). The system permits rapid up and down changes in the temperature with speeds in excess of 10 K s^{-1}. A more detailed description of crystal cooling and flow-cell techniques may be found elsewhere (Bartunik 1991).

Rapid stepwise reheating may be achieved with lasers. T-jumps by 5–10 K may be produced with a pulsed excimer laser emitting at or in the near ultraviolet. Alternatively, dyes with broad absorption bands in the visible range may be diffused into the enzyme crystal and then pumped with dye lasers or flash lamps. In general, dye concentrations in the crystal may be so small (less than 1%) that the diffraction experiment is not affected, even if the dye binds to the enzyme as a competitive inhibitor, like proflavin to chymotrypsin or trypsin.

T-jumps followed by rapid quenching to the initial low temperature may in theory be used for promoting an enzymatic reaction from one specific step to the next along the pathway. Such a combination of techniques may permit to study a series of different intermediates subsequently at a constant low temperature.

Optical spectroscopy and monitoring

Enzyme kinetics in solution and in the crystalline environment may differ substantially (see, for example, Merli & Rossi 1986). Therefore, optical spectroscopy on single crystals before X-ray diffraction experiments may be useful in determining suitable conditions for accumulating intermediates, for example in the case of substrates with photochromic leaving groups, dyes acting as competitive inhibitors, or photochromic coenzymes.

Optical monitoring during diffraction experiments may be useful for interactive optimization of experimental conditions. Broad-band spectroscopic measurements through capillary tubes as they are used in X-ray diffraction suffer from image distortions and scattering. Monochromatic monitoring may provide better conditions for quantitative applications, e.g. in the determination of the concentration of active ATP liberated in a hexokinase crystal by laser pulse photolysis (Heinecke *et al.* 1992).

We developed a microspectrophotometer for time-resolved transmission measurements on single crystals at temperatures down to 100 K (Heinecke *et al.* 1992). Spectra may be recorded either with incident white radiation and a linear diode-array spectrometer (280–750 nm), or with monochromatic radiation and a photomultiplier (350–950 nm). Using a flow cell for solvent exchange, conditions for the trapping of enzyme–substrate intermediates in single crystals have been investigated.

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Laue monitoring

The sensitivity of Laue diffraction patterns to crystalline disorder (see below) may be used to detect changes in cell parameters or in the lattice periodicity which will in general accompany conformational changes. An application of Laue monitoring is described below.

4. Crystal structural analysis of catalytic intermediates

(a) *Space and time averaging*

X-ray diffraction techniques average over all molecules in the irradiated crystal volume and over all structural states that are populated during the exposure time. In studies of enzyme kinetics, electron density maps will in general correspond to a superposition of a number of states possibly including stable initial and final states in addition to intermediate states. Substrates may be bound to the enzyme in different productive or non-productive modes. Steps along the reaction pathway may be reversed, e.g. if diffusion of products away from the active site is hindered by the molecular packing. Furthermore, active site fluctuations may be essential to enzyme catalysis, e.g. in the case of serine proteases (Daggett *et al.* 1991). Then, enzyme intermediates might exist in more than one structural form, or conformational isomers may possibly interconvert. Some experimental evidence has been provided by ^{13}C NMR studies of acyl-chymotrypsin (McClement *et al.* 1981; McWhirter *et al.* 1985).

Unequivocal interpretation and refinement of coexisting crystal structures in general will not be possible, unless they differed in atoms or groups of atoms with distinct locations and small thermal fluctuations. Deconvolutions into structural components may further be complicated by the presence of unknown distorted binding geometries as they are expected to occur in transient states of enzyme reactions. In practice, accumulation of a specific intermediate with high occupancy is of central importance for a determination of its structure by diffraction techniques.

For crystallographic studies, the lifetime of the accumulated intermediate must be long compared with the time required for diffraction data collection. These conditions may be fulfilled by applying time-resolved diffraction techniques under cryoconditions.

