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Can Laue catch Maxwell?: observation of short-lived species by Laue X-ray crystallography

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Now that the Laue method has been established as a tool for protein crystallography, the main problem involved in any prospective time-resolved X-ray diffraction study is one of chemistry. The reaction or process in question must be initiated on a timescale that is fast compared with its kinetics. For most biochemical events photochemistry is the most suitable trigger, but not all substrates can be caged for photochemical release. This problem can be solved by the novel strategy of caging the enzyme with a photoreversible covalent inhibitor. The logic of this method will be discussed and its application to a time-resolved study of the reaction of a suicide substrate with the protease gamma chymotrypsin shown. The question of real-time crystallographic 'movies' of enzymatic reactions can now be considered. It seems likely that following a reaction in real time in a single experiment will be very difficult if not impossible in most cases, in part because even a synchronized process will rapidly become asynchronous in a protein crystal, and also because it will be very difficult to know exactly what species one is observing at any instant unless one has extremely high resolution. It seems that the best use of the Laue technique will be to study unstable species that can be accumulated in the crystal under defined conditions for short periods of time. An entire reaction sequence can then be obtained as a series of individual steps, each of which is obtained from a separate experiment.

1. Introduction

The dynamic properties of proteins have been used to interpret kinetic data of enzyme reactions and protein–ligand interactions, and to evaluate the spectroscopic data obtained with proteins. X-ray crystallographic data can put this mobility into a structural framework, providing evidence for the intermediate spatial changes that accompany conformational changes induced by interactions with other molecules (Ringe *et al.* 1990).

The path whereby such a conformational change occurs is normally inaccessible to direct structural observation. Even when no final conformational change is observed as a consequence of, say, a ligand interaction, there may be temporary adjustment to a structure that allow an optimal fit to occur. In the case of enzymatic reactions, the adjustment of a structure to the presence of a substrate, subsequent intermediates, and the product can usually only be surmised from structures of inhibitors bound to the enzyme; which may, or may not, mimic any one of these

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complexes. Although the details of transient conformational changes that occur during a catalytic cycle are not generally available from structural data, it is widely felt that such changes hold the key to an understanding of the catalytic power and unique specificities of enzymes. The Laue method has the potential that such structural changes might be observed when they accompany the formation of a transient intermediate that accumulates during the course of an enzyme-catalysed reaction (Hajdu & Johnson 1990).

To observe a structure at high resolution crystallographically, several criteria must be fulfilled. Probably the most crucial is the requirement for a uniform sample throughout the crystal: every molecule (and there are about 10^{17} of them!) must be in the same state as every other molecule. For the time-resolved observation of stable complexes, this criterion is not a problem: the complex either crystallizes or is formed by diffusion of the stable ligand into a crystal and, once formed, does not undergo any further changes during the lifetime of the crystal. Experience suggests that the minimum occupancy of a ligand that will give rise to interpretable electron density is probably about 25–35%. For the observation of short-lived species, however, all molecules must interact, react, and release a ligand at the same time and at the same rate. Any important movements that occur as the protein adjusts in response to a ligand must all take place simultaneously throughout the crystal. These requirements for synchrony are difficult to meet in systems that are characterized by a Maxwell–Boltzmann distribution of states with a broad width.

2. Time and synchrony

Figure 1 summarizes some of the timescales that must be taken into consideration when a time-resolved experiment is being planned. The most striking disparity is between the time required for a small ligand to diffuse into a crystal (10–1000 s) (Bishop & Richards 1968) and the time required for data acquisition (less than 1 s). The rate-limiting step in such an experiment would therefore seem to be the rate at which an intermediate species can be generated. Unfortunately, if diffusion is slower than intermediate lifetimes, during a Laue experiment the molecules in a crystal will be a heterogeneous mixture of reactants, intermediates and products. It would seem that diffusion cannot be used to generate the species of interest in most instances. Consequently, a more sophisticated approach is called for, which will require some clever chemistry to overcome these problems.

The development of the Laue method allows us to assume that if an intermediate has a lifetime longer than a few milliseconds, we can collect data on a comparable timescale with current technology. The problem that remains, as indicated above, is the much more difficult problem of synchronization; of ensuring that the desired intermediate occupies the active sites of most of the enzyme molecules in the crystal lattice during the Laue data collection period.

