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Conference Paper

Catalysis in Penicillin G Amidase – a Member of the Ntn (N Terminal Nucleophile) Hydrolase Family*

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The Ntn (N terminal nucleophile) hydrolases are a new family of hydrolytic enzymes with a characteristic fold in their catalytic domain. These enzymes act on a range of substrates, cleaving amide or ester bonds by a nucleophilic reaction. The catalytic nucleophile is the oxygen in serine or threonine side chains, or sulphur in the cysteine side chain. The active site is often created by autocatalytic cleavage at the nucleophile-containing catalytic residue. As a result of cleavage a free alpha amino group is generated which is an essential component in the enzyme's catalytic structure. The crystal structure of the precursor of the enzyme penicillin amidase has been determined, revealing the stereochemistry at the scissile bond prior to autocatalytic cleavage.

Key words: serine hydrolase, nucleophilic attack, Ntn-hydrolase, autocatalysis.

INTRODUCTION

All chemical reactions in biological systems are essentially catalytic in nature. They are carried out by enzymes which, in addition to providing the specific local stereochemistry that stabilises the transition states in the reaction, also select the specific substrates. This combination of properties gives biological systems the capacity to regulate these complex and highly varied metabolic and functional processes. The requirements for catalysis

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impose strict stereochemical conditions at the active site. It turns out that evolution has independently selected some catalytic structures several times. And it also turns out that the differently organised catalytic sites carrying out equivalent chemistry share chemical and structural features.

Over the last thirty years, and especially in the last ten years, there have been a great many enzyme crystal structures determined. They have revealed the spatial arrangements and chemical interactions in atomic detail and have set the scene for analysing the general principles of catalytic organisation in proteins in general. In this paper I will concentrate on an enzyme penicillin G amidase (PGA) which belongs to the Ntn hydrolase family, a hydrolytic enzyme. The hydrolytic enzymes include the most famous catalytic structure, the so-called charge-relay system, or the catalytic triad, Asp-His-Ser, first observed in chymotrypsin.[1] Generally the hydrolytic enzymes employ nucleophilic attack usually by a seryl oxygen on the carbonyl carbon of the scissile bond. The nucleophilic capacity in this enzyme class is generated by a precise stereochemical arrangement of side chain and main chain groups. The most well-known catalytic structure is the catalytic triad Asp-His-Ser, already referred to, whose H bonds and electrostatic interactions promote the nucleophilic capacity in the seryl OG in a range of serine hydrolases.[2] These three side chains have distinct properties. The Asp carboxylate accepts a H bond from His; the His acts as a base and abstracts the protein from the Ser; the serine becomes a powerful nucleophile on losing this proton. When the substrate peptide bond arrives in these enzymes it is positioned through contacts in the specificity pocket and active site, to favour approach by the nucleophilic OG. In this complex several events happen in concert. The proton leaves the OG and the scissile bond moves into the transition state conformation. This then breaks down into the acyl intermediate which in its turn is attacked by a water, now given nucleophilic capacity by the Asp : His structure, still in place. On the collapse of this second tetrahedral intermediate, the product leaves and the free enzyme is regenerated.

In the last decade protein crystallographic research has revealed that there is a wide range of catalytic structures in the serine proteases in which the three components of the classic catalytic triad Asp-His-Ser vary. The Asp is replaced by Glu and carbonyl O; the His by Lys and α amino groups and the Ser by Thr and Cys.[3] Generally speaking the enzymes in each of the different families are closely related in their structures; their sequences, though varying a great deal, usually readily identify the enzyme concerned. And their catalytic structures in particular are very similar in their side chain conformation and 3 dimensional arrangement. This generalisation obviously does not include the specificity pockets.

