Volume 285, number 1, 115–119 FEBS 09911 July 1991

ADONIS 001457939100620G

X-Ray structure of the antibiotic bacitracin A*

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Received 29 April 1991

Bacitracins are a group of widely used peptide antibiotics. There has been interest in determining the three-dimensional structure of the bacitracins. However, solution studies indicate significant flexibility in their structure and to date native bacitracins have resisted attempts at crystallisation despite considerable efforts over a number of years by several groups. Here we report the first three-dimensional X-ray structure of a bacitracin, complexed to a subtilisin proteinase. X-Ray diffraction data were collected using synchrotron radiation in combination with the Image Plate Scanner system. The complex structure including two enzymes, two bacitracins, 220 water molecules and two Ca^{2+} ions was refined by restrained least-squares to a crystallographic R factor ($=\Sigma\{|F_o-F_c|\}/\Sigma\{F_o\}$) of 16.3% at 2.0 Å.

Antibiotic peptide; X-ray structure; Synchrotron radiation

1. INTRODUCTION

The bacitracins are a group of widely used broad spectrum antibiotics [1–3]. They are a set of closed related cyclic dodecapeptides, termed bacitracin A, B, C, D, E, and F, produced by a strain of *Bacillus licheniformis* [4,5]. This article refers only to bacitracin A (Fig. 1). The bacitracins have certain special structural features. Firstly there are four right-handed D-amino acids alternating with the usual L-amino acids along the peptide chain. Secondly, the several charged side chains enhance the possibility of interaction with other proteins. Thirdly, there is a thiazoline ring close to the N-terminus. The thiazoline ring is though to be synthesized by a cyclic condensation of the isoleucylcysteine moiety during the process of bacitracin biosynthesis [6,7]. Fourthly, the side chain of L-Lys-6 forms a link to the chain of L-Asn-12, producing a cyclic structure. Bacitracins are widely used in chemotherapy because of their antibacterial action mainly due to their inhibition of specific enzymes during bacterial cell wall biosynthesis in Gram-positive bacteria [8–10]. They are in fact multifunctional peptides and have a number of other interesting properties. For example, they can interact with the large groove of DNA by means of hydrogen bonds [11]. Bacitracin A can also chelate a single zinc ion. The cyclic structure and the presence of four D-amino acids, are assumed to give them special conformation(s) explaining their resistance to hydrolysis by the majority of proteinase, and to be a potential inhibitor towards proteinases. The kinetic constant for the inhibition (K_i) of subtilisin enzymes by bacitracin is relatively low and lies in the range of 5 mM as determined with Suc-2-Ala-Phe-*p*-NPA as substrate [12]. This explains also the use of bacitracin A as a sorbent in affinity chromatography based on its interacting selectively as inhibitor with certain hydrolytic enzymes, including the subtilisins [13,14]. This property has been exploited here to co-crystallise bacitracin A with Savinase, an alkaline subtilisin from *Bacillus lentus* [15]. The enzyme is highly homologous to subtilisins Carlsberg and BPN′ [16–19]. The native structure has been refined at 1.4 Å resolution [20]. Bacitracin A and zinc-bacitracin A both inhibit this enzyme by forming stoichiometric 1:1 non-covalent complexes. The molecular weights of the two complexes were analysed in solution by centrifugation and ultra-filtration. The results gave an unusually high molecular weight, twice the molecular weight of one 1:1 complex.