(b) *Choice of diffraction method*

Detailed investigation of enzyme–substrate interactions requires determination of interatomic distances with an accuracy of a few tenths of an ångstrom, hence measurement of structure factor amplitudes to high resolution (less than 2 Å). Initial phases may usually be derived from structural models of stable states. The choice of the diffraction method used depends first of all on the degree of *crystalline order*. Initiation of the reaction in the crystal, or conformational changes, often causes a broadening in the crystal mosaicity. Spontaneous re-annealing has been observed in a number of experiments; however, the re-ordering occurred on relatively long timescales of several minutes to a few hours. Figure 1 shows subsequent disordering and partial re-ordering in Laue patterns of hexokinase P2 following rapid diffusion of glucose into the crystal.

White-beam Laue methods may only be applied if the crystal mosaic spread angles stay within a few tenths of a degree (Bartunik & Borchert 1989; Bartunik *et al.* 1992). The resolution functions or 'Intensitätsbereiche' (von Laue 1960) of a mosaic crystal

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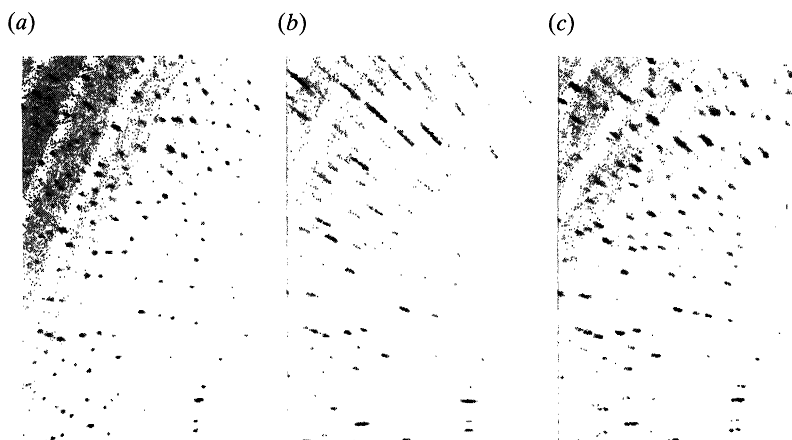

Figure 1. White-beam Laue pattern of hexokinase P2 recorded (*a*) before, (*b*) 1 min and (*c*) 3 min after rapid diffusion of glucose into the crystal.

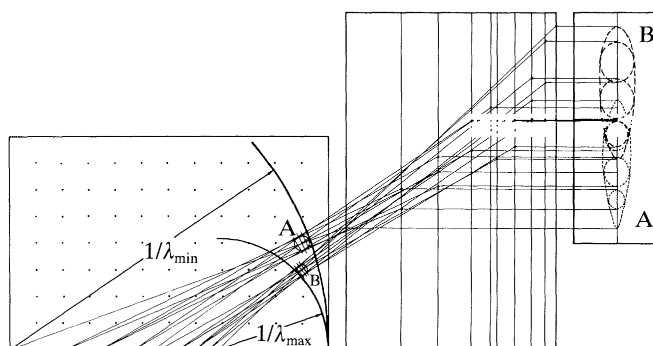

Figure 2. Laue diffraction geometry in the case of spherical resolution functions and overlapping reflection spots.

are extended and give rise to overlapping reflection spots on the detector surface (figure 2). Separation of the contributions from overlapping neighbouring reflections to the total intensity in a streak has been tried on the basis of profile-fitting techniques (Shrive *et al.* 1990). However, the total intensity in the overlap region can only be divided up into contributions from different reflections and scaled according to their wavelength dependence, if the (in general anisotropic) resolution functions and the incident intensity distributions can be modelled reliably. The problem is complicated further by the fact that the crystal mosaic spread and its anisotropy may change during an enzymatic reaction. An experimental solution is provided by narrowing of the incident wavelength bandpass. Optimum adjustment of the bandpass is feasible with scanning Laue techniques (Bartunik & Borchert 1989). Bandwidths of 5–10% may be obtained from undulators or from monochromator systems based on mosaic crystals or multilayers. Laue data recorded with a 6% bandpass using a graphite double monochromator may yield structure factors of adequate accuracy, despite the strongly varying spectral distribution in the incident intensity and the requirement to estimate the partialities (Bartunik *et al.* 1992). Reduced-bandwidth Laue diffraction or monochromatic crystal rotation techniques with bandwidths around 0.1% may tolerate rather large mosaic spread angles up to

