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Direct crystallographic observation of an acyl-enzyme intermediate in the elastase-catalyzed hydrolysis of a peptidyl ester substrate: Exploiting the "glass transition" in protein dynamics ☆,☆☆

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Abstract

The crystal structure of the acyl complex of porcine pancreatic elastase with its peptidyl ester substrate *N*-acetyl-ala-ala-ala-methyl ester ($Ac(Ala)_3OMe$) has been determined at 2.5 Å resolution. The complex was stabilized by exploiting the "glass transition" in protein dynamics that occurs at around −53 °C (220 K). Substrate was flowed into the crystal in a cryoprotective solvent above this temperature, and then the crystal was rapidly cooled to a temperature below the transition to trap the species that formed. The use of a flow cell makes the experiment a kinetic one and means that the species prior to the rate determining transition state has a chance to accumulate. The resulting crystal structure shows an acyl-enzyme intermediate in which the leaving group is absent and the carbonyl carbon of the C-terminal alanine residue is covalently bound to the gamma oxygen of the active site

☆ Dedicated to the memory of Miriam (Mimi) Hasson, our student, colleague, and friend. A scientist of rare ability whose curiosity and drive never got in the way of her humanity, she was always as interested in beautiful results from other labs as she was from her own. We think she would have enjoyed the work described in this paper, as it touches on a subject dear to her heart: the structural basis of the catalytic power and specificity of enzymes. We miss her.

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serine. The ester carbonyl shows no significant distortion from planarity, with the carbonyl oxygen forming one hydrogen bond with the oxyanion hole. The tripeptide is bound in an extended antiparallel β-sheet with main chain residues of the enzyme. The geometry and interactions of this acyl-enzyme suggest that it represents a productive intermediate. To test this hypothesis, the same crystal was then warmed above the glass transition temperature and a second data set was collected. The resulting electron density map shows no sign of the substrate, indicating hydrolysis of the intermediate followed by product release. This experiment provides direct evidence for the importance of dynamic properties in catalysis and also provides a blueprint for the stabilization of other short-lived species for direct crystallographic observation.

Keywords: Crystallographic observation; Acyl-enzyme intermediate; Elastase-catalyzed hydrolysis; Peptidyl ester substrate; Glass transition; Protein dynamics

1. Introduction

Determining the high resolution crystal structure of short-lived intermediates formed by enzyme reactions using normal crystallographic techniques is normally impossible due to the short lifetime of most intermediates relative to the long time needed to collect X-ray data. Therefore, new experimental techniques have been developed to deal with this problem [16]. Laue diffraction, which enables data to be collected on a sub-second timescale, has been successfully used to trap and structurally observe the metastable complex of GTP with H-ras [21]. In favorable circumstances, productive enzyme-substrate complexes can sometimes be observed directly at room temperature over normal data collection times by use of flow techniques to establish a pseudo-steady-state, as in the case of D-xylose isomerase [11]. In other cases the best strategy is to extend the lifetime of the desired species by the use of sub-zero temperatures [1]. Often more than one of these techniques must be employed simultaneously; examples include the structure determination of the photolyzed state of carbonmonoxymyoglobin [22] and intermediates in the catalytic pathway of cytochrome P450 [23].

The low temperature crystallographic techniques have as their basis the fact that the rates of most chemical reactions are temperature-dependent. The Arrhenius equation relating reaction rates to temperature predicts that an enzymatic reaction having an energy of activation of 15 kcal/mol will be slowed by a factor of 10^4 on decreasing the temperature from +25 to −50 °C (298–223 K). This simple fact forms the basis of cryoenzymology: at a suitably low temperature, the rate of the slowest step in an enzymatic reaction becomes negligible compared with the rates of the remaining steps, allowing the intermediate before the slowest step to be trapped. Knowledge of those kinetic parameters of an enzyme reaction that control the formation and breakdown of the different reaction intermediates can be used to select conditions of pH, substrate type and temperature such that the desired species will accumulate [5].

Although attractive conceptually, this method presents several problems. First, it requires finding a cryoprotective solvent that does not alter the reaction mechanism and in which the enzyme crystals are stable and the substrate is soluble [14]. Second, if flow techniques are needed—as they often will be—the temperature of the experiment must be above that of the freezing point of the cryoprotectant, limiting the range of temperature that can be accessed. The final, and most serious, problem is related to the second: for

many enzyme reactions, the activation energy is so small that Arrhenius theory predicts that extremely low temperatures will be required to stabilize any intermediate for a time long enough to allow conventional monochromatic X-ray data collection.

An attractive candidate for such studies is the class of enzymes known as the serine proteases. These ubiquitous enzymes hydrolyze both ester and amide substrates by a multistep mechanism in which the eponymous serine residue in the active site (Ser195 in the standard numbering scheme for mammalian pancreatic serine proteases) carries out a nucleophilic attack on the carbonyl carbon of the scissile amide bond (Fig. 1). The acyl-enzyme intermediate resulting from loss of the leaving group is metastable until attack on the same carbon by a water molecule hydrolyzes it off in the final step of the reaction. Since breakdown of the acyl-enzyme is rate-determining for ester substrates, they are usually preferred in experimental studies aimed at trapping the intermediate.