There are two solutions to this problem of obtaining homogeneous crystalline species and, therefore, clean electron density maps. One is to slow down the rate at which an intermediate reacts further, allowing it to accumulate, and thereby bringing its lifetime within the range of diffusion lifetime. The other is to use a chemical or physical trigger to cause all the species in a crystal to react simultaneously to generate the desired complex after diffusion is complete. Neither method is ideal for every system, but it should be possible to find conditions, often involving combinations of both methods, that are suitable for many systems.

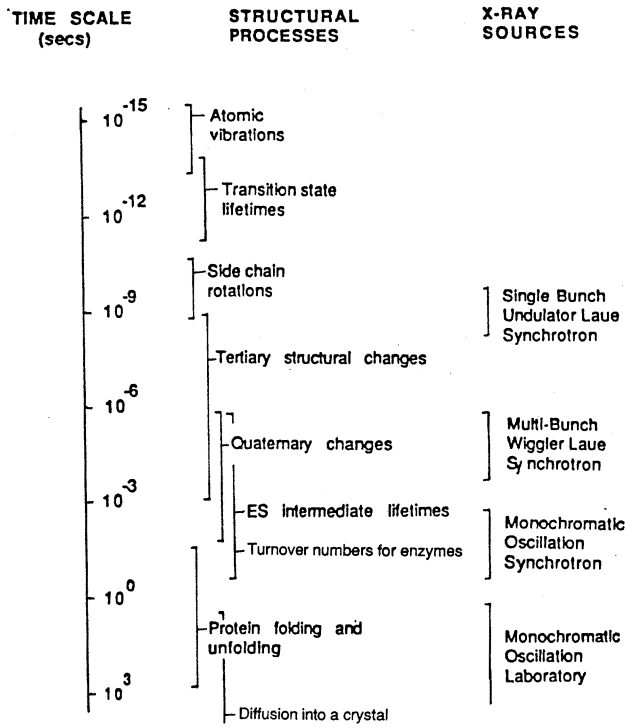


Figure 1. Some timescales of interest in biological systems.

The first method relies on finding a catalytic process that is slow under normal conditions, or conditions under which a normally fast process is slow. If it is slow enough, diffusion can then be used as a trigger. Some examples that fall into this category are abnormal substrates for which turnover is slow (slow-reacting mutant enzymes) or conditions of temperature, pH or additives under which a catalytic process is slow. It is imperative that the normal catalytic mechanism remain unaltered; a condition sometimes hard to achieve. The other difficulty with these examples is the issue of saturation on the protein. For most slow reactions, the poor rate reflects poor occupancy of the ligand at the binding site. A data-set collected under these conditions will also reflect that poor binding and will therefore not be useful. Ideally, a system that belongs to this category should consist of a substrate which binds tightly, turns over slowly, forms intermediates with long lifetimes, and releases product slowly. This does not sound like most enzyme–catalysed reactions.

3. Triggering

Triggering a reaction in the crystal would seem to be a more promising means of generating short-lived species on an enzyme, with high occupancy. A number of ways are available for triggering a reaction. For instance, temperature jump or pH jump can be used.

pH jump may be a very effective way of triggering a reaction in solution. Most enzymes are active only in a relatively narrow range of pH. Consequently, a very large change in pH is rarely required to go from a state in which an enzyme is inactive to one in which the enzyme is active. Hydronium ions diffuse through a crystal

lattice on a timescale of less than a second. Alternatively, photoactivatable acid or base compounds can be used to produce a rapid *in situ* change of pH. Photoreactivation for these compounds usually falls into a timeframe from 0.1 s to 1 s comparable with hydronium ion diffusion times.

Temperature jump triggering is possible at the rate of 20 °C s^{-1}, given a suitable cryostat around the crystal. At sufficiently low temperatures, a reaction may be slowed down enough that diffusion is no longer rate limiting, allowing the accumulation of unreacted substrate in the active sites of a crystalline enzyme. Increasing the temperature would then provide the thermal energy required to start the reaction *in situ*. How large a temperature jump would be required for such a jump start? If a rate reduction of one half for every 10 °C is accepted as an initial estimate, cooling of the sample to 70 °C below ambient is needed to reduce the rate by a hundred fold. Return to ambient temperature would take 3–4 s at the above temperature jump rate, assuming crystal thermal conductance is ideal. Of course, a small rise in temperature would be sufficient to initiate the reaction in some of the molecules in the crystal, but once again the Maxwell–Boltzmann distribution implies that the rapid conversion of most of the molecules from their quenched state would require a large rise in temperature. Otherwise, we would expect asynchrony from the beginning. The exact numbers will differ for different systems, and for some crystalline enzymes favourable conditions may be found, but temperature jump would seem unlikely to be a general method with present technology.