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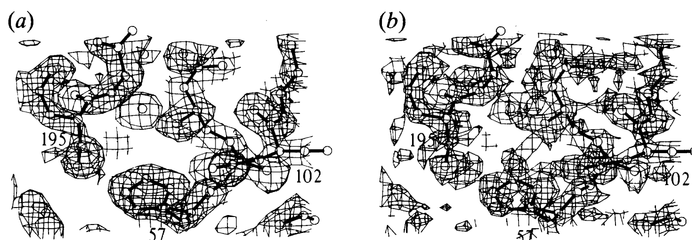

Figure 3. Effect of the completeness of diffraction data at low resolution on the contrast in $2F_O-F_C$ difference Fourier maps calculated for BP trypsin at 1.8 Å resolution. (*a*) monochromatic, (*b*) Laue data (from Bartunik *et al.* 1991).

Table 1. *Time-resolved diffraction methods*

bandwidth	method	number of exposures	time/s per exp.	time/s total
1–1.5 Å	white-beam Laue	1–3	0.001[a]	—
1–1.5 Å	scanning Laue	1–3	3[a]	—
5%	reduced-bandwidth Laue[b]	30	0.01[a]	—
0.1%	monochromatic			
	(1° rotation)	90	1	90[c]
	(0.1° rotation)	900	0.4	360[c]

[a] Defined by time required for crystal re-orientation.
[b] With graphite or multilayer monochromators.
[c] Defined by time required to clear image or change cassette.

about 2–3 degrees; furthermore, the heat input into the sample and the effects of radiation damage are considerably reduced as compared to broaden-bandpass Laue techniques.

Monochromatic diffraction data tend to be of higher quality than (white-beam) Laue data. This is due to a higher signal-to-noise ratio and more reliable scaling of structure factors. Furthermore, Laue structure factor sets possess a systematic *lack of completeness* at low and medium resolution which results from inefficient sampling of reciprocal space near its origin. This lack of completeness degrades the contrast in electron density maps (Bartunik *et al.* 1992); an example is shown in figure 3. Inclusion of relatively few model structure factors may improve the contrast substantially; however, such a procedure may introduce a bias.

(*c*) *Timescales of diffraction data collection*

The incident wavelength bandwidth defines to a great extent the timescale of diffraction data collection using synchrotron radiation. Table 1 gives an overview over the time resolution which may be reached on a wiggler beamline with double-focusing X-ray optics on a second generation synchrotron-radiation source like DORIS. Except for white-beam Laue diffraction, the time required for full data collection is at present essentially defined by the speed of detector data handling. The total times indicated in table 1 may be reached with parallel data handling techniques, which are being developed.

In the case of the white-beam Laue technique and high-symmetry space groups, one single exposure may be sufficient provided that the effect of crystal mosaicity

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may be neglected (Clifton *et al.* 1991). If Laue diffraction patterns have to be taken at a number of different crystal orientations, e.g. to obtain sufficiently high completeness at low resolution, the total time required is practically defined by the speed of crystal rotation; thus, it will not be shorter than several seconds.

It should be noted that the scanning Laue method offers high data collection speeds in the range of 10 s for an entire data-set. With rapid intermediate data read-out during the scan, the same signal-to-noise ratio may be achieved as in monochromatic diffraction measurements.