Experiments based on this idea and designed to determine the high-resolution crystal structure of a productive complex of the serine protease elastase with an ester substrate showed that the substrate (Fa-LeuOMe) and a similar inhibitor (Fig. 2) could not react with crystalline elastase at a temperature of −45 °C (228 K), but could do so rapidly at a temperature of −26 °C (247 K). [4] This result is similar to that obtained from studies of ribonuclease A, in which crystalline ribonuclease A did not bind substrate or inhibitor at −61 °C (212 K but was found to bind either rapidly at −45 °C (228 K) [18]). If substrate binding and turnover both follow Arrhenius behavior with temperature, assuming typical activation energies for both processes, one would expect to see, at the most, a 5 to 10-fold reduction in rate of binding and catalysis on going from −26 to −45 °C (247–228 K). But the behavior we observed was very different: binding had become many orders of magnitude weaker. The only property that changes so dramatically over such a small tempera-

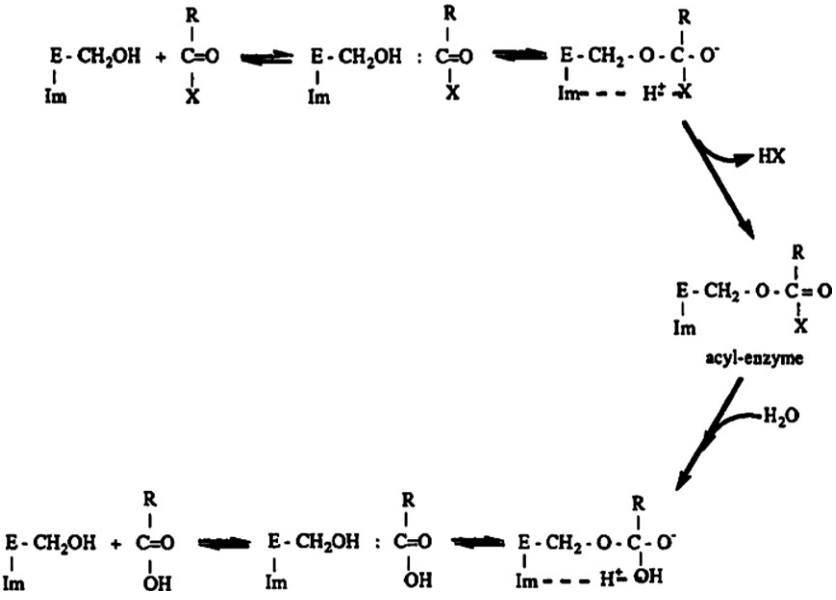
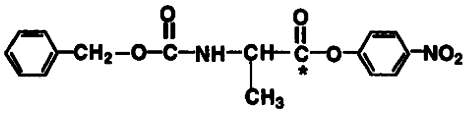
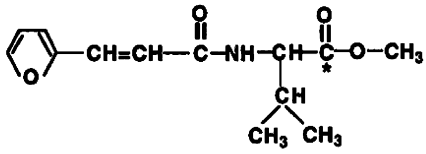


Fig. 1. Schematic of the general mechanism for serine protease catalysis of ester and amide bond cleavage. "Im" is the imidazole group of the histidine residue always found with the eponymous serine in the active site.

N-carbobenzoxy-L-alanyl-p-nitrophenol ester (ZAP)



N-(2-furyl)-acryloyl-L-Leu-methyl ester(Fa-LeuOMe)



Acetyl-alanyl-alanyl-alanyl-methyl ester (ALA3)

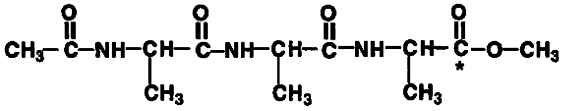


Fig. 2. Structures of elastase inhibitors and substrates referred to in this work.

ture range is protein dynamics, which undergoes what we and others have called a "glass transition" precisely in this region [19].

That many motions in all proteins "freeze out" at around −53 °C (220 K) is well established. When the dynamic properties of different proteins are plotted as a function of temperature, biphasic behavior is observed (Fig. 3), with a broad transition centered around −40 to −50 °C (233–223 K); this transition is called the "glass transition" because it displays many of the characteristics of the transition seen when a melt solidifies to a glass: it is broad, dependent on the rate of cooling, and causes certain properties to change by many orders of magnitude over a small temperature range. Molecular dynamics simulations suggest that above the "glass" transition temperature, collective motions of both bonded and non-bonded groups of atoms (e.g., dihedral transitions involving backbone atoms and side

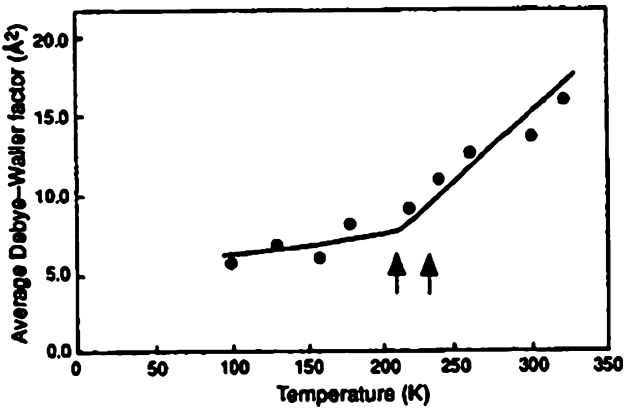


Fig. 3. Graph of the average Debye–Waller factor (*B*-factor) for all atoms for crystalline bovine pancreatic ribonuclease A at nine temperatures spanning the "glass transition" at around 200 K. Data taken from [25].

chains) occur rapidly; below the "glass" transition temperature, simple harmonic vibrations of individual atoms predominate, possibly due to coupling of protein collective motions to a freezing out of motions in the bound layer of solvent around the macromolecule [12,26].