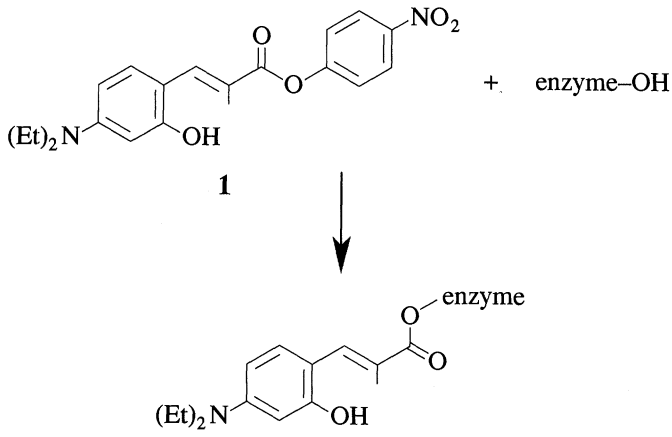
Both temperature jump and pH jump require that the crystal withstand rapid changes in environment. In our experience few do, and the Laue method is notoriously intolerant of even slight crystal imperfections. A milder form of this strategy is the diffusion of metal ions into a crystal. Many metalloenzymes utilize a loosely held metal ion during catalysis, either as part of the chemical mechanism of the reaction or as a form of glue to bind the substrate to the active site. Thus an apoenzyme in a crystal that is bathed in a mother liquor containing the substrate may be activated by the diffusion of metal ion, a process which takes at the most a few seconds. For slow enzymes, this method has some promise, but once again the presence in the crystal of a distribution of states is guaranteed, this time by the relatively long diffusion time. Unless the desired E–S complex or intermediate that forms once the metal binds has a lifetime much longer than the diffusion time, molecules near the surface of the crystal will become out of synchronization with those near the centre very quickly. Maxwell wins again!

4. Photochemical initiation

As indicated by the timescales for these triggering mechanisms, a more rapid trigger is still needed to take full advantage of data collection capabilities at the millisecond or faster limit, which is needed for even moderately fast enzymes. Photochemical activation is the obvious solution. Photochemical processes in solution occur at rates that fall into the nanosecond to microsecond range. Thus, such a process would seem to be the answer to a Laue crystallographer's dream. If a substrate can be released in less than a microsecond, compared with the reaction rate that is instantaneous. We would then know time-zero precisely, and could use data collection rates on the same timescale as the rate of the enzyme reaction. The approach to such a system could be from either of two directions: a caged substrate made available for reaction, or a caged enzyme released for reaction by photolysis of

a crystal with the appropriate substrate-saturated mother liquor present. Caged substrates have been used successfully with the H-ras p21 system. The experiment described in this present paper falls into the second category: the activation of a caged enzyme in the presence of a substrate.

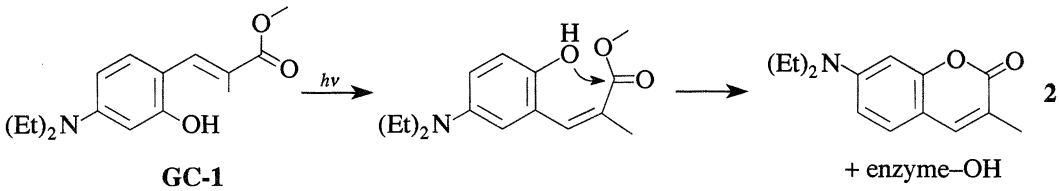
Ned Porter and his associates (Turner *et al.* 1987) have designed a series of photoreversible inhibitors for the serine proteases:

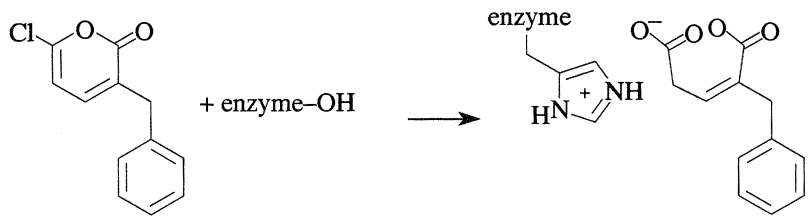


GC-1

These compounds fall into the mechanism-based inactivator class of inhibitors, in that the inhibition of the enzyme is dependent upon turnover of the enzyme. The structure of the compound is based upon the chemical framework of the cinnamate group, which can be obtained stereospecifically in one of two stereochemical forms. Exposure of the target protease to one of these enantiomeric inhibitors in solution results in rapid, irreversible inactivation of the enzyme. Irradiation of the complex produces isomerization around the double bond, followed by return of catalytic activity. Crystallographic analysis of the complex between one such inhibitor (**1**) and gamma chymotrypsin shows that the complex (**GC-1**) (Stoddard *et al.* 1990*a*) is a covalent adduct to the active site serine as a stable acyl enzyme (see above). The stability of this complex seems to result from the configuration of the acyl group relative to the oxyanion hole. The carbonyl oxygen of the inhibitor is oriented away from the protein, towards the solvent. Consequently, the stabilization of the negative charge on this oxygen, which develops during formation and breakdown of the deacylation transition state, by interaction with the residues of the oxyanion hole is prevented, and the inhibitor does not deacylate at any appreciable rate. Upon irradiation with ultraviolet (UV) light at a suitable

wavelength the cinnamate isomerizes and deacylates, presumably via a non-enzymatic mechanism (Stoddard *et al.* 1990*b*):





Model acyl derivatives of this type of compound hydrolyze by intramolecular attack of the aromatic hydroxyl group on the ester carbonyl carbon atom after photoisomerization. Such a reaction is possible on the enzyme, although protein–ligand interactions would be expected to reduce the rate. Intramolecular deacylation would be expected to generate a coumarin product, which might form a transient complex with the enzyme. Since even the solution deacylation rate is slow, there may be an interesting consequence: it may be possible to study the deacylation of the inhibitor directly, at atomic resolution, by Laue diffraction. Alternatively the inhibitor may be used to block the active site of the enzyme in the crystal while substrate is diffused in, in the dark. Unblocking of the active site photolytically might then make it possible to observe the initial steps in an enzyme-catalysed reaction.

An analysis of the timescales of these reactions (Porter & Bruhnke 1989) should provide the framework within which such experiments are feasible and outline the effectiveness of these types of triggering reactions. Simultaneous initiation of catalysis by the enzyme in the crystalline state by the cinnamate derived inhibitors described above depends on a number of factors involving the actual time required for release of the enzyme and the time for accumulation of the species of interest. Isomerization of the inhibitor and formation of the coumarin (**2**) product from the inhibited complex in solution, occurs on a submillisecond timescale. This should be fast enough to trigger the accumulation of a rate-limiting intermediate in the deacylation and inhibitor release steps in the crystalline enzyme. In experimental terms, observation of a concerted accumulation of such an intermediate as a consequence of the synchronized activation of the enzyme depends on the thermodynamic properties of the system and the time-course of the reaction to be followed. The Maxwell–Boltzmann energy distribution for a population of a given molecular species states that the percentage of molecules that possess enough total energy to cross a kinetic barrier decreases exponentially in relation to the absolute magnitude of the barrier. If one assumes an approximate overall energy of activation

for serine proteases of 10–15 kcal mol^{-1}† and a time course for the chemical event in the picosecond range (bond-making or bond-breaking), then formation of the initial chemical intermediate will not occur quantitatively over the lifetime of that intermediate, and the yield of this species will decrease as the lifetime of the intermediate decreases. If more than one intermediate occurs during the course of a reaction, then for each kinetic barrier present in the reaction pathway the yield of a given intermediate will decrease as a multiplicative function of the chemical efficiency of each step. Therefore the probability of forming and observing a transient intermediate state in the crystal is strictly dependent on the efficiency of the triggering reaction, the magnitude of the kinetic barrier leading to any one intermediate, the corresponding energy state of the catalytic system, the lifetime of the intermediate, and the ability to collect sufficient data within a single turnover of the enzyme in the crystal. As an example, observation of the GTP–ras p21 Michaelis complex (half-life 20 min) following release of the caged enzyme is possible after initiation by flash photolysis (Schlichting *et al.* 1990). Observation of an acylated intermediate in the chymotrypsin-catalysed hydrolysis of an ester substrate (half-life 0.01–1 s in solution) is a more difficult problem.