5. Applications

(a) *Native protein structures*

A number of low-temperature structures have been refined at high resolution. These include sperm-whale met-myoglobin at a number of temperatures between 80 K and room temperature (Parak *et al.* 1987), BP (bovine pancreatic) trypsinogen at 103 K and at 170 K (Walter *et al.* 1982), BP trypsin (Bartsch & Bartunik 1988), and rat trypsin (Earnest *et al.* 1991). Some results of these studies are of interest in the present context, since they indicate that the use of (different) cooling procedures and cryosolvents does not adversely affect the tertiary structures. The myoglobin and the rat trypsin experiments involved shock-freezing in undercooled propane or ethane. In the other cases, the solvents were stepwise exchanged against (up to 70%) aqueous methanol under cooling.

In all cases, comparisons of ambient and low-T structures showed close agreement in the positional parameters. In the example of BP trypsin, the root mean square (RMS) deviation in the atomic coordinates was about 0.30 Å. Most of the water molecules which were located in the room-temperature structure are visible also in the low-T structure at identical positions (within 1 Å). Further water molecules (71) and many atoms of side chains which are disordered in the room-temperature structure could be located in electron density in the low-T structure, due to a decrease in the temperature factors. For example, all water molecules contained in the P1 binding pocket were observed. Five methanol molecules were located, but none of them near the active site. The hydrogen-bonding scheme in the active site region remained unchanged.

In most investigations, the diffraction limit was not substantially affected by cooling. This may indicate that a considerable part of the total disorder in the crystal structure is static. There was no indication of any structural changes which could have been caused by cold denaturation (Privalov 1989; Tamura *et al.* 1991).

(b) *Study of an acyl-enzyme intermediate at subzero temperatures*

The reaction pathway of serine protease catalysed hydrolysis of amide and ester substrates involves the formation of an acyl-enzyme intermediate. Formation and decay of this intermediate proceed via tetrahedral transition states (Fersht 1985; Bode & Huber 1986). Crystal structures of acyl-enzyme complexes were determined for indoleacryloyl-α chymotrypsin (Henderson 1970), and guanidinobenzoyl trypsin (Mangel *et al.* 1990). These studies involved substrate analogues with low turnover rates at room temperature; in both cases, the bulkiness of the substrates induced distortions in the active-site geometry. Serine proteases remain active at subzero temperatures in a number of suitable cryosolvents both in solution (Douzou 1971; Fink & Ahmed 1976; Compton *et al.* 1986) and in the crystalline state (Fink & Ahmed

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1976). For the case of crystalline elastase and ester substrates, kinetic constants were determined in 70% aqueous methanol; it was estimated that approximately stoichiometric concentrations of acyl-enzyme could be obtained at temperatures between 220 and 230 K (Fink & Ahmed 1976). Petsko and coworkers (Alber *et al.* 1976) applied crystallographic techniques in an investigation of the reaction of crystalline porcine pancreatic elastase (PPE) with N-carbobenzoxy-Ala-p-nitrophenylester at temperatures above 200 K using 70% aqueous methanol as cryosolvent. They obtained evidence for a trapped acyl-enzyme intermediate; however, the resolution (3.5 Å) was not sufficient for structural interpretations. Xuong and coworkers repeated the experiment (Xuong *et al.* 1981) in collaboration with the group of Petsko; despite the fact that diffraction data to 1.8 Å were included in this study, the substrate part of the electron density was not significantly better defined than in the previous 3.5 Å maps.

We investigated the reaction of PPE with the productive substrate Boc–Pro–Ala–Ala–OMe (from J. C. Powers) by cryocrystallography at high resolution. The diffraction experiments were carried using synchrotron radiation at the focusing beamline X31/DORIS. The experiment was carried out in two parts; first, a suitable temperature range was determined applying Laue monitoring, then diffraction data for crystal structure analysis were collected with monochromatic techniques. In the first part of the experiment, we cooled the PPE crystal to 220 K under stepwise exchange of the solvent against cryosolvents containing up to 70% aqueous methanol and diffused the substrate into the crystal over a period of several hours. At a series of temperatures increasing in steps of 5–10 K, scanning Laue diffraction patterns were recorded with a FAST area detector (Enraf–Nonius). Each scan over a wavelength range of 1.0 Å took about 10 s. Initially, the patterns indicated high crystalline order. At temperatures around 250 K, the diffraction pattern suddenly turned streaky. This indicated a broadening in the crystal mosaicity which might have been caused by conformational changes involved in the formation of Michaelis complex. The crystal lattice partly reordered within about 10 min at constant temperature. In the second part of the experiment, the cooling and solvent exchange procedure was repeated with a new PPE crystal under similar conditions. Structure factors were measured both at 244 K and 264 K using the monochromatic crystal rotation method; the crystal mosaic spread (of about 0.35°) was too broad for broad-bandpass Laue methods. A full account of the processing, refinement and modelling, and an interpretation of the results in terms of the catalytic mechanism will be given in a future paper.