Therefore, we assume that the glass transition exists for elastase, and under the conditions of our experiment, this transition temperature is not lower than about −50 °C (223 K). How can we combine the cryocrystallography technique with the dynamic properties of proteins to design an experiment to trap, e.g., the acyl-enzyme intermediate? It is known that below the glass transition, substrate could not react with the enzyme [18,4]. The experiment done at −26 °C (247 K) aimed at trapping the acyl-enzyme formed by the hydrolysis of an ester substrate by elastase shows that at this temperature, enzyme can react with substrate in the crystal, and the temperature of −26 °C (247 K) is high enough for turnover to occur. The experiment with ribonuclease A shows that once inhibitor is bound to the enzyme at a temperature above the glass transition, it cannot be washed off after being cooled to below the transition temperature due to rigidification of the protein. Based on this information, the final strategy used to trap the acyl-enzyme intermediate successfully involved formation of the acyl-enzyme at −26 °C (247 K; a temperature above the glass transition) followed by cooling the crystal down to −50 °C (223 K; a temperature below the glass transition) to stabilize the acyl-enzyme. The natural substrate of elastase is a protein (elastin) consisting of repeated stretches of alanines and other small amino acids interspersed with stretches of lysines. Therefore, a trialanyl methyl ester was used as the substrate to mimic elastin and determine the effect of chain length on the stabilization of the acyl enzyme.

The fundamental thesis of transition-state theory as applied to enzymes is that an enzyme functions by furnishing a template complementary to the substrate in its transition-state configuration. In other words, enzymes bind the transition state of a substrate much tighter than they do the substrate itself. Accordingly, elastase, like other serine proteases, should have a high affinity for species that resemble the putative tetrahedral intermediate. The way elastase binds such species strongly is to use a pair of hydrogen bonds from the backbone NH's of Ser203(195) and of Gly201(193) to the negatively charged carbonyl oxygen in the tetrahedral intermediate. This has been seen in a series of covalent inhibitor-serine protease complexes in which the inhibitor assumes a tetrahedral geometry with an oxygen or hydroxyl group similarly placed in the oxyanion binding site e.g., [20,7,24]. The structure of a productive enzyme-substrate complex observed in this study allows us to answer the question: what are these hydrogen bonds like in the acyl-enzyme intermediate? It has long been believed that such hydrogen bonds should exist for the acyl-enzyme intermediate, and even speculated that such hydrogen bonds might be stronger in the transition-state like tetrahedral intermediate than in other intermediates, thereby effectively lowering its free energy and favoring its formation.

2. Materials and methods

2.1. Materials

Porcine pancreatic elastase (PPE) was purchased from Worthington Biochemical and was used without further purification. Native PPE was crystallized by the method of vapor diffusion using sitting drops. The crystals were grown in 18–24 μL drops, containing

1.4 mM sodium sulfate and 100 mM sodium acetate at pH 5. The PPE concentration varied from 6.8 to 8.3 mg/ml. The reservoir consisted of 15 mL of 100 mM acetate buffer at pH 5. Substrate acetyl-L-alanyl-L-alanyl-L-alanyl-methyl ester ($Ac(Ala)_3OME$) was purchased from Sigma Biochemical and was used without further purification.

2.2. Cryoprotection of elastase crystals, substrate binding, data collection, and data processing

The procedure for protecting elastase crystals at low temperature by transferring them to 70% MeOH-30% aqueous buffer solution was followed as described [4]. Briefly, a crystal of elastase was mounted in a flow cell [15], allowing substrate to flow past the crystal continuously. The crystal was bathed in 70% MeOH–30% aqueous solution containing 10 mM sodium acetate and 1 mM sodium sulfate at a proton activity (pH*) of 6.7 [8]. The flow cell was mounted on a Siemens P3 single-counter four-circle diffractometer equipped with a modified LT-1 low temperature device and a cryostat designed and constructed specifically for this project. The substrate used was $Ac(Ala)_3OMe$. For this substrate, k_{cat}/K_m is reported as $170 \times 10^3\ M^{-1}\ s^{-1}$; K_m was estimated as 0.43 mM [6]. $Ac(Ala)_3OMe$ was dissolved in a solution containing 70% methanol, 10 mM sodium acetate and 1 mM sodium sulfate (pH* 6.7) at a final concentration of 3.0 mM. To avoid problems due to spontaneous hydrolysis, the solution was made fresh twice daily. The crystal of elastase in the flow cell/cryostat system was first cooled to −26 °C (247 K). For substrate binding at −26 °C (247 K), a flow rate through the flow cell of about 10 mL/day was used. A small set of medium intensity reflections at high resolution was measured at regular intervals. Time-dependent intensity changes were observed and used to monitor binding. After the binding monitor curve had leveled off (Fig. 4), presumably indicating saturation, the crystal was cooled to −50 °C (223 K) to stabilize the expected covalent acyl-enzyme intermediate against further breakdown [18], and a complete set of crystallographic data to 1.85 Å resolution was collected using the background-peak-background (ω-scan) method. During data processing, absorption, linear radiation decay and Lorentz-polarization corrections were applied. Refinement was carried out with X-PLOR [2] with manual rebuilding performed between refinement cycles on an Evans and Sutherland graphics station running FRODO [9]. The initial phases were calculated using the coordinates of native elastase to 1.65 Å resolution in 56% MEOH at −45 °C (228 K) [4]. Water molecules were taken from the initial model, additional water molecules were added using the program Waterhunter (Shigetoshi Sugio, unpublished result). All water molecules are refined and checked individually for their fit into difference electron density maps with coefficients $(2F_{obs} - F_{calc})$ displayed at the 1.5 σ level. An energy-minimized initial model for substrate was built using QUANTA (Molecular Simulations Inc.). An Evans & Sutherland PS300 graphics system and the program FRODO were used for modeling of substrate into the difference Fourier electron density at the active site. The final refinement results are given in Table 1.

