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Structural Studies of Penicillin Acylase

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Abstract

Penicillin acylases are used in the pharmaceutical industry for the preparation of antibiotics. The 3-D structure of Penicillin G acylase from *Escherichia coli* has been solved. Here, we present structural data that pertain to the unanswered questions that arose from the original strucutre. Specificity for the amide portion of substrate was probed by the structure determination of a range of complexes with substitutions around the phenylacetyl ring of the ligand. Altered substrate specificity mutations derived from an in vivo positive selection process have also been studied, revealing the structural consequences of mutation at position B71.

Protein processing has been analyzed by the construction of site-directed mutants, which affect this reaction with two distinct phenotypes. Mutations that allow processing but yield inactive protein provide the structure of an ES complex with a true substrate, with implications for the enzymatic mechanism and stereospecificity of the reaction. Mutations that preclude processing have allowed the structure of the precursor, which includes the 54 amino acid linker region normally removed from between the A and B chains, to be visualized.

Index Entries: Penicillin acylase; 3-D structures; site-directed mutagenesis; substrate binding; autocatalytic processing; precursor.

Introduction

Penicillin acylases are used industrially for the enzymatic removal of amide-linked groups from β-lactam antibiotics to yield 6-aminopenicillanic acid. This β-lactam nucleus can be modified into a range of semi-synthetic penicillins. The chemoselectivity of Penicillin G acylase (PGA) for phenylacetyl groups has been used for a variety of reactions, including the resolution of racemic mixtures and as a specific deprotecting agent during peptide synthesis. The enzyme from *Escherichia coli* is a 86kDa heterodimer of A and B chains (209 and 557 amino acids, respectively), which are

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processed from a single precursor protein by the excision of a 54 amino acid spacer peptide. The 3-D structure *(1)* disclosed many details of this unusual protein, including the structural basis for competitive inhibition by phenylacetic acid and the presence of a calcium-binding site, as well as revealing the active site residue to be serine at position 1 of the B chain leading to the concept of an N-terminal nucleophilic residue whose catalytic capacity is enhanced by its own α-amino group. This arrangement has since been recognized in a wide range of proteins, which form an emerging superfamily, the Ntn hydrolases *(2)*.

The increasing use of PGA in the fine chemicals industry and a pharmaceutical need to expand the substrate range of the enzyme, for instance, toward cephalosporins, prompted a series of 3-D structural studies to examine the chemoselectivity and binding properties of the enzyme. Such information is crucial for any attempts to remodel the active site in a designed fashion.

Altered Specificity Mutants

PGA has a marked specificity for phenylacetyl groups on the acyl portion of substrates, but the amide portion can be replaced by a variety of substituents without loss of hydrolytic activity. If an amino acid is used to replace the β-lactam ring, a positive genetic screen can be used to select from a random mutant pool for activity in *E. coli* strains auxotrophic for that amino acid *(3,4)*. Only altered enzymes with the ability to cleave the substrate will release the required amino acid and so allow cell growth. In this way, it was hoped that a compendium of PGA residues important for substrate binding could be defined. Surprisingly, challenges by quite different substrates, e.g., those that mimic the structures of cephalosporin C or cephalexin, lead to alterations of a single amino acid, at position Phe B71 *(5)*. We have solved the structure of B71 Leu *(6)*, which has a very modest activity against glutaryl-L-Leu (k_{cat} 1/s, k_{cat}/K_m 55/s/*M*), but represents an increase of at least 10,000-fold compared to the wild-type enzyme.

The 3-D structure of PGA B71 Leu mutant emphasizes the importance of hydrophobic interactions within the binding site, and provides a rationale for the finding that mutant selection with a variety of substrates leads to alterations at the same site. Loss of stacking interactions leads to the hydrophobic side chain of Phe B256 flipping from its position in the wild-type structure into the void, which forms one face of the S1' binding site (Fig. 1). This leads to a drastic alteration of the binding surface visible to the substrate, so it is likely that the mutation leads to changes in the surface complementarity between enzyme and substrate rather than altering a specific interaction.