Both structures (244 K/264 K) were refined independently with X-PLOR (Brünger 1990) on the basis of the room-temperature structure of PPE (Meyer *et al.* 1988). The substrate atoms and the side chains of His 57 and Ser 195 were excluded from the calculations. The current R factors at 2.0 Å resolution are 16.0% for the 244 K structure, and 15.1% for the 264 K structure. Outside the active-site region, the enzyme structure is practically identical to the room-temperature structure. Difference Fourier maps show strong density which flows continuously into the density for the Ser 195 OG (figure 4). The two alanines are in P1 and P1′ respectively. The P1 carbonyl oxygen forms a hydrogen bond to the nitrogen of Gly 193. The P1 carbon is covalently bound to the OG (1.5 Å). The shape of the difference density (extending to 8 σ) indicates an approximately planar binding geometry around the carbon. These results indicate that the acyl-enzyme intermediate has been trapped. For the first time, direct evidence has been obtained for the formation of a covalent

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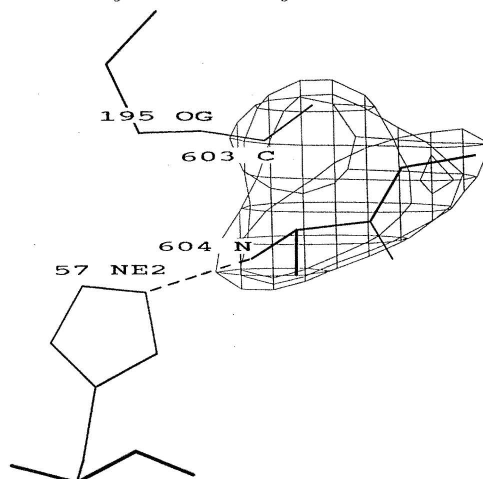

Figure 4. Acyl-elastase and product. Part of the F_O–F_C difference Fourier map near Ser 195 and His 57. F_C was calculated without the substrate.

bond between OG and C; this discards the hypothesis of Komiyama & Bender (1979) which was based on a direct donation of the proton to the nitrogen.

The first product remained bound to the enzyme, possibly due to the molecular packing in the crystal. The P1′ nitrogen is within hydrogen-bonding distance to the NE2 of the imidazole ring of His 57. One may assume that the scissile bond is re-formed during a certain percentage of the total timescale of the diffraction data collection. Then, a Michaelis complex (with smaller population) and the acyl-enzyme may coexist in the crystal.

Determination of kinetic constants for the investigated reaction in solution is in progress in the laboratory of Professor J. C. Powers. Location of the Boc and the proline of the substrate into existing significant but interrupted difference density has not been possible in a unique way. The proline in S2 may be at various positions along an extended hydrophobic surface situated at the entrance to the binding site of elastase. Furthermore, it may be that conformations with different degrees of tetrahedral distortion around the P1 carbon coexist. Both aspects of structural flexibility could explain the weak density for this part of the substrate.

