

# *Actinomadura* R39 D-Ala-D-Ala Carboxypeptidase

## DATABANKS

*MEROPS* name: D-Ala-D-Ala carboxypeptidase (*Actinomadura*-type)

*MEROPS* classification: clan SE, family S13, peptidase S13.002

*Tertiary structure*: Available

*Species distribution*: superkingdoms Archaea, Bacteria

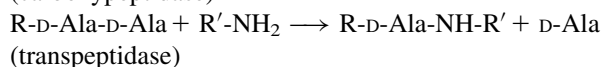
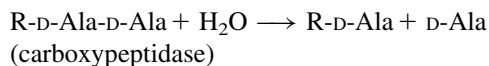
*Reference sequence from*: *Actinomadura* sp. R39 (UniProt: P39045)

## Name and History

After the discovery of the penicillin-sensitive *Streptomyces* R61 D-Ala-D-Ala carboxypeptidase ([1], see Chapter 766), a similar soluble enzyme was found in the culture medium of *Actinomadura* R39 (formerly *Streptomyces* R39) [2]. Like its R61 counterpart, it catalyzed transpeptidation reactions when supplied with adequate substrates [3–6]. Inactivation by  $\beta$ -lactam antibiotics results from the formation of a covalent bond with the essential serine residue [7].

## Activity and Specificity

The catalyzed reactions are:



where R-D-Ala-D-Ala and R'-NH<sub>2</sub> are the donor and acceptor substrates, respectively.

The enzyme also exhibits a so-called endopeptidase activity, *i.e.* it can release a C-terminal residue bearing a large and complex side chain, which is in fact a facet of its carboxypeptidase activity.

## Substrate Specificity

Good activity is observed if R = N- $\alpha$ -acetyl-L-Lys, N- $\alpha$ , N- $\epsilon$ -diacetyl-L-Lys and N- $\alpha$ -acyl-*meso*-A<sub>2</sub>pm-(L). Peptide specificity studies on the R39 enzyme have shown that the enzyme strongly interacts with a structural element consisting of the dipeptide *meso*-A<sub>2</sub>pm (L)-D-Ala, which is a direct mimic of the stem peptides from the *Actinomadura* organism (R = A<sub>2</sub>pm in the reaction described above). It seems very likely that the major substrates *in vivo* also contain this structural element, *i.e.* these substrates have a non-cross-linked N-terminus. These could be either monomers (carboxypeptidase activity) or oligomers/polymers (endopeptidase activity) [8].

The C-terminal residue can be replaced by Gly (10% efficiency), D-Glu (100%) or D-Leu (75%). Replacement of the penultimate D-Ala residue by D-Leu, Gly or L-Ala yields substrates on which the enzyme is not significantly active [2,9].

The only good acceptors are glycine and D-amino acids, but the latter may have complex side chains. For instance, they may be derivatives of *meso*-A<sub>2</sub>pm in which the amino and carboxyl groups on the D center are free while those on the L center are engaged in peptide bonds. The best substrates closely mimic the donor and acceptor parts of the peptides in the nascent *Actinomadura* peptidoglycan [5]. When supplied with compounds containing both a D-Ala-D-Ala C-terminus and an adequately positioned amino group, such as the 'natural' pentapeptide L-Ala- $\gamma$ -D-Glu-(L)-A<sub>2</sub>pm-(L)-D-Ala-D-Ala, where A<sub>2</sub>pm is the *meso* isomer, the enzyme catalyzes the formation of dimers [6]. The DD-peptidase also acts as an esterase and a thioesterase on compounds of general structure R-Xaa-Y-CH(R')-COOH, where Y is O or S [10] and Xaa is Gly or a D residue, preferentially D-Ala. These compounds are also utilized in transacylation reactions. Among these, the best reported substrate is Bz-D-Ala-S-CH<sub>2</sub>-COOH ( $k_{\text{cat}} = 6 \text{ s}^{-1}$ ,  $k_{\text{cat}}/K_{\text{m}} = 330\,000 \text{ M}^{-1} \text{ s}^{-1}$ ). The structural requirements for the R group and stereospecificity of the

thioesters are somewhat modified when compared to those for the peptides [11].

A detailed study of the reactions of 3-(D-cysteiny) propanoyl-D-alanyl-D-alanine and 3-(D-cysteiny) propanoyl-D-alanyl-D-thiolactate, catalyzed by the enzyme, showed DD-carboxypeptidase, DD-transpeptidase and DD-endopeptidase activities. These results confirm the specificity of the enzyme for substrates exhibiting an N-terminal R''-CO- group whose R'' chain contains a free D-amino acid center at the end of a rather long hydrophobic chain (for instance R'' = <sup>-</sup>OOC-CH(NH<sub>3</sub><sup>+</sup>)-CH<sub>2</sub>-S-CH<sub>2</sub>-CH<sub>2</sub>-CO) and indicate a preference for extended D-amino acid leaving groups. It was concluded that 'a specific substrate for this enzyme, and possibly the *in vivo* substrate, may consist of a partly cross-linked peptidoglycan polymer where a free side chain N-terminal un-cross-linked amino acid serves as the specific acyl group in an endopeptidase reaction' [8].

## Mechanism

In the hydrolysis reaction, the enzyme follows the classical acyl enzyme pathway of serine proteases:



where ES\* is the acyl enzyme. With the peptide Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala, acylation is the rate-limiting step, and the acyl enzyme cannot be detected. By contrast, deacylation is strongly rate limiting with most thioesters [12].

