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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