Catalytically Inactive Mutants

To further characterize the S1' binding site for the amide portion of substrates, site-directed mutagenesis experiments were designed to make

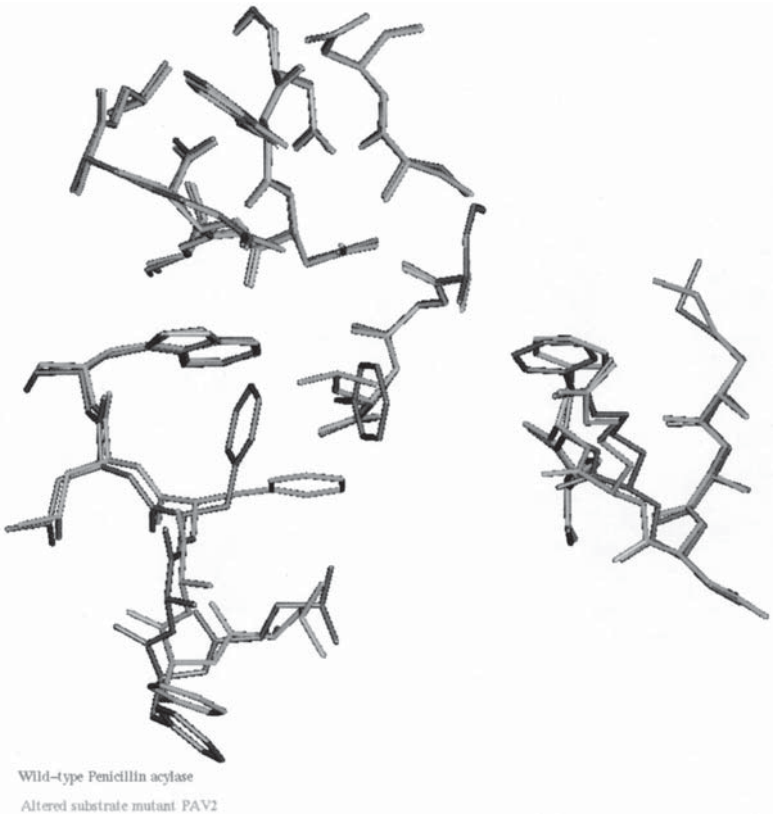

Fig. 1. Conformation changes associated with the altered specificity mutant PAV2 (Phe B71 to Leu, **Green)** compared to the wild type enzyme **(Blue)**. The binding pocket shape is remodeled by the concomitant change in the position of Phe B256 and the entrance to the specificity pocket is modulated by slight changes in the position of Phe A146 (right of picture).

a catalytically inactive enzyme for structural studies of ES complexes. A number of candidate residues were selected for mutation, which exhibited two distinct processing phenotypes.

Most alterations around the active site lead to the accumulation of precursor protein due to the intimate association of the development of catalytic activity and the requirement for processing to reveal the active site Ser at the N-terminus of the B-chain. The nucleophilicity of the Ser -OH is enhanced by its own α-NH_2 group; this ensures that catalytic competence is only achieved post-processing. Alterations at the processing site also lead to precursor production *(9)*. The 3-D structure of a PGA T263G mutant precursor has recently been solved (*7*; Hewitt et al., in preparation), clearly showing that the spacer peptide, which is normally excised, occludes the active site (Fig. 2). The inaccessibility of the active center and the fact that alterations at positions distant to the processing site, e.g., Arg B263, also lead to precursor formation (JAB, unpublished observations) strongly support the accumulating evidence that processing occurs via an autocatalytic pathway.

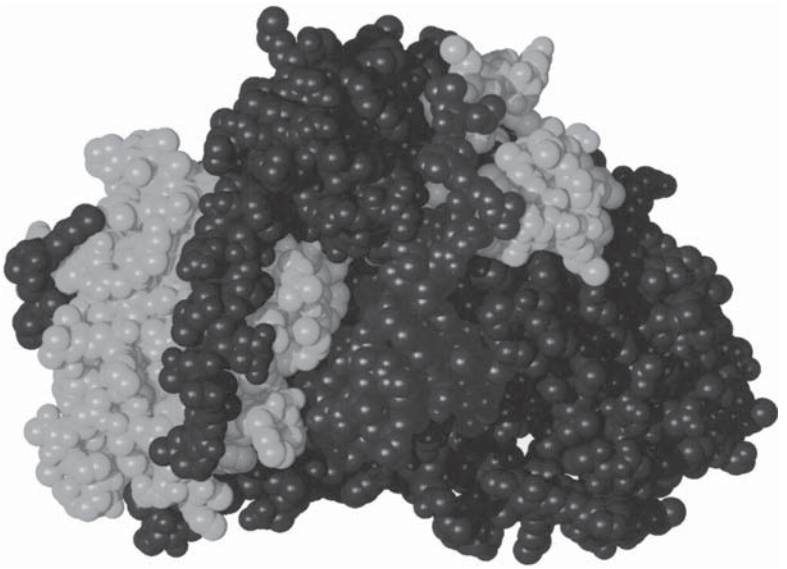

Fig. 2. Space-filling model showing the position of the spacer peptide **(Red)** between the A chain **(Green)** and the B chain **(Blue)** in the PGA precursor structure (Hewitt et al., in preparation). The spacer occludes the active site Ser, barely visible at the centre of the figure. This work is the result of collaboration with the group of Volker Kasche, Hamburg-Harburg TU, Germany.