2.3. Control experiment—proof that the acyl-enzyme formed at low temperature is productive

In order to test whether the acyl-enzyme formed at low temperature is productive, a control experiment was performed by trying to wash the substrate out of the enzyme at

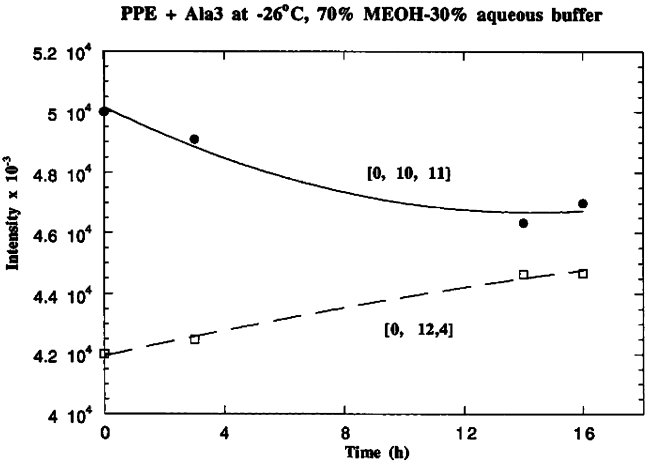


Fig. 4. Graph of the changes in intensity over time of two high-resolution 0kl reflections of elastase as substrate is added to the crystal via the flow cell.

higher temperature. After collecting $Ac(Ala)_3OMe$ data, the temperature was raised to −20 °C (253 K). and substrate-free cryosolvent (70% MeOH-30% aqueous buffer) was flowed through the crystal at a flow rate of about 10 mL/day for about 24 h. Then X-ray data to 2.5 Å resolution were collected. Data collection and processing were accomplished as described above. Refinement was carried out with the program X-PLOR [2]. The initial phases were calculated using the coordinates of native elastase to 1.65 Å in 56% MEOH at −45 °C (228 K) [4] from which the atoms of the side chains of the active site residues Ser203 (195), His60 (57) and the atoms of active site solvents were omitted. These phases together with data from 10 to 2.5 Å resolution ($I/\sigma(I) > 1$) gave an initial model with an overall starting *R*-factor of 26.4%. A difference Fourier electron density map with coefficients ($2F_{obs} - F_{calc}$) was calculated. The water molecules were checked in this map. Only the water molecules with electron density at the 1.5 σ contour level or better were retained. Refinement was continued with X-PLOR and brought the *R*-factor down to 21.5%. At this stage, a difference Fourier electron density map with coefficients ($F_{obs} - F_{calc}$) was calculated. There was no electron density which could be interpreted as substrate. However, three lobes of electron density were found in the active site region which correspond to the positions of three water molecules in the native structure. Two of these three electron densities are too large to be a water molecule, especially since the shape of the density is ellipsoidal rather than spherical. So they were interpreted as two methanol molecules and one water molecule. An energy minimized initial model for methanol was built using QUANTA (Molecular Simulations, Inc.). An Evans & Sutherland PS 300 graphics system and the program FRODO [9] were used for modeling of methanol into the difference Fourier electron density at the active site. Further refinement was continued with X-PLOR using individual *B*-factors. Most of the ordered water molecules observed in the structure of the acyl-enzyme were found to be close to their corresponding positions in the native structure. Additional water molecules were found using the program Waterhunter (Shigetoshi Sugio, unpublished) and checked in difference Fourier electron density maps. The final refinement results are given in Table 1.