5. Experimental strategy

This report will now detail the approach we have taken toward this problem and the results which we have obtained thus far. The general strategy is that of caging the enzyme: blocking of the active site of chymotrypsin by a photoactivatable cinnamate inhibitor. Diffusion of a substrate, actually another mechanism-based inactivator of the enzyme, into the crystal lattice before photolysis makes this compound available to the active site as soon as the first inhibitor is released. Thus photolysis of the cinnamate-inactivated crystalline enzyme should initiate catalysis by unblocking the active site simultaneously throughout the crystal. The observed species at later times should be one for which decay is the rate-limiting step in the reaction of the enzyme with the new substrate. The entire procedure was monitored by collection of sequential Laue X-ray diffraction patterns.

Preliminary experiments were performed to characterize the initial and photolytic release of the cinnamate bound at the active site of chymotrypsin (Stoddard *et al.* 1990*b*). Photolysis of the inhibited enzyme is facile in solution and was therefore expected to be straightforward in the crystal. However, in practice, the wavelength of the absorption maximum is critical to this process in the crystal. If this maximum is too close to the absorption maximum of the protein, the photolysis becomes so inefficient that no isomerization occurs at all, implying that all of the radiation is absorbed by the protein in unspecified ways and very little reaches the derivative bond, which should isomerize. The isomerization reaction was monitored by measuring the intensity of several reflections which were expected to be sensitive to the presence of the cinnamate derivative (figure 2).

Immediately upon irradiation, crystals of chymotrypsin inhibited with the cinnamate show a sharp dip in the integrated intensity of the reflections being monitored. The intensity returns to a new base-line value within a minute. The temporary loss of diffraction from the crystal occurs simultaneously with the initiation of photolysis. The effect seems to be caused by a transient disordering of the crystal that is not related to the isomerization reaction, but is instead related to

† 1 cal = 4.184 J.

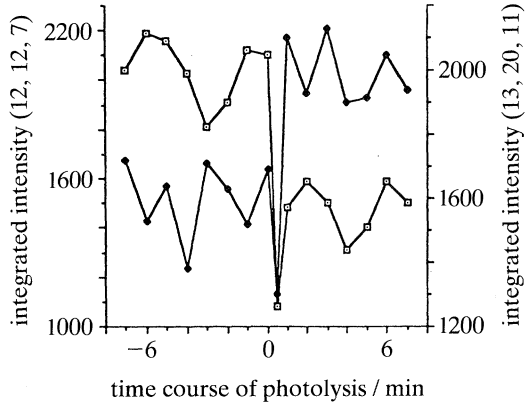


Figure 2. Crystallographic flow cell data for the (**GC-1**) flash experiment. The crystal was irradiated for a total of 10 min. The same transient loss of diffraction intensity is seen for both reflections measured during flash initiation, and both reflections return to new base-line values within one min (one measurement).

the initiation of irradiation, and can be reproduced by repeated opening and closing of the shutter used to control the flux of light impinging on the crystal. The transient disordering lasts less than 30 s, after which the crystal behaves normally. The disordering was also observed in the diffraction pattern when data collection occurs simultaneously with flash photolysis. A transitory streaking of the reflections occurs which again disappears within 30 s. It is not known whether this effect is general for all crystalline proteins, although similar experiments performed on ras p21 have produced the same streaking in the diffraction pattern. The return of any reflection to a stable intensity value is faster than the rate at which repeated measurements can be made with the equipment which we have been using.

To determine if the inhibitor had been released, a complete monochromatic data-set was collected on the crystal in the flow cell after illumination. The electron density map of the photolyzed cinnamate–enzyme derivation showed an almost complete absence of any density in the active site region. The protein density clearly terminates at the γ-oxygen of serine-195 and the specificity pocket is practically devoid of any electron density (see figure 3*c* for comparison). A small fragment of electron density that is observed may be due to remains of unphotolyzed cinnamate inhibitor or may be due to bound water molecules or salt ions from the solution. The presumed product of the photolysis reaction, the coumarin (**2**) formed by intramolecular deacylation, is not observed in the specificity pocket or in any other region of the map. The γ-oxygen has rotated toward the now vacant binding pocket. With the exception of the γ-oxygen of the active site serine residue, the other residues of the protein show very little change in position or orientation relative to the inhibited enzyme.