6. Conclusions

The developments in time-resolved X-ray diffraction using synchrotron radiation have greatly improved the experimental conditions for crystal structure analysis of catalytic intermediates. Insertion-device beamlines at operating storage rings and efficient area detectors make it feasible to measure by monochromatic diffraction techniques an entire set of structure factors to high resolution within a total exposure time of a few minutes. This is based on the assumption that the presently applied data handling systems are replaced by state-of-the-art technology involving, for example, multiprocessor arrays. With scanning Laue or reduced-bandwidth Laue methods, timescales in the range of 10 s may be reached. These diffraction techniques minimize the effects of radiation damage and heating of the sample and are applicable also to crystals with broadened mosaicity. Such timescales will in many cases not be adequate for investigations of productive intermediates, unless the turnover rates are slowed down either by adjustment of the pH or by cooling to

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subzero temperatures. Cooling represents the preferable solution, since it exerts a much smaller effect on the charge distribution of the enzyme molecule provided that proper cryosolvents and buffers are used. Taking the short X-ray exposure times into account, cooling to moderately low temperatures (greater than 250 K) will often sufficiently prolong the lifetimes of intermediates for crystal structure analysis. This broadens the choice of cryosolvents. In particular, salt solutions like ammonium acetate may possibly be used, avoiding some of the drawbacks of conventional organic cryosolvents.

Cryotechniques provide a means for initiating enzymatic reactions in crystals. This may require rather low temperatures, if the volume diffusion is slow, as in the case of large substrates. As a possible alternative, caged metabolites and *in situ* activation by pulse photolysis may be used. Other initiation techniques like pressure jumps or external (alternating) electric fields should be further developed for applications to single crystals. White-beam Laue diffraction techniques may be applicable in specific cases when the crystal mosaicity remains small despite initiation of an enzymatic reaction in the crystal and the occurrence of conformational changes. More often, they may be useful for monitoring purposes with the aim of detecting changes in crystal disorder that may indicate structural transitions. Laue or optical monitoring provides a means of interactive optimization of experimental conditions. Considering the relative complexity of enzyme structural kinetics and the rather short time slots available at synchrotron beamlines for any individual experiment, such interactivity as well as rapid processing are of considerable practical importance.

The high-resolution crystal structure analysis of a catalytic intermediate in the hydrolysis of Boc–Pro–Ala–Ala–OMe by elastase was based on the application of cryoenzymology, Laue monitoring and rapid diffraction data collection. It provided for the first time direct evidence for the formation of a covalent O–C bond between a serine protease and a productive substrate. Further, it showed that the bonding geometry around the substrate carbon is essentially planar. This demonstrates that cryocrystallography may in fact yield detailed structural information on intermediate states. On the other hand, the study illustrates some of the limitations of X-ray diffraction methods. Products may possibly not diffuse away from the active site, due to the crystalline environment, and different states may coexist. The averaging over space and time may lead to electron density distributions which may not be interpreted unequivocally. This may be considered as a general feature of crystal structure analysis of unstable states. It will therefore be of importance to check hypotheses based on crystallographic studies of enzyme reactions by other spectroscopic methods under similar conditions. Of particular interest are spectroscopic techniques which may be applied both in solution and in the crystal over a broad range in temperature; such techniques include ^{13}C NMR (Mackenzie *et al.* 1984; Tobias & Markley 1986) and time-perturbed angular correlation (Butz *et al.* 1982). Further theoretical studies including quantum-mechanical calculations are needed to derive models for distorted binding geometries which may be characteristic for structural intermediates.

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Discussion

J. Hajdu (*Oxford University, U.K.*). Low temperature studies on reaction intermediates give meaningful results only if Arrhenius behaviour of the system can be assumed. Unfortunately this assumption does not necessarily hold, as the shape of the potential well for the ligands changes during cooling. This is due to the fact that the state of ionization of relevant side chains on the protein (and on the ligand) changes differentially on cooling, just like the strength of hydrophobic/hydrophilic interactions, etc., changes in different ways as a function of temperature. This may change the process giving structures that are relevant at that temperature only, but may not be relevant to the physiological process.

H. D. Bartunik. The relevance of the structural conformations and of the reaction pathways under investigation has to be checked for each individual system. This refers to all studies of enzymatic reactions under essentially non-physiological conditions, in particular at low temperatures or in crystalline environment. The relevance of reaction pathways under cryoenzymological conditions has been investigated with spectroscopic techniques for a number of entirely different enzymes, in several cases both in solution and in the crystal.