Table 1
Data collection and refinement statistics

Crystal environment		
Sample/substrate	$Ac(Ala)_3OMe$ (3 mM)	After warming
Reaction temperature	−26 °C (247 K)	−20 °C (253 K)
Data collection temperature	−50 °C (223 K)	−20 °C (253 K)
Cryosolvent	70% MeOH/30% Aq	70% MeOH/30% Aq
Data collection		
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit cell	$a = 51.81$ Å $b = 58.08$ Å $c = 74.74$ Å $\alpha = \beta = \gamma = 90°$	$a = 51.81$ Å $b = 58.08$ Å $c = 74.74$ Å $\alpha = \beta = \gamma = 90°$
Molecules/asymmetric unit	1	1
Resolution (Å)	11–1.85	10–2.5
Total No. of reflections	19,696	7855
Completeness of measured data (%)	100	97
Refinement statistics		
Resolution range (Å)	10–1.85	10–2.5
R-factor	0.19	0.15
No. of reflections ($I/\sigma(I) > 2$)	13725	5654
Completeness of $>2\sigma$ data (%)	70	69
Geometry (rms)		
Bond lengths (Å)	0.015	0.015
Bond angles (°)	3.0	3.0
Average *B*-factor (Å^2)		
Main chain	10.5	16.1
Side chain	14.0	19.0
Waters	22.3	22.8
Substrate	29.1	19.9 (MeOH)
No. of protein atoms	1822	1822
No. of substrate atoms	18	0 (4 MeOH)
No. of waters	149	129

3. Results and discussion

3.1. Observation of a productive enzyme-substrate intermediate

Elastase is a two domain protein with the catalytic triad located at the domain interface (Fig. 5). An electron density map with coefficients ($2F_{obs} - F_{calc}$) at 1.85 Å resolution from data collected from a crystal through which substrate had been flowed at −26 °C (247 K), followed by cooling of the system to −50 °C (223 K), clearly shows that substrate $Ac(Ala)_3OMe$ has reacted with the active site Ser203 (195) and has formed a covalent bond with O–γ, as indicated by contiguous electron density between the carbonyl carbon of the C-terminal alanine and the sidechain hydroxyl oxygen atom of Ser 203(195). The distance between these atoms in the final refined model is 1.4 Å, indicating a covalent bond . There is also clear electron density for the rest of the substrate, excluding the methoxide leaving group, which apparently has been displaced, as expected (Fig. 6a).

On the other hand, the electron density map with coefficients ($2F_{obs} - F_{calc}$) at 2.5 Å resolution calculated from the same crystal through which substrate-free mother liquor

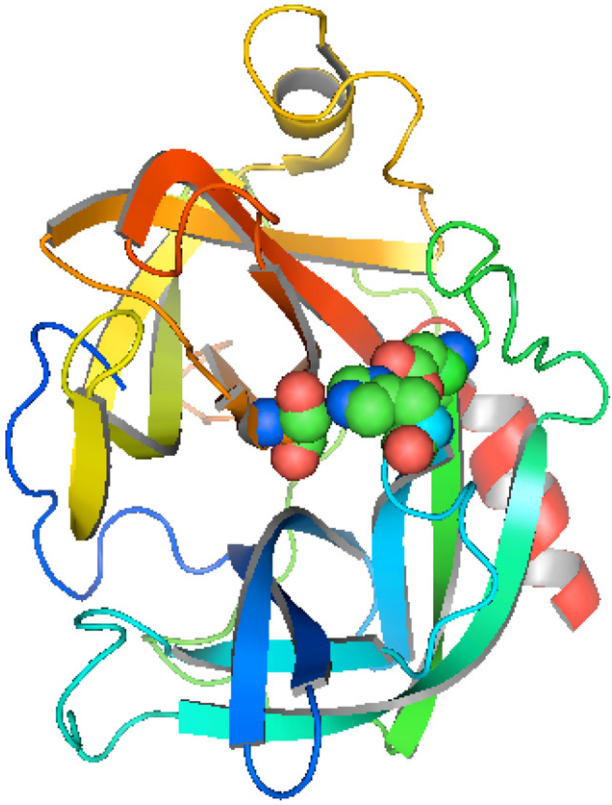


Fig. 5. Ribbon diagram of the backbone structure of elastase. The residues of the catalytic triad are shown in stick representation and the bound alanyl tripeptide is shown in space-filled form and atom typed.

had been flowed after warming of the system to −20 °C (253 K) is very different. The acyl-enzyme that was evident at −50 °C (223 K) is clearly gone, as indicated by a disappearance of the strong, contiguous electron density observed at the lower temperature. Instead there is clear electron density for two methanol molecules and one water molecule (Fig. 6b).

These two experiments indicated that the structure formed when elastase was exposed to the peptidyl ester substrate was that of a covalent acyl-enzyme intermediate, and this acyl-enzyme intermediate formed at low temperature is productive, as it will hydrolyze off the enzyme at higher temperature.

The models of the acyl-enzyme intermediate and final enzyme (after deacylating) show negligible changes to the original native elastase model. The rms deviations for alpha carbons between the native enzyme and acyl-enzyme, and between the acyl-enzyme and the control structure, are 0.2 and 0.2 Å, respectively. Elastase is a relatively rigid enzyme and does not undergo any large conformational changes when it reacts with substrate. Collective atomic motions that are required for specific substrate binding are evidently small and highly localized, or do not produce a final structure that differs significantly from the unbound state. We hypothesize that most of the collective motions that are important for catalytic activity are involved with displacement of water molecules from the active site when peptide substrates bind, or in specific motions that assist the atoms