2. MATERIAL AND METHODS

Zinc-bacitracin A was purchased from Aldrich-Chemie and the *Bacillus lentus* Savinase was provided by NOVO-NORDISK (Copenhagen, Denmark). Crystals of the zinc-bacitracin/Savinase complex were grown by vapour diffusion [21]. A 5:1 molar ratio of inhibitor and enzyme was used. Crystals were grown at 18°C at 0.5 M citrate, pH 6.0, 12% polyethylene glycol 8000, 0.5 M NaCl and 5 mM $CaCl_2$ using a precipitant and buffer concentration of 50% of the well concentration [24]. For X-ray studies, single crystals were mounted in 0.7 mm diameter thin-walled glass capilarry tubes. The crystals grow in space group C2 with cell dimension $a=105.4$ Å, $b=60.6$ Å, $c=95.1$ Å, $\beta=117.3°$ for the zinc-bacitracin/Savinase complex ($V_m=2.64$ Å^3/Da). X-Ray data to 2.0 Å resolution were collected at the storage

*Coordinates of bacitracin A, as well as the coordinates of the whole complex will be deposited in the Brookhaven Protein Data Bank.

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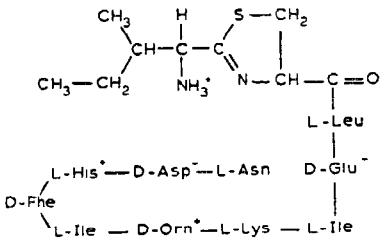


Fig. 1. The chemical formula of Bacitracin A. The molecular weight is 1450 dalton. The region presented in detail consists of an isoleucine and a cysteine residue, condensed to form a thiazoline ring rather than the usual peptide linkage. The charges shown are for the molecule at neutral pH.

ring DORIS at DESY using the EMBL image plate scanner. The intensity data have a merging R_{sym} factor, defined as $R_{sym} = \Sigma\Sigma [I - I_i]/\Sigma\Sigma I_i$ where I is the mean value of i intensity observations I_i of 6.5%. The structure was solved by molecular replacement. The special features of bacitracin were taken into consideration as additional restraints in the refinement. The final R value was 16.3% for all data in the range of 2.0–8.0 Å. The model contains all atoms of the enzyme and of the bacitracin excluding the N-terminal residue Ile-1 for both chains I and II because of very weak electron density. 220 solvent molecules and two Ca^{2+} ions bound to the enzyme were included. The RMS deviations from ideal values for bond lenghts, peptide planes, chiral centres and bond angles are 0.018 Å, 0.01 Å, 0.223 and 2.3°, respectively. Details of the structure analysis will be published later. The biochemical experiments to analyse whether the zinc is still bound to the bacitracin in solution were performed by spectroscopy in the Chemistry Department of the University of Hamburg.

3. RESULTS

The complex of Savinase with zinc-bacitracin A was crystallized and the structure refind (Fig. 2). The model provides the first crystal structure of a bacitracin and shows a novel mode of inhibition of a subtilisin, fundamentally different to that observed with other natural or synthetic inhibitors to date. The complex is formed from two enzyme molecules (referred to as A and B) linked by two bacitracin molecules (I and II) (Fig. 3 and 4). Each bacitracin A molecule binds non-covalently to the catalytic site of one Savinase and the substrate binding region of the second, such that the active sites of the two molecules face one another. The interaction of bacitracin with the active site is through the D-Glu-4 side chain forming hydrogen bonds to the catalytic site atoms NE1 of His-64 and OG of Ser-221 (the numbering is that of the reference structure subtilisin BPN'), to the oxyanion-holeforming side chain of Asn-155, the main chain N of Ser-221 and to the carbonyl oxygen of Asn-218 (Fig. 2). The links to the second enzyme are through direct H-bonds with the substrate binding region or bridges via water molecules. For both enzyme molecules the structure remains as in the native protein, and the geometry of the active site is not perturbed. The hydrogen bonding in the active site is as found in the active conformation and shown in Figs. 2 and 4.

