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Crystallization and X-ray Diffraction Study of the *Streptomyces* K15 Penicillin-binding DD-Transpeptidase

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The 262 amino acid residue long DD-transpeptidase/penicillin-binding protein of *Streptomyces* K15 has been crystallized at room temperature by using the hanging drop vapour diffusion technique. The crystals belong to the orthorhombic space group $P2_12_12_1$, with unit cell parameters $a = 46.4$ Å, $b = 54.1$ Å and $c = 108.3$ Å. They contain one protein molecule per asymmetric unit and diffract to about 1.9 Å. X-ray data have been collected to 2.0 Å from a native crystal. The previously published amino acid sequence of the protein has been corrected at positions 71, 72, 113, 114 and 156.

Keywords: DD-transpeptidase; penicillin-binding protein; mass spectrometry; crystallization; X-ray analysis

The serine DD-peptidase/penicillin-binding protein (PBP \dagger) of *Streptomyces* K15 was synthesized in the form of a 291 amino acid residue precursor possessing a cleavable 29 amino acid residue signal peptide (Palomeque-Messia *et al.*, 1991). After translocation through the plasma membrane, the 262 amino acid residue protein remains attached to the outer face of the membrane though lacking transmembrane anchors. Overexpression in *Streptomyces lividans* results in the export of a substantial amount of the synthesized protein in the culture medium. The purified water-soluble enzyme requires 0.5 M NaCl to remain soluble. It is indistinguishable from the detergent-extracted wild-type enzyme with respect to thermostability, enzyme activity on *R*-D-alanyl-D-alanine-terminated peptides such as di-acetyl-L-Lys-D-Ala-D-Ala and penicillin-binding capacity (Palomeque-Messia *et al.*, 1992).

The *Streptomyces* K15 DD-peptidase/PBP possesses the active site defining motifs characteristic of the penicilloyl serine transferases family: the tetrad S35TTK (with S35, the essential serine residue), the triad S96GC (assumed to be analogous

to the usual SXN or YXN motifs) and the triad K217TG. Sequence similarity searches and hydrophobic cluster analysis show that this enzyme and the DD-peptidases/PBPs 5 and 6 of *Escherichia coli* and 5 of *Bacillus subtilis* (and the analogous SpoIIA protein) are structurally related and have similarity in polypeptide folding with the β -lactamases of class A (Palomeque-Messia *et al.*, 1991).

At variance with the aforementioned DD-peptidases/PBPs, which are essentially hydrolases, the *Streptomyces* K15 enzyme catalyses transfer of the *R*-D-alanyl moiety of carbonyl donor peptides to structured amino acceptors with a much higher efficacy than to water and, therefore, behaves almost as a strict DD-transpeptidase (Nguyen-Distèche *et al.*, 1986). Determination of its three-dimensional structure should shed light on the mechanism through which the high M_r PBPs catalyse peptide crosslinking during wall peptidoglycan synthesis.

Streptomyces K15 was purified to 95% homogeneity according to the technique described by Palomeque-Messia *et al.* (1992) and Granier *et al.* (1994). The mass spectrum, after conversion by the Max Ent program, revealed the presence of several molecular species, one of which had a mass of 27,473.5 Da (Figure 1). This value corresponded to the theoretical value of 27,474 Da of the revised

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\ddagger Abbreviations used: PBP, penicillin binding protein; PEG, polyethylene glycol.