Some mutations, e.g., Ser B1 to Cys, allow processing but yield inactive protein. The side chain of Asn B241 is a major contributor to the putative oxyanion hole, which stabilizes the transition state of the enzymatic reaction. The mutation B241 Ala leads to a protein that is correctly processed but is catalytically impaired. Coupled with improvements in crystallization and cryocrystallography *(10)*, this has allowed the direct visualization of a structure at 1.4 Å resolution which approximates to the Michaelis–Menten ES complex (*6*; McVey et al., in preparation).

The substrate benzylpenicillin is intact (Fig. 3) and is extremely well defined by the electron density, despite the fact that the amide portion (6-amino penicillanic acid) on its own is a weak noncompetitive inhibitor with binding constants in the millimolar range. The substrate is pinned between Phe A146 and Phe B71, reinforcing the suggestion of their importance by the directed evolution approach for the selection of altered substrate specificity, particularly as Phe A146 seems to undergo a major shift in position on substrate binding (Fig. 4). This system, utilizing chiral substrates, can now be used to examine stereospecificity at the S1' binding site.

The Phenylacetyl Binding Pocket

Further evidence of flexibility within the binding site has been obtained from a structural study designed to probe the major specificity-determination pocket. This is a small enclosure lined with hydrophobic

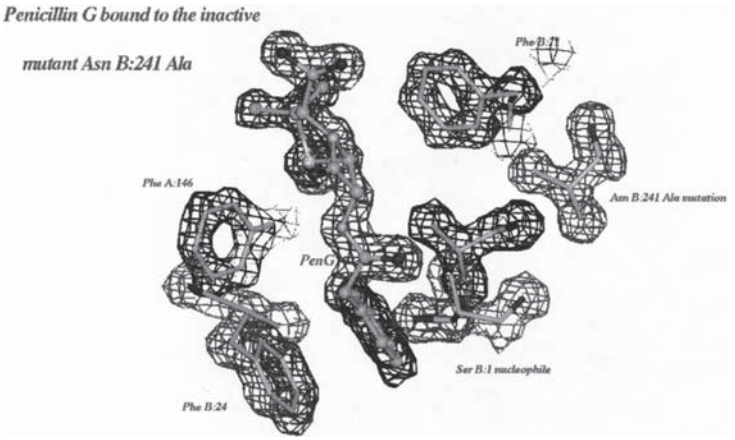

Fig. 3. Electron density in the active site region of the ES complex between the inactive mutant B241 Ala and benzylpenicillin (PenG).

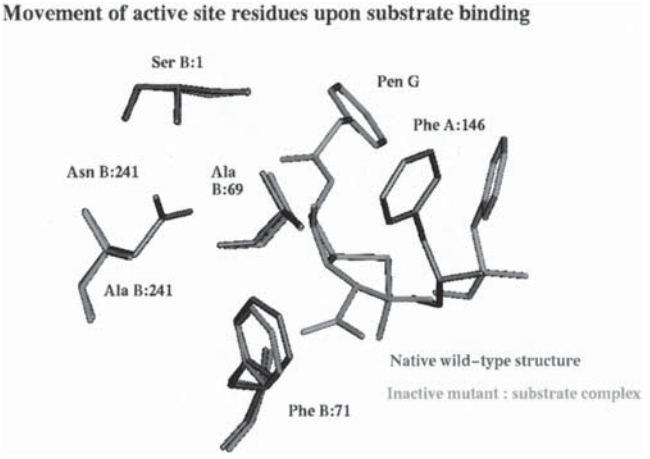

Fig. 4. Overlay of wild-type **(Blue)** and ES complex structure **(Green)** showing the movement of Phe A146 on binding of substrate (PenG).