## Assays and Influence of Physicochemical Conditions

The hydrolysis of peptides is monitored by a colorimetric, discontinuous assay of the released D-alanine [13], while that of the thioesters can be followed directly at 250 nm [10]. Transpeptidation was formerly quantified by high-voltage paper electrophoresis [3], but HPLC techniques are now preferred [14]. The enzyme presents a broad optimum pH between 5.5 and 9.5. Transpeptidation is favored by high pH, high ionic strength and high and low acceptor and donor concentrations, respectively ([5] and unpublished results of the authors).

## Inhibitors

Antibiotics of the  $\beta$ -lactam family are very efficient transient inactivators that acylate the active-site serine according to the pathway described above for the substrates, but no P1 is formed (due to the  $\beta$ -lactam structure) and the deacylation step is very slow ( $10^{-3}$ – $10^{-6} \text{ s}^{-1}$ ), so that an inactive acyl enzyme accumulates [15,16]. Among the presently known enzymes, the R39

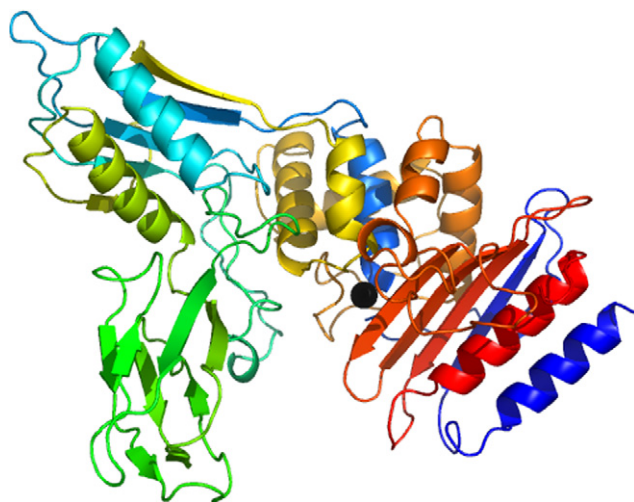
DD-peptidase is one of the most penicillin sensitive. With some but not all  $\beta$ -lactams, the deacylation step does not yield the expected penicilloic acid but involves the cleavage of the C5-C6 bond of the antibiotic [17].

Boronic acids were also found to be excellent reversible inhibitors [18,19]. In particular, (D- $\alpha$ -aminopimelylamino)-D-1-ethylboronic acid, designed to be a peptidoglycan-mimetic transition state analog inhibitor of R39, was found to be a potent inhibitor of the peptidase with a  $K_i$  value of  $32 \pm 6$  nM [19]. Since it binds some 30 times more strongly than the analogous peptide substrate, the boronate may well represent a transition state analog.

## Structural Chemistry

Several X-ray structures of R39 have been determined, in its apo form [20] or in complex with different inhibitors or peptides [19,21,22].

The structure of R39 is composed of three domains: a penicillin-binding domain and two domains of unknown functions (Figure 771.1). The latter two domains are inserted in the penicillin-binding domain, between the SXXK and SXN motifs, in the way of ‘Matryoshka dolls’. The penicillin-binding domain shares its overall fold with other penicillin-binding proteins and the serine  $\beta$ -lactamases: the R39 active site is located in an extended groove between a five-stranded antiparallel  $\beta$ -sheet and a cluster of  $\alpha$ -helices. The structures with peptidoglycan mimetic peptide, cephalosporin or boronic acid have confirmed the importance of the A<sub>2</sub>pm side chain for the affinity of substrates and inhibitors with R39.



**FIGURE 771.1** Structure of the R39 DD-peptidase (PDB 1W79). The active serine is shown as a black sphere.

## Preparation

The enzyme was initially extracted and purified from culture filtrates of the original *Actinomadura* R39 strain [23]. Much higher yields were later obtained when the cloned gene was reintroduced into the R39 strain itself by electroporation of the pDML15 plasmid [24].

## Biological Aspects

The physiological role of the R39 DD-peptidase is unclear. Homologous proteins have a preferential endopeptidase role *in vivo* but they are not essential for cell survival.

## Distinguishing Features

Among the penicillin-binding proteins (PBPs), the R39 enzyme is remarkable for its high activity on simple peptides and thioesters, its ability to catalyze the formation of dimers and its high sensitivity to most  $\beta$ -lactams. This latter property has been utilized in the design of a sensitive method for the assay of  $\beta$ -lactams in biological fluids [25].

## Related Enzymes

The sequence of the enzyme can be aligned with those of *Bacillus subtilis* PBP-4a [26] and of *E. coli* PBP-4 (Chapter 769) with 46 and 19% of identical residues, respectively. The X-ray structures of these proteins have confirmed their structural similarity with R39 [27,28]. Although the three proteins share some enzymatic similarities, their sensitivities to penicillins and cephalosporins can be very different. For instance, the *B. subtilis* PBP-4a reacts with benzylpenicillin 200-fold more slowly than the R39 enzyme. It is related to the *Streptomyces* R61 DD-peptidase (Chapter 766) by its high activity against -D-Ala-D-Ala terminated peptides.

## Further Reading

The properties of DD-peptidases and their structural and functional relationships with  $\beta$ -lactamases have been considered in detail by Frère & Joris [29], Joris *et al.* [30], Frère *et al.* [31], Ghuysen [32] and Jamin *et al.* [33]. Extensive details on the low molecular mass PBPs are given in a recent review [34].

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