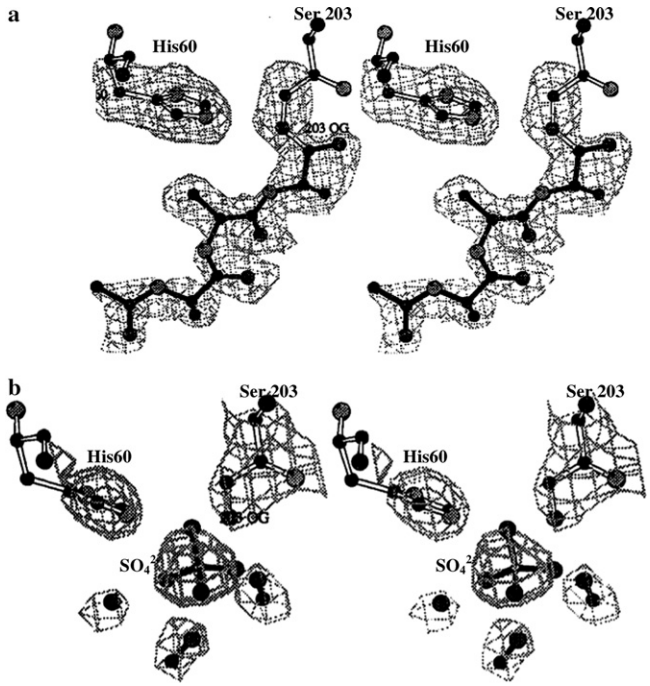


Fig. 6. (a) Stereo diagram of the electron density map in the region of the active site of elastase after substrate was added at elevated temperature and the crystal was then cooled below the "glass transition" temperature. The model of *N*-acetyl-$(Ala)_3$ has been built into this electron density as a covalent adduct at Ser203. (b) Stereo diagram of the electron density map in the region of the active site of elastase after deacylation of the intermediate by warming of the crystal above the "glass transition" temperature. No electron density is observed for the substrate, but density suggestive of two bound methanol molecules can be seen instead.

of the enzyme-substrate complex in passing over the various activation energy barriers in this multi- step reaction pathway. It is the freezing out of these motions that presumably stabilizes the acyl-enzyme intermediate indefinitely below the glass transition temperature [18,19].

3.2. Structural determinants of catalytic power

The structure elements participating in catalysis for elastase include: (1) the catalytic triad (Asp108(102), His60(57), Ser 203(195))—the numbering of residues in this paper is given by the porcine elastase sequence first, followed by the corresponding residue number in the sequence of the homologous protease chymotrypsin in parenthesis; (2) the oxyanion hole used to stabilize the negative charge on the oxygen of the putative tetrahedral intermediate by hydrogen bonding from two backbone –NH groups; (3) an extended polypeptide binding site on the acyl-group side of the scissile bond (S subsites); (4) specificity sites for side chain binding, and (5) weaker binding sites for substrate backbone and side-chain groups on the leaving group side (S′ subsites). The role of the catalytic triad has been well established through decades of crystallographic and other studies [17]. Elastase, like other serine proteases, might better be described as a histidine protease since the serine hydroxyl

is more dispensable for catalysis than is the imidazole ring. The function of the serine hydroxyl is to act as a nucleophile, attacking the carbonyl carbon of the scissile bond in the substrate. This role can be filled by a water molecule if the serine is absent, albeit at greatly reduced rate. The histidine has several functions. It activates the serine hydroxyl by forming a hydrogen bond to it, protonates the leaving group to assist in the formation of the acyl enzyme intermediate, and then serves as a general base to activate the water molecule that hydrolyzes this intermediate. Loss of the histidine is catastrophic for catalysis in the ''serine'' proteases.

The S′ subsites are rarely observed structurally since P′ residues that bind in S′ subsites are on the leaving group side of substrate, and all the serine proteases are designed to release these residues efficiently. Therefore, the cryocrystallographic studies have been concentrated on understanding the binding mode of groups in the oxyanion hole and S subsites.

The species with the highest affinity in the reaction between elastase and a substrate is expected to be the tetrahedral intermediate because it is thought to be closest to the transition state of the reaction. This intermediate is stabilized by a pair of hydrogen bonds from the backbone NH's of Ser203(195) and of Gly201(193) to the negatively charged oxygens. However, it is not clear whether the acyl-enzyme intermediate is stabilized in the same way, or whether such hydrogen bonds might be weaker in the acyl-enzyme than in the transition state-like tetrahedral intermediate. The difference in stabilization of the acyl-enzyme intermediate may effectively lower its free energy relative to other intermediates, thereby favoring its formation.