THE NTN HYDROLASES

The Ntn hydrolases are a recently identified enzyme family whose unique characteristics make it quite distinctive.[4] First the catalytic group is a N-terminal residue which can be Ser, Thr or Cys, showing a variation not seen in other families. Secondly the N-terminal α amino group acts as the base in the catalytic structure – the first time this has been observed. Thirdly there is a characteristic organisation of secondary structure in the catalytic domain. The catalytic residue in this structure is sited in a β strand situated in a 5 stranded β-sheet; this sheet, generally rather flat, is packed against a second β-sheet. Above and below these two sheets sit two pairs of α-helices. It turns out that the connectivities between the individual elements of secondary structure are often largely preserved, suggesting some members share a common evolutionary origin. Finally this catalytic structure is generated by autocatalytic cleavage.[5]

THE CHEMICAL REACTION OF PENICILLIN G AMIDASE NTN-HYDROLASES

The Ntn-hydrolases all carry out nucleophilic attack on the carboxy carbon in an amide bond. The substrates however vary from peptides (the proteasome), amino acid side chains, (Glutamine amido transferase GAT), amidated sugars (Aspartyl glucosyl amidase AGA) and amides (Penicillin G amidase PGA, Penicillin V amidase PVA).

PGA, illustrated in Figure 1(a), removes the phenyl side chain of penicillin G, hence its name. Its real substrate is not known but probably the enzyme has a rôle in breaking down aromatic and non-polar molecules as a food/energy source. The molecule is unusual for a bacterial protein in that it is synthesised as a single chain precursor whose autocatalytic cleavage produces two chains, an A chain of 209 amino acids and a B chain of 557 amino acids.[6] The catalytic domain is constructed exclusively from the B chain.[7] The active site is situated at the base of a large cavity whose walls are mostly formed by the B chain. Both the β sheets in the catalytic domain are extended by additional strands from the A and B chains, giving the molecule a roughly pyramidal shape.

Inspection of the catalytic B1 Ser shows its OG makes well-defined H bonds to the B23 NH and to two water molecules.[7] One of these, labelled in Figure 2(a), is also H bonded to the α-amino group and to B23 carbonyl O, completing for both these atoms a tetrahedral H bonding pattern. The α amino N also contacts the B241 amide O of the Asn side chain and OE1 of B23 Glu.

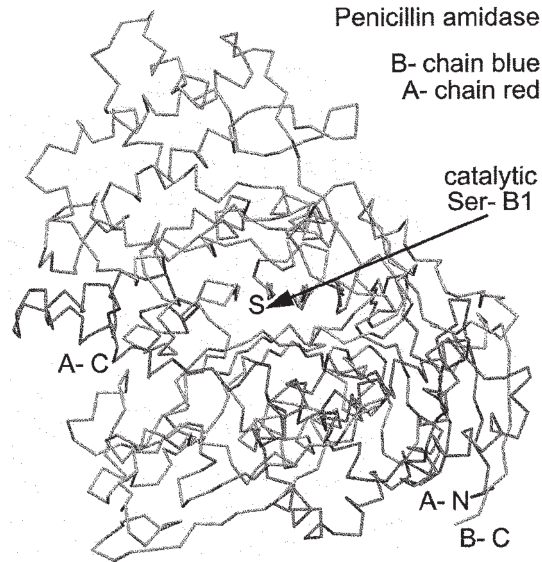

Figure 1(a). The PGA molecule shown as Cα–Cα bonds. The A chain is red, the B is blue. The catalytic and oxyanion hole residues are shown in green.

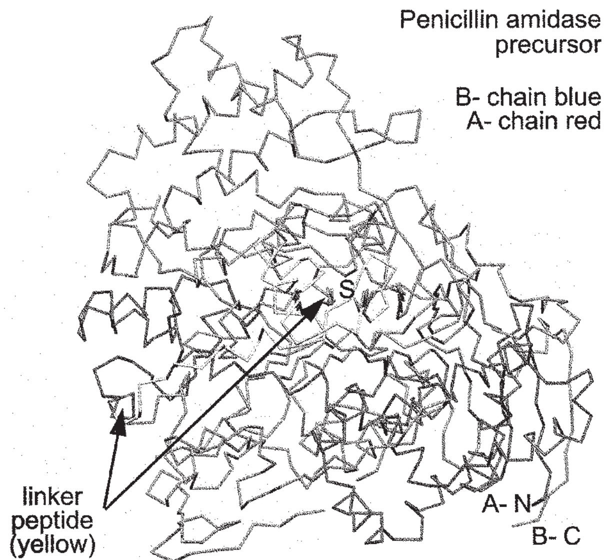

Figure 1(b). The precursor PGA molecule represented as in (a). The linker peptide is yellow.