These results can be compared to analogous experiments done in solution (Stoddard *et al.* 1991). The activity of the inhibited enzyme before irradiation is *ca.* 3% of the photoactivated chymotrypsin activity. Although it is difficult to quantitate precisely the recovery of activity after photolysis relative to native enzyme (because the acylated enzyme is purified over a gel filtration column), the enzyme regains more than 90% of its specific activity after irradiation. With a suicide substrate (pyrone) present, the enzymatic activity then decreases as a

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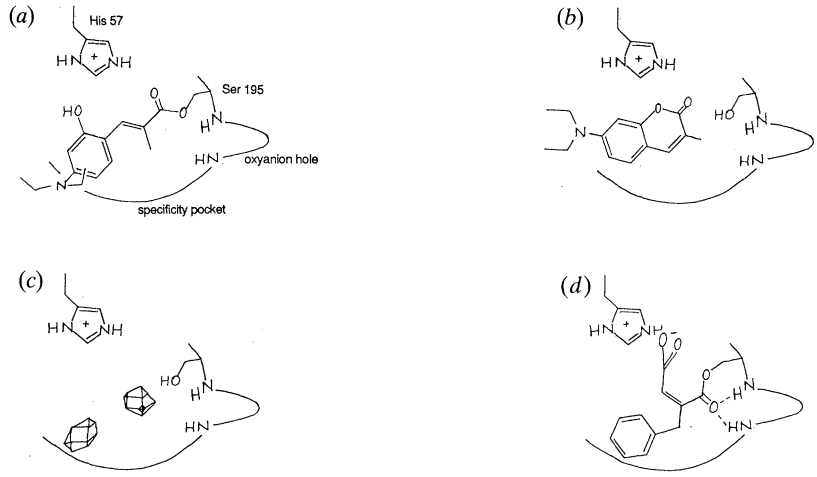

Figure 3. Reaction sequence for the light-induced deacylation of the enzyme–cinnamate complex (**GC-1**) followed by binding and reaction of pyrone (3) to give the enzyme–pyrone derivative complex (**GC-2**). (*a*) Structure of the cinnamate bound to chymotrypsin ($t = 0$ min). (*b*) Structure of the photolysis product ($t = 5$ min). (*c*) Structure of the free enzyme ($t = 6$ min). (*d*) Structure of the pyrone-bound complex ($t = 3$–24 h).

function of time with an approximate first-order rate constant for inactivation of 1.06×10^{-2} min^{-1} and a half-life of 65 min. In the absence of pyrone, photolysis produces a rapid recovery of activity with no subsequent decrease.

Having established the feasibility of the photolysis reaction in the crystal the time-resolved experiment is now ready.

A crystal of g-chymotrypsin inactivated with *trans-p*-diethyl-amino-*o*-hydroxy-α-methylcinnamate (**1**) was mounted in the dark in a capillary filled with mother liquor containing 3-benzyl-6-chloro-2-pyrone (**3**). A series of single-exposure Laue diffraction patterns were collected over a period ranging from $t = 0$ min (before irradiation) to $t = 24$ h. 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 exposures produced a series of structures (figure 3) which have been interpreted according to the scheme on page 278.

6. Results

Examination of the protein difference electron density maps shows the clear presence of the cinnamoyl group before photolysis (figure 3*a*) and the expected final acyl pyrone (figure 3*d*) derivative at $t = +24$ h. Free enzyme (figure 3*c*) is observed as soon as 1 min after photolysis, persisting more than 20 min after irradiation. These structures compare favourably in quality and in detail with those determined previously by monochromatic crystallography (Ringe *et al.* 1986). The $t = 5$ min difference map (figure 3*b*) shows electron density in the active site aromatic binding pocket that has the approximate dimensions and appearance of a weakly bound, 10-membered bicyclic coumarin ring. This map contains no definitive features of the diethylamino substituent and the ring orientation is therefore difficult to model unambiguously in the pocket. A frame-by-frame analysis of this first enzymatic 'movie' is shown in figure 3.