Parts of bacitracins I and II differ significantly in conformation in the complex. For both, the two N-terminal residues and the thiazoline ring are somewhat disordered with rather weak electron density. From residue 3 onwards the electron density is well defined. In bacitracin I the main chain lies in a extended conformation from residue 4 to 8 leading enzyme A to B. The side chain of D-Phe-9 fits smoothly into the substrate binding site formed by the strands 99–104 and 125–131 of Savinase, which normally bind the main chain of the

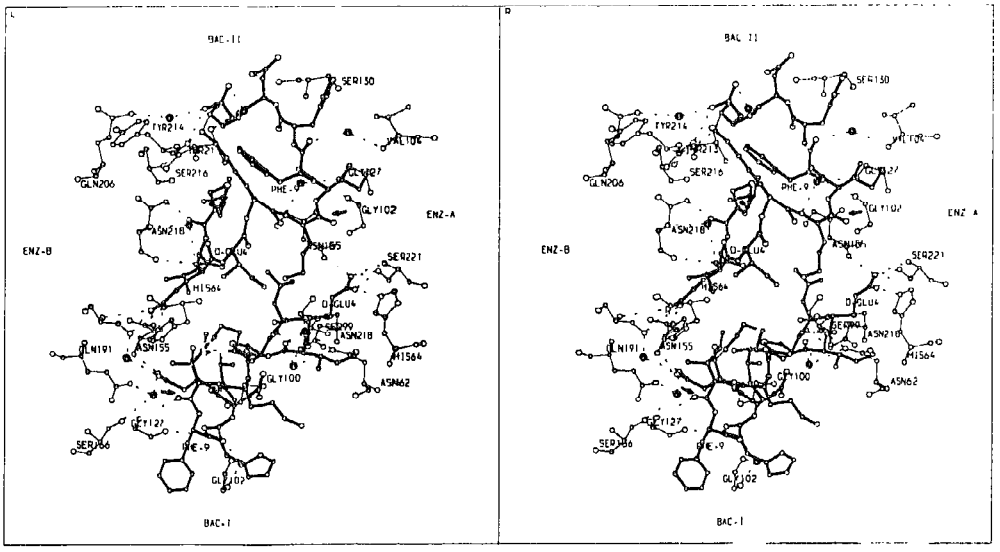
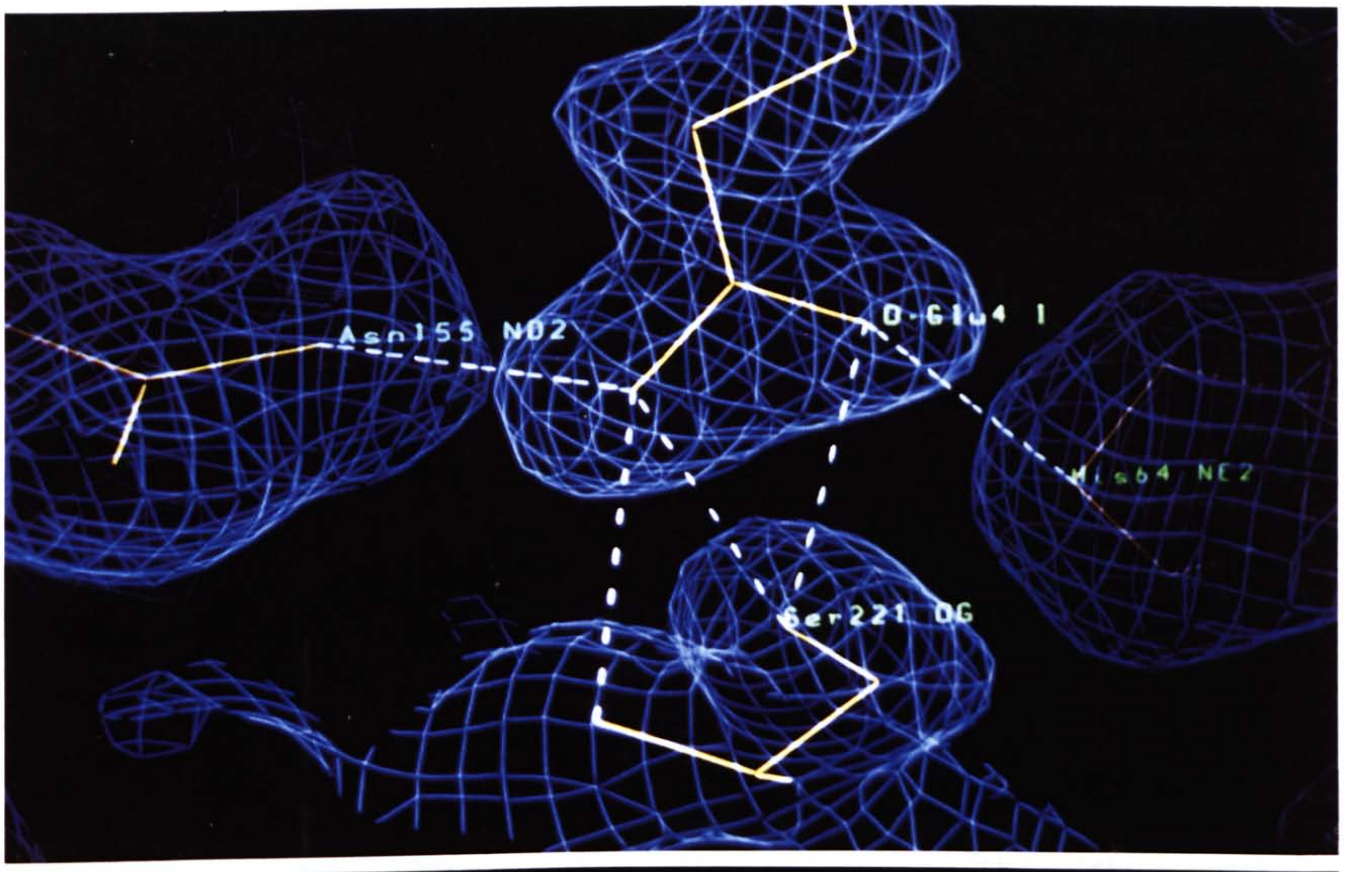