The X-ray analysis of the enzyme reacted with such covalent inhibitors as phenyl boronate – a substrate analogue, shows them bonded to B1 Ser OG in a tetrahedral structure. This mimics the transition state and identifies the interactions and their stereochemistry that stabilise its tetrahedral geometry. There are two H-bonds to the main chain, one to the NH_2 of the B241 Asn and a water molecule, seen in Figure 2(c) (unpublished data).[8]

The nucleophilic capacity of the Ser OG is generated by its interactions with its adjacent alpha-amino group acting as a base, kept in the unproto-

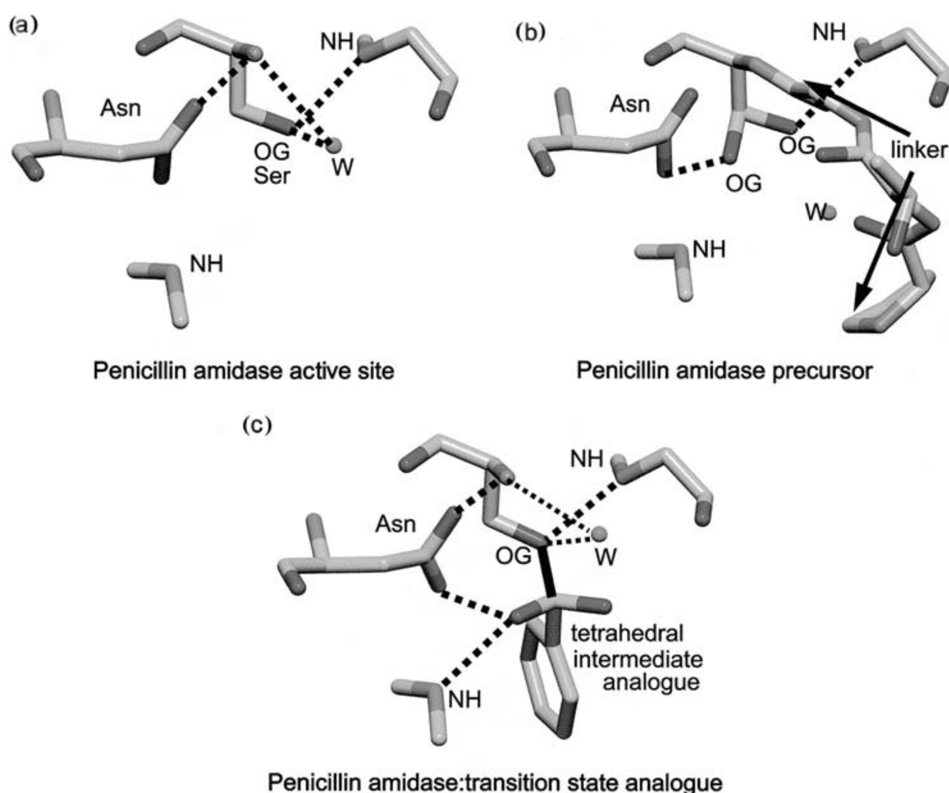

Figure 2. The catalytic site and oxyanion hole (a) in PGA, (b) the PGA precursor and (c) the PGA complex with a boronate transition state analogue. Dotted lines indicate H bonds. In PGA these connect to the α-amino group (from Asn and W), the Ser OG (from W). In the PGA precursor the two Ser OG conformations H bond respectively to Asn and to a main chain NH which forms part of the oxyanion hole. The linker peptide is marked by an arrow. In the PGA transition state analogue complex there are H bonds for two of the Os to two main chain NH functions and to the Asn NH_2. The OG bond to the boron is shown as a heavy line.

nated state in the free enzyme by its H-bonds to two carbonyl Os. There are two possible mechanisms for the initial reaction. First, direct attack by the Ser OG on to the scissile peptide's carbonyl C. The second mechanism is indirect, it involves the deprotonation of the water molecule by the alpha-amino group that sits between the alpha-amino group and the Ser OG. This OH ion acting as a virtual base, in turn deprotonates the OG and promotes it into an effective nucleophile.[7]

Thus although the stereochemistry in the catalytic machinery of the Ntn-hydrolases is largely contained in one residue, its general organisation of a base interacting with a OG, is chemically equivalent to that of the His and Ser in the catalytic triad.