(*a*) *Structure of the cinnamate bound to chymotrypsin* ($t = 0$ min) (*figure 3a*)

This inhibitor shows a bound geometry identical with that observed previously. The compound is covalently bound to the active-site serine with a bond distance of 1.5 Å†, a bond angle of 125° through the carbonyl carbon, and a trans geometry through the α-carbon double bond. The inhibitor has a planar geometry. The hydroxyphenyl ring is found in the specificity pocket flanked by the backbone of residues 190–195 and 214–217. The *para* amino nitrogen is visible and participates in a polar interaction with the backbone oxygen of residue 217 as previously observed. The acyl oxygen points away from the oxyanion hole due to the lack of rotational freedom about the sp^2 hydrodized carbon.

(*b*) *Structure of photolysis product* ($t = 5$ min) (*figure 3b*)

The diffraction pattern collected concurrently with photolysis shows streaking of the individual spots on the film. This streaking disappears in subsequent exposures, consistent with a temporary internal disordering of the crystal lattice during the flash, as observed in earlier monochromatic studies of photolysis. This disorder caused a significant deterioration in the final statistics for the data set and the refinement. Nevertheless, examination of the resulting F_o-F_c map shows protein density that is still clean and free of noise but disjointed throughout the map relative to other time points. Examination of the active site shows that the nucleophilic serine γ-oxygen is free of covalently bound inhibitor and has moved by 0.8 Å relative to its position in the cinnamate-bound complex, with a 35° rotation about the C^{α}–C_{β} bond. The specificity pocket shows the presence of density that is of the approximate size and shape of a bicyclic coumarin ring. This density is found in a planar orientation similar to that of the phenyl ring of the cinnamate but appears to be rotated somewhat relative to the cinnamate density. The density is of poor quality, with an absence of density for the diethylamino group, making the structure difficult to model unambiguously.

(*c*) *Structure of free enzyme* ($t = 6$ min) (*figure 2c*)

The electron density map is fit well by a model identical to that determined previously from monochromatic data collection long after photolysis. The serine side chain is unchanged from the $t = 0$ time point, with clear density that terminates at the γ-oxygen. The specificity pocket shows density at a 2σ contour level that appears to reflect the presence of bound solvent, salt, or a small amount of non-photolyzed inhibitor. Lowering the contour level close to the noise level of the map, however, shows density corresponding to a low occupancy of non-covalently bound peptide that is found in the active site of crystalline chymotrypsin (Almo *et al.* 1992; Dixon & Matthews 1989), and that is not displaced by the cinnamate ester during the crystal soak. This density is present at the next three time points ($t = 10$, 15 and 25 min) (not shown).

(*d*) *Structure of pyrone-bound complex* ($t = +3$ *to* $+20$ h) (*figure 3d*)

By 3 h after flash, density is apparent that is consistent with pyrone derivative bound (**GC-2**) in a conformation similar to that reported previously (Ringe *et al.* 1986). At 24 h, difference maps clearly reveal fully bound pyrone derivative in the

† 1 Å = 10^{-10} m = 10^{-1} nm.

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active site. This density is distinct from that observed for bound cinnamate, due to the non-planar sp^3 carbon extending from the phenyl ring, which is bound in the specificity pocket. The inhibitor is covalently bound to the serine with a bond length of 1.8 Å and exists as an extended molecule that is formed after ring opening, hydrolysis, and formation of a polar interaction with His 57 as described previously. Initial modeling of the density was performed by a right body superposition of model coordinates for bound pyrone from the previous structure. The distance from the terminal carboxyl oxygens of the inhibitor to the $\epsilon 2$ nitrogen of His 57 is *ca.* 3.0 Å.

This series of structures gives a running set of 'snapshots' documenting the time-course of the two reactions being followed. Thus, the triggering has been solved, although the results are not yet perfect. However, it is clear that synchronization is still a problem, if the reaction is followed over long time periods. Even if the triggering were perfect, synchronization decays rapidly due to the distribution of energies of the reactive species in the crystal. A combination of photolysis and low temperature – i.e. rapid triggering and accumulation of intermediates trapped by an energy barrier – would seem to be the most likely strategy for the successful observation of short-lived species in multi-step reactions like this one.

7. Conclusion

Thus we could conclude that the best use of the Laue technique at present is to study such unstable species that accumulate in the crystal under defined conditions for short periods of time. An entire reaction sequence is best obtained as a series of individual steps, each obtained from a separate experiment. Splicing together these individual 'scenes', which is how movies are actually made anyway, produces a complete sequence. In other words, if you can't win against Maxwell, don't even join the race.

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