G. A. Petsko (*Brandeis University*, *Massachusetts*, *U.S.A.*). How long did you flow in substrate before the low temperature elastase data collection was started? Did you continue to flow substrate through the crystal during data collection?

H. D. Bartunik. Flowing substrate through the crystal started 8 h before data collection. It continued during the measurement of both data-sets which were taken from the same crystal.

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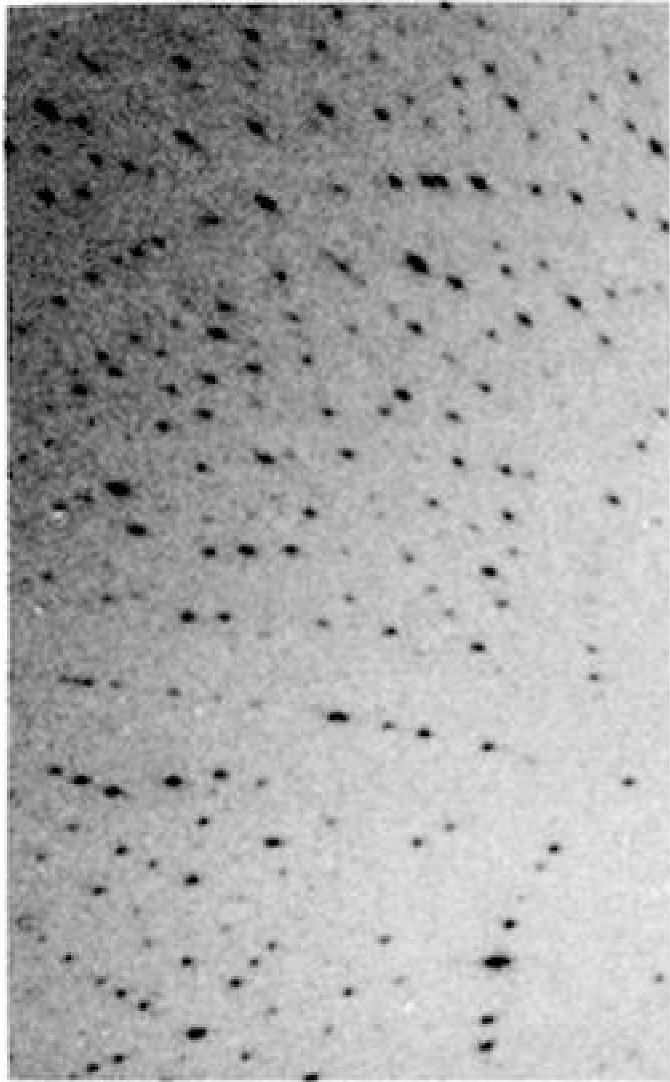
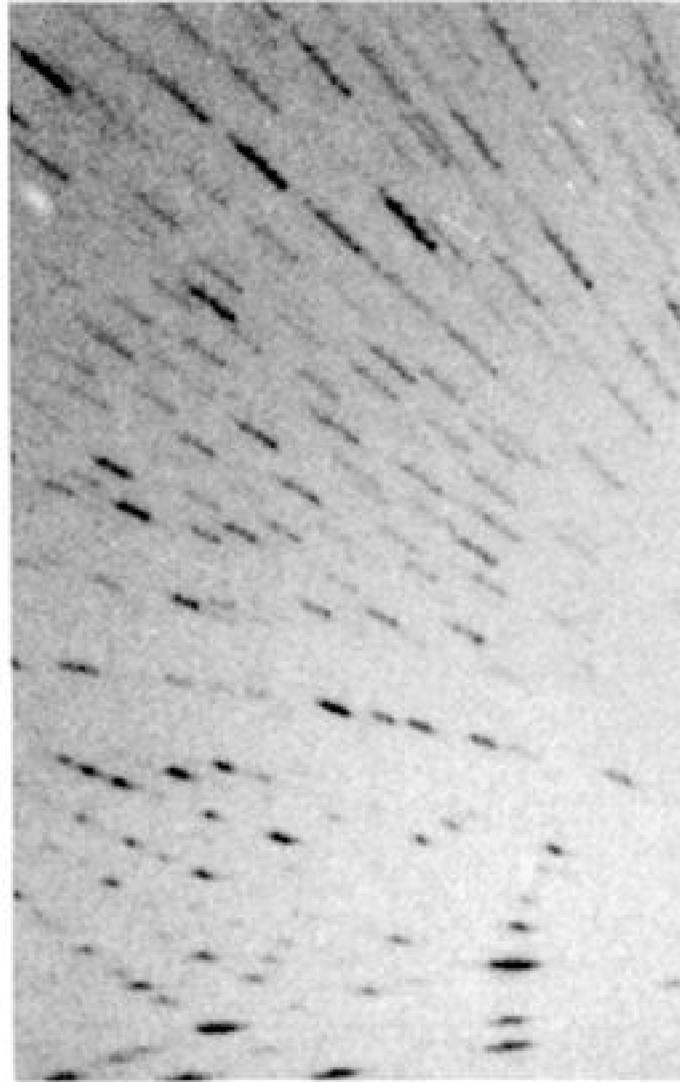
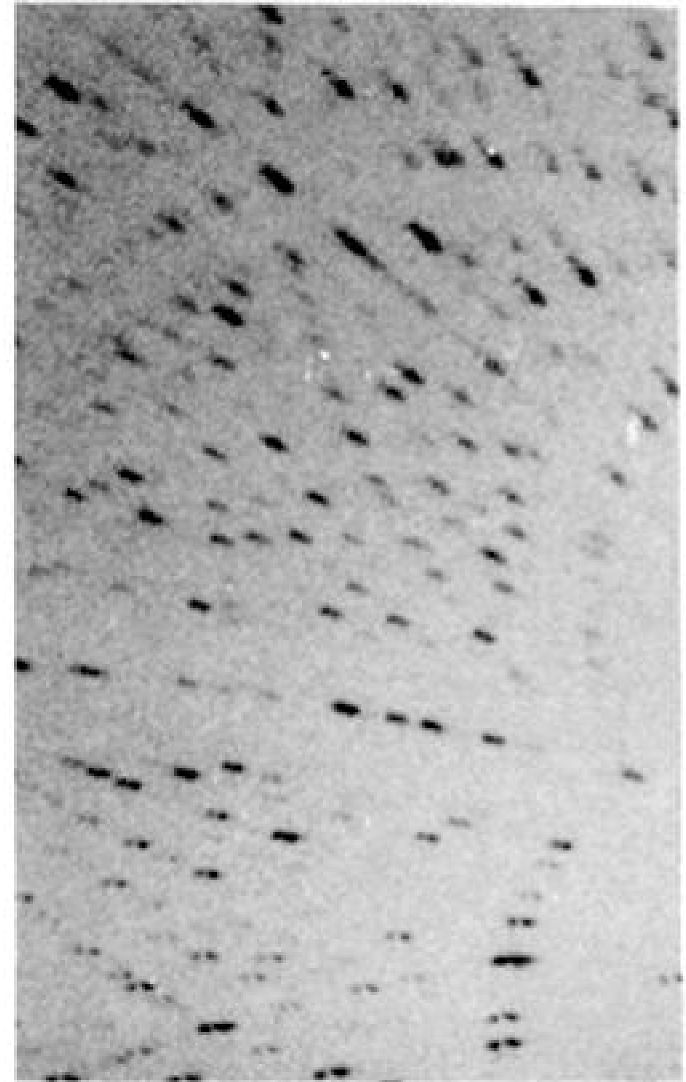
(a) 

(b) 

(c) 

igure 1. White-beam Laue pattern of hexokinase P2 recorded (a) before, (b) 1 min and (c) 3 min after rapid diffusion of glucose into the crystal.

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