AUTOCATALYTIC ACTIVATION

The activation of PGA is achieved by the removal of a 54 amino-acid connecting peptide which links the A and B chains together in the inactive precursor molecule. Normally this processing happens quickly.[8] Mutations at certain residues near B1 Ser however slow down conversion sufficiently for crystallisation experiments to be possible.[8] The mutation B263 SerGly has been prepared and the unconverted enzyme crystallised and its structure determined with data extending to 1.8 Å spacing.[8] A crystallographic analysis shows that the connecting peptide is folded compactly into the large active site cavity, completely burying the scissile peptide between B1 Ser and the linking peptide, as is shown in Figure 1(b). This observation provides a convincing structural basis for the biochemical experiments that demonstrate the processing is autocatalytic.[9]

Detailed inspection of the scissile peptide bond and its environment in the mutated precursor molecule is the obvious step to understand the chemistry. But the precursor is not the native molecule, there is a mutation at one of the critical residues, the Ser (B-1) at the linker peptide's C-terminus. Thus the local side chain and possibly main chain conformations and the surrounding water structure may differ from the native molecule. Nonetheless with care comparison can be made and possible conclusions arrived at. Figure 2b shows that the atoms in the B1 Ser and the other residues involved in catalytic activity generally have very similar positions to their equivalents in the mature, active enzyme. There are several important differences however. The first is that the catalytic seryl OG has two conformations in the crystal, one of which corresponds to the OG in the active enzyme. The second OG conformation puts it in a position appropriate for a direct nucleophilic attack on the carbonyl of the scissile bond and this is obviously relevant to processing. Inspection of the environment about the OG

shows however there are no groups that can act as a base to extract the proton. Thus the reaction is presumably driven by the physical approach of the OG to the peptide brought about by the local structure.[10,11]

The autocatalytic reaction will require a structure to stabilise the tetrahedral transition state. This can be identified from the crystal structure as the main chain NH (Gln B286) and the side chain NH_2 of Asn B241. There is a guanidinium group from Arg B263, which is also in a position to interact with the transition state O's. The role of this residue is confirmed by its mutation which prevents processing. The subsequent collapse of the tetrahedral intermediate produces the acyl intermediate. This will be cleaved by the action of a water molecule whose nucleophilic character will be promoted by the newly released alpha-amino group. Thus the study on PGA has revealed a new catalytic machinery for amide hydrolysis and identified the environment capable of autocatalytic removal of its precursor peptide. In both processes important questions remain, but knowing the 3-dimensional structures will be a valuable guide for future chemical and enzymological research.

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SAŽETAK

Kataliza u penicilin G amidazi – pripadniku Ntn (N-terminal nukleofil) familije hidrolaza

Guy G. Dodson

N-terminal-nukleofil-hidrolaze (Ntn-hidrolaze) nova su familija hidrolitičkih enzima s karakterističnim zavojem u katalitičkoj domeni. Ti enzimi djeluju na niz supstrata, cijepajući nukleofilnom reakcijom amidne ili esterske veze. Katalitički je nukleofil kisikov atom u serinskom ili treoninskom ogranku ili sumporov atom u cisteinskom ogranku. Aktivno mjesto često nastaje autokatalitičkim cijepanjem u katalitičkom ostatku koji sadržava nukleofil. Kao rezultat cijepanja nastaje slobodna α-amino-skupina koja je bitna komponenta u katalitičkoj strukturi enzima. Određena je kristalna struktura prekursora enzima penicilin-amidaze. U strukturi se jasno razabire stereokemija veze koja se cijepa neposredno prije autokatalitičkog cijepanja.