3.3. Recognition of peptide substrates

In the electron density map for the acyl-enzyme intermediate formed by the reaction of elastase and $Ac(Ala)_3OMe$ (Fig. 7), the electron density for the ester group is a trigonal, planar feature, indicating no distortions within the limits of error at this resolution. The carbonyl oxygen atom of the C-terminal alanine sits in the oxyanion hole, but it is forming only one hydrogen bond, with the main chain NH of Gly201(l93) (2.6 Å). The distance between this carbonyl oxygen and the amide of Ser203(195) is 4.0 Å, too far to be considered a hydrogen bond. Although the donor–acceptor distance of the hydrogen bond between the Gly201 (193) amide nitrogen and the substrate oxygen in the oxyanion hole is consistent with that expected for a ''short, strong'' or ''low barrier'' hydrogen bond [13] at the resolution of this structure determination we cannot state with certainty that this hydrogen bond actually falls into that class. In the previously observed structure of an acyl-enzyme formed by the reaction of elastase and the non-polypeptide substrate *N*-carbobenzoxy-L-alanine-*p*-nitrophenyl ester (ZAP; [4]), the carbonyl oxygen atom of the single alanine residue in ZAP sits in the oxyanion hole in that structure as well, forming a strong hydrogen bond with the main chain NH of Gly201(193) (2.5 Å), and a weak hydrogen bond with Ser203(195) (3.0 Å). In light of the above discussion, we believe that in the acyl-enzyme intermediate, the carbonyl oxygen of the scissile bond only needs to form a hydrogen bond with one of the backbone amides that comprise the oxyanion hole, and that one is with the main chain –NH of Gly201 (193). In the ensuing formation of tetrahedral geometry for the carbon atom when it is attacked by water during deacylation, there is abundant evidence that the substrate rearranges to form hydrogen bonds with both main chain NH's of Gly201(193) and Ser203(195) to stabilize the negative charge

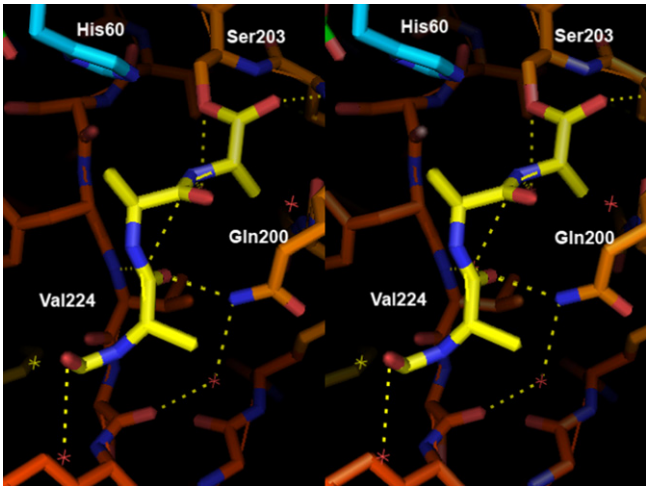


Fig. 7. Stereo view of the final refined structure of the acyl-enzyme adduct showing the interactions made by this peptide substrate with the active site of the enzyme. The bound tripeptide is in atom colors with the carbons yellow; the enzyme structure has orange carbons and lighter shading. Probable hydrogen bonds are indicated by dashed lines when the donor-to-acceptor atom distance is less than 3.5 Å.

on the oxygen of the ensuing tetrahedral intermediate. These interactions also serve to polarize the carbonyl group in order to facilitate attack by nucleophillic water.

In the model for productive binding of acyl residues of a substrate, the backbone of the polypeptide chain to be cleaved is believed to form an antiparallel β-sheet with the main-chain atoms of residue 222(214)–224(216), with the side-chains binding in specific S-pockets. For the acyl-enzyme formed by the reaction of elastase and $Ac(Ala)_3OMe$, the main chain atoms of acyl residues did indeed form an antiparallel β-sheet with the main-chain atoms of residues 222(214)–224(216). This sheet includes hydrogen bonds between the amide of the P1 alanine residue and the carbonyl oxygen of Ser222(214) (3.1 Å), the carbonyl oxygen of the P2 alanine residue and the side chain nitrogen of Glu200(192) (2.9 Å), and the carbonyl oxygen of the P3 alanine residue and the amide of Val224(216) (2.9 Å). The methyl side chain of the P1 alanine residue binds in the P1 pocket, forming a good hydrophobic interaction with Val224(216). The methyl side chain of the P2 alanine residue binds underneath the His60(57) into the P2 pocket. Comparison of the binding modes of the two acyl-enzyme intermediates, formed by ZAP and $Ac(Ala)_3OMe$, shows that the substrate parts bind very differently. Even so, ZAP and $Ac(Ala)_3OMe$ have similar k_{cat} and K_m values. This result probably means that elastase is able to accommodate different binding modes in its enzyme reaction.

After this work was completed [3] but before this paper was submitted, two additional studies of the elastase reaction have appeared that are relevant to the results presented here. Katona et al. [10] examined the structure of a complex of elastase with a tripeptide C-terminal carboxylate inhibitor and found it to bind as an acyl-enzyme. In accord with our results, the tripeptide makes the same backbone interactions, binding as an extended antiparallel β-sheet with the oxygen of the acyl enzyme hydrogen bonded in the oxyanion hole only to the amide of Gly201 (193). Wilmouth et al. [27] reported the structure of a cryogenically trapped acyl-enzyme intermediate in the hydrolysis of a hexapeptide

substrate, β-casomorphin-7, by elastase. Their structure also shows the same backbone interactions, but with symmetrical hydrogen bonding to the oxyanion from the amides of both Gly201 (193) and Ser203 (195). The ester carbonyl shows no significant distortion away from planarity, in agreement with the geometry observed here.

4. Summary and conclusions

We were surprised to find that the introduction of additional residues into the substrate for elastase had no effect on the geometry of the acyl-enzyme intermediate. It would appear that interactions in the S1 subsite alone are sufficient to establish a productive geometry for this intermediate, at least for ester substrates. The results of this study also show that the mode of binding of polypeptide substrates, observed here for the first time, is the same as that predicted from studies on the binding of polypeptide inhibitors, both covalent and noncovalent.