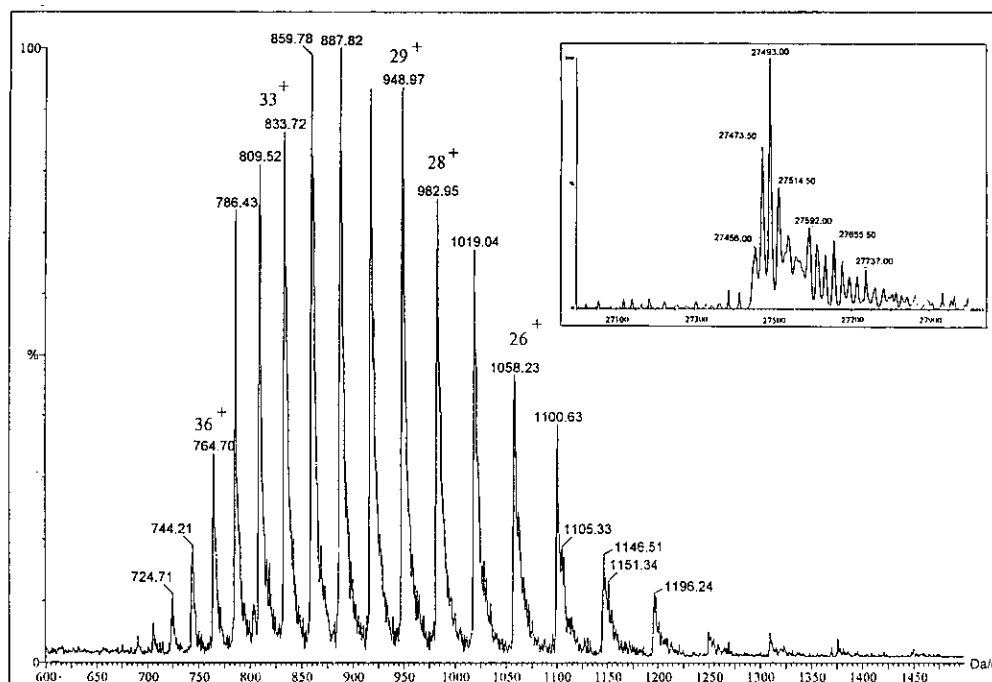


Figure 1. Electron spray ionization mass spectrum of the *Streptomyces* K15 penicillin-binding DD-transpeptidase. The inset represents the molecular mass values deduced from the M/Z peaks by the Max Ent software. The spectrum was obtained using a BIO Q mass spectrometer. The sample was dissolved in 50% (v/v) acetonitrile containing 1% (v/v) formic acid. The capillary voltage was 4 kV and the sample cone voltage 50 V.

amino acid sequence (from gene sequencing) in which Lys71 has been replaced by Asn, Pro72 by Ala, Gln113 by Thr, Ala114 by Arg and His156 by Asp. The other peaks of the mass spectrum were, most likely, sodium adducts of the protein because of the mass values themselves and the repetitive pattern of their occurrence.

Optimal conditions for crystallization were searched for by using the hanging-drop vapour diffusion technique at 20°C. The enzyme solution was concentrated by filtration through a 10 kDa exclusion membrane using a Millipore Ultrafree-MC concentrator, to a final concentration of 15 mg protein/ml. Polyethyleneglycol (PEG) of varying molecular masses and NaCl were investigated as precipitating agents. Micro to medium-sized crystals were obtained within a few days in 10 μ l drops containing 10 μ g protein/ml of 50 mM Tris·HCl (pH 5.6), 0.4 M NaCl, 10 mM NaN₃, 0.2 mM dithiothreitol, 15% (w/v) PEG4000 or 8000, equilibrated against a 1 ml-reservoir well containing 30% (w/v) PEG4000 or 8000 in the same buffer. Larger bipyramidal crystals were grown by the macroseeding technique after a few weeks. Successive seedings were needed to obtain a typical prismatic crystal with maximal dimensions of 0.6 mm \times 0.5 mm \times 0.3 mm. The maximal content of the drop solutions was 7.5 mg protein/ml and the drop solutions were equilibrated against 25 to 28% (w/v) PEG8000. Crystals of the same shape but of smaller size were obtained when PEG was omitted and the NaCl concentration increased to 4 M. A crystal was transferred to the mother liquor solution (without NaCl) containing the chromogenic β -lactam nitrocefin at a

final concentration of 1 mM. The crystal turned red within 30 minutes, indicating that the endocyclic β -lactam amide bond was hydrolysed.