residues, adjacent to the active site Ser B1, into which access is limited by the residue Phe A146. A series of –OH and $–NO_2$ substituted phenylacetic acid derivatives have been used as ligands in complex with PGA, and the structures analyzed to correlate their kinetic inhibition constants *(8)*. Generally, increased steric bulk and hydrophobicity of substituents leads to decreased binding and hence associated higher inhibition constants. An interesting exception is *m*-NO_2 phenylacetate, which is a relatively good inhibitor due to an extra H-bond forming capability, in agreement with other kinetic data *(11)*. The structures fall into two distinct subsets. One set, typified by *p*-OH phenylacetate, is identical to the EI complex with phenylacetic acid. The other set is typical of di-substituted ligands, e.g., 3,4-

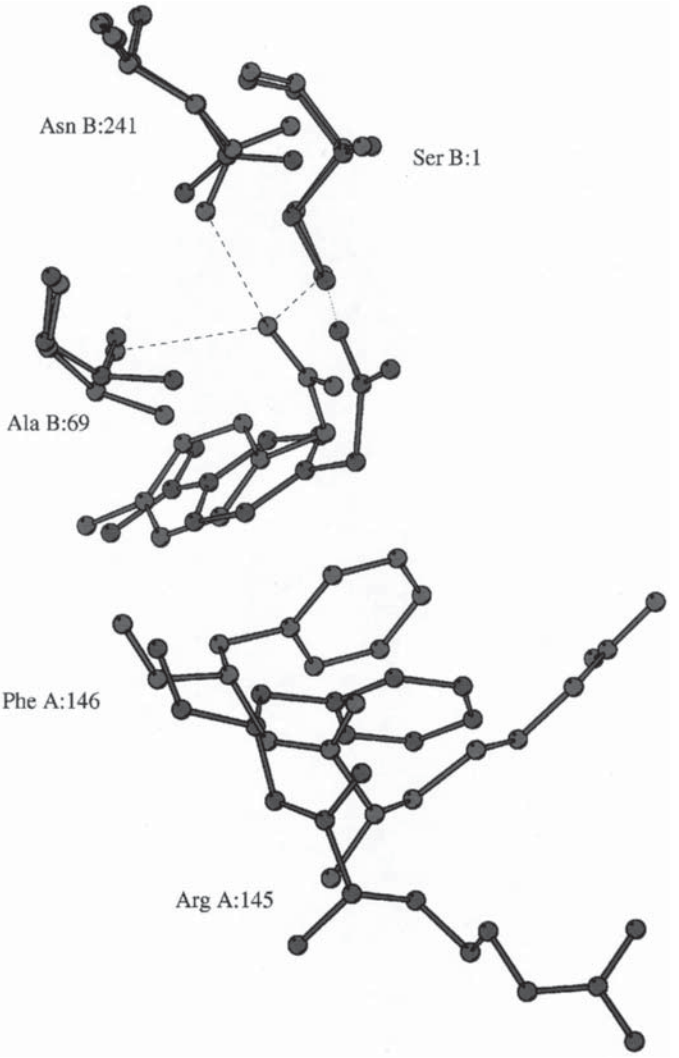

Fig. 5. Overlay of representative ligand positions in the complexes from Subset 1 (*p*-OH phenylacetate, **Red**) and Subset 2 (*p*-NO_2 phenylacetate, **Blue**), showing the large side-chain conformational change of residues Arg A145 and Phe A146 on binding of inhibitors.

dihydrophenylacetate. These ligands bind with their carboxyl groups shifted out from the pocket and are associated with a large change in the side chain positions of Arg A145 (5.7 Å) and Phe A146 (2.6 Å) in a similar manner to the conformational changes seen on binding of substrate to inactive mutant (Fig. 5).

Evidence from all three threads above suggest that there are two alternate conformations of residues A145 and A146 and that flexibility in the residues A139 to A151 may serve to transmit or propagate these structural changes from the active site. The dual energetically favourable states may

represent a switch between an active and inactive form of the enzyme, with ramifications for autocatalytic processing. It is likely that the binding of calcium is the trigger for processing. As *E. coli* PGA is periplasmic and there are very low levels of free calcium within the cell, this would ensure that processing occurs only after translocation. The calcium-binding site would seem to be strategically placed for such a role, with calcium coordination to residues B73, B75 and B76 near the S1' site and A152. A careful analysis of the precursor and processed forms in the absence of calcium, allied with site-directed mutagenesis experiments, are planned to test this hypothesis.

Acknowledgments

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