Fig. 4. Stereo-view of the binding of the two bacitracin molecules (labelled I and II) to the active sites of the enzymes (A and B). The bacitracins are shown in thick lines, and in thinner lines those residues of the enzyme involved in the interaction. The linkage between $N\zeta$ of L-Lys and the C atom of L-Asn is also shown in thinner lines. Solvent molecules are indicated as circles. Hydrogen bonds are dashed. The positions where the two bacitracin peptides begin to differ in conformation are marked with arrows. The specific recognition and binding apears to be in the active site region.



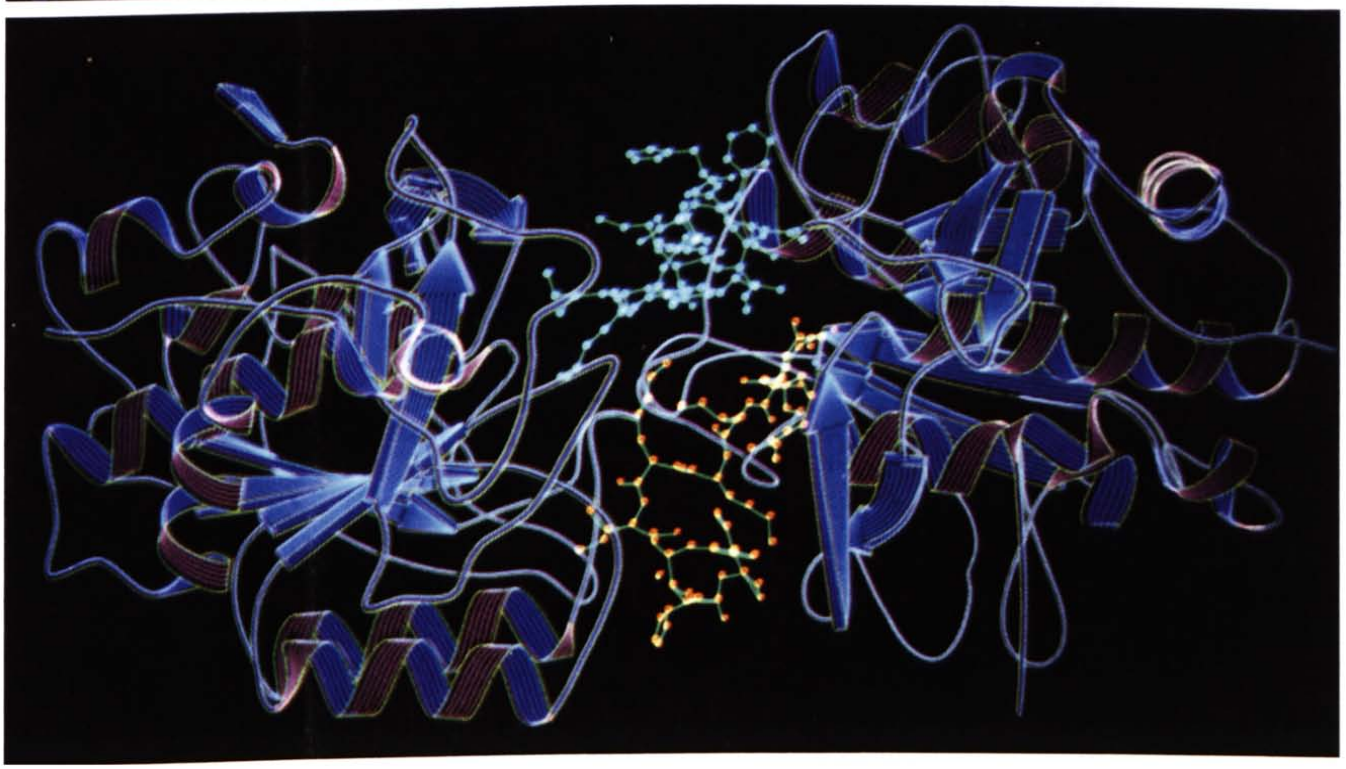


Fig. 2. View of the ($2F_o-F_c$) electron density showing that part of the bacitracin A chain interacting with the active site of the enzyme.

Fig. 3. A ribbon plot of the bacitracin/Savinase complex.

substrate or inhibitor by forming an antiparallel β-structure. The last three residues of bacitracin I form a bend towards the P1 site of the same enzyme. The C-terminal residue Leu-12 makes the ϵ linkage with L-Lys-6.

Bacitracin II lies in a similar position in the active site of enzyme molecule B. Up to residue 8 the conformation is extended as in bacitracin I. However, the conformation of the loop of residues 8–11 is nearly opposite to that in molecule I (Fig. 4). The D-Phe-9 side chain is not located in the substrate binding region but is directed away from the surface of the enzyme. The side chain of His-10 instead forms a weak hydrogen bond with the Ser-130 in this region of the enzyme. Even though the chain folding of the two bacitracins is different the contacts to contracts to the enzyme are rather similar. All contracts via H-bonds are summarised in Table I.

4. DISCUSSION

No zinc ions could be located in the three-dimensional structure, even though zinc-bacitracin was used in the co-crystallisation. A sample of zinc-bacitracin was dissolved in crystallisation buffer and dialysed against the same buffer. Spectroscopic analysis indicated that zinc remained complexed to bacitracin in solution and was not removed by dialysis. NMR techniques [22–25] indicated that at pH 5.0 three groups in the bacitracin bind to the zinc cation: the thiazoline ring, Glu-4, and His-10. In the complex described here these groups lie far away from one another, two of

Table I

The whole direct hydrogen bond interaction of the two bacitracin chains with the two enzyme molecules is listed together with the hydrogen bonds via water molecules

Bacitracin			Enzyme			
Atom	Residue/Chain		Atom	Residue	Molecule