For X-ray analysis, the crystals were mounted in thin-walled glass capillaries, the tips of which were equipped with 1 cm long paper filter fragments soaked in the mother liquor, and sealed with wax. X-ray precession photographs of the crystals (obtained with either PEG4000/8000 or 4 M NaCl) indicated that they were orthorhombic and belonged to the space group $P2_12_12_1$. The unit cell parameters were $a = 46.4$ Å, $b = 54.1$ Å and $c = 108.3$ Å. On the basis of a molecular mass of 27,474, and assuming that four protein molecules occurred per unit cell (which is equivalent to 1 molecule/asymmetric unit), the packing density was equal to 2.47 Å³/Da with a solvent concentration of approximately 51%. These values fall well in the range of protein crystals (Matthews, 1986).

The data were collected at 20°C to 1.9 Å resolution using a Siemens X100 area detector. The X-ray source was a graphite-monochromated Cu α radiation produced by a Rigaku RU-200 rotating anode generator operating at 50 kV and 90 mA with a 0.3 mm fine-focus cathode. The detector was at a 130 mm distance with a 2θ angle of 25°. The data collection step size was of 0.3° between frames with a count time of 60 s/frame. With two crystal orientations, a total of 900 frames were collected. Indexing, integration and scaling of the intensity data were carried out using the XENGEN processing program (Howard, 1991). The data collection statistics are shown in Table 1. The completion of the data is 90% at 2.5 Å and 80% at

Table 1
Data collection statistics

Resolution shell lower limit (Å)	Average reflection intensity	Average $I/\sigma(I)$	No. of reflections in $I/\sigma(I)$ ranges								No. of reflections	No. of observations	R_{sym}^\dagger
			0	<2	<5	<10	<20	<40	<60	>60			
3.52	1016.06	75.918	26	72	79	114	226	507	486	1565	3075	9373	0.031
2.79	476.41	29.251	47	186	225	373	643	754	364	378	2970	8339	0.055
2.44	218.08	12.930	81	421	453	619	753	514	104	29	2974	7198	0.095
2.22	152.41	8.397	149	600	632	729	614	241	44	4	3013	6426	0.114
2.06	107.64	5.390	230	805	681	583	332	105	9	0	2745	4854	0.139
1.94	65.85	3.028	154	606	308	220	68	7	1	0	1364	1920	0.150
Totals	373.73	24.968	687	2690	2378	2638	2636	2128	1008	1976	16,141	38,110	0.041

$^\dagger R_{\text{sym}} = \sum_h \sum_i |I_{hi} - I_h| / \sum_h \sum_i I_h$, where I is the scaled intensity of the i th measurement of reflection h or its equivalent, and I_h is the average intensity of reflection h .

2.0 Å. Beyond 2.0 Å, the diffraction is weak, with only 56% of the data in a 2.06 Å to 1.94 Å resolution shell having amplitude $I > 2\sigma(I)$.

The *Streptomyces* K15 enzyme contains two cysteine residues at positions 98 (of the assumed active-site defining motif SGC) and 223, one of which only, can be labelled with 5,5'-dithio-bis-(2-nitrobenzoate) or *p*-chloromercuribenzoate (*p*CMB). The enzyme binds *p*CMB in a 1:1 molar ratio, resulting in a drastic decrease of the peptidase activity and penicillin-binding capacity (Leyh-Bouille *et al.*, 1987). This property is being exploited in order to prepare a mercuric derivative suitable for the phase determination using isomorphous replacement and anomalous scattering data. The *Streptomyces* K15 enzyme is the fourth PBP and the first strict DD-transpeptidase that has been crystallized. The *Streptomyces* R61 PBP, a secretory DD-carboxypeptidase/transpeptidase, is of known three-dimensional structure (Kelly *et al.*, 1989). The structure of the *E. coli* PBP5, which is essentially a DD-carboxypeptidase (Ferreira *et al.*, 1988), and that of the high M_r PBP2X of *Streptococcus pneumoniae*, which is one of the lethal target sites of penicillin in this organism (Charlier *et al.*, 1993), are being resolved.

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