Taken together with the results from our earlier experiment on ZAP binding to elastase, these experiments validate the use of the glass transition temperature as a general approach to trapping intermediates in enzyme-catalyzed reactions. The difficulties associated with finding suitably fluid cryoprotective solvents and substrates that are sufficiently soluble at low temperatures in such solvents suggest that the detailed procedure described herein may not be suitable for all cases. Rather, for many enzymes, it would seem likely that useful results could be obtained by simply exposing the crystalline enzyme to substrate rapidly at near room temperature, followed by extremely fast cooling to below the glass transition temperature. Diffusion of many substrates into the lattices of crystalline enzymes should be fast enough to allow reasonable occupancies to accumulate, although it might be necessary to use slow substrates or non-optimal pH values in order to buy enough time to build up the intermediate before turnover becomes a problem.

References

[1] T. Alber, G.A. Petsko, D. Tsernoglou, Nature 263 (5575) (1976) 297–300.
[2] A.T. Brunger, J. Mol. Biol. 203 (3) (1988) 803–816.
[3] Ding, X. Studies of the Mechanism of Elastase via X-ray Crystallography. Ph.D. Thesis, Brandeis University (1995).
[4] X. Ding, B.F. Rasmussen, G.A. Petsko, D. Ringe, Biochemistry 33 (31) (1994) 9285–9293.
[5] P. Douzou, G.A. Petsko, Adv. Protein Chem. 36 (1984) 245–361.
[6] A. Gertler, T. Hofmann, Can. J. Biochem. 48 (3) (1970) 384–386.
[7] R. Huber, D. Kukla, W. Bode, P. Schwager, K. Bartels, J. Deisenhofer, W. Steigemann, J. Mol. Biol. 89 (1) (1974) 73–101.
[8] G. Hui-Bon-Hoa, P. Douzou, J. Biol. Chem. 248 (13) (1973) 4649–4654.
[9] T.A. Jones, J.Y. Zou, S.W. Cowan, Kjeldgaard, Acta. Crystallogr. A. 47 (Pt 2) (1991) 110–119.
[10] G. Katona, R.C. Wilmouth, P.A. Wright, G.I. Berglund, J. Hajdu, R. Neutze, C.J. Schofield, J. Biol. Chem. 277 (24) (2002) 21962–21970.
[11] A. Lavie, K.N. Allen, G.A. Petsko, D. Ringe, Biochemistry 33 (18) (1994) 5469–5480;
T.D. Fenn, D. Ringe, G.A. Petsko, Biochemistry 34 (2004) 6464–6474.
[12] R.J. Loncharich, B.R. Brooks, J. Mol. Biol. 215 (3) (1990) 439–455.
[13] D. Neidhart, Y. Wei, C. Cassidy, J. Lin, W.W. Cleland, P.A. Frey, Biochemistry 40 (8) (2001) 2439–2447.
[14] G.A. Petsko, J. Mol. Biol. 96 (3) (1975) 381–392.
[15] G.A. Petsko, Methods Enzymol. 114 (1985) 141–146.
[16] G.A. Petsko, D. Ringe, Curr. Opin. Chem. Biol. 4 (1) (2000) 89–94.
[17] L. Polgar, Cell Mol. Life Sci. 62 (19-20) (2005) 2161–2172.

[18] B.F. Rasmussen, A.M. Stock, D. Ringe, G.A. Petsko, Nature 357 (6377) (1992) 423–424.
[19] D. Ringe, G.A. Petsko, Biophys. Chem. 105 (2-3) (2003) 667–680.
[20] A. Ruhlmann, D. Kukla, P. Schwager, K. Bartels, R. Huber, J. Mol. Biol. 77 (3) (1973) 417–436.
[21] I. Schlichting, S.C. Almo, G. Rapp, K. Wilson, K. Petratos, A. Lentfer, A. Wittinghofer, W. Kabsch, E.F. Pai, G.A. Petsko, R.S. Goody, Nature 345 (6273) (1990) 309–315.
[22] I. Schlichting, J. Berendzen, G.N. Phillips Jr., R.M. Sweet, Nature 371 (6500) (1994) 808–812.
[23] I. Schlichting, J. Berendzen, K. Chu, A.M. Stock, S.A. Maves, D.E. Benson, R.M. Sweet, D. Ringe, G.A. Petsko, S.G. Sligar, Science 287 (5458) (2000) 1615–1622.
[24] L.H. Takahashi, R. Radhakrishnan, R.E. Rosenfield Jr., E.F. Meyer Jr., Biochemistry 28 (19) (1989) 7610–7617.
[25] R.F. Tilton, J.C. Dewan, G.A. Petsko, Biochemistry 31 (1992) 2469–2481.
[26] D. Vitkup, D. Ringe, G.A. Petsko, M. Karplus, Nature Struct. Biol. 7 (1) (2000) 34–38.
[27] R.C. Wilmouth, K. Edman, R. Neutze, P.A. Wright, I.J. Clifton, T.R. Schneider, C.J. Schofield, J. Hajdu, Nat. Struct. Biol. 8 (8) (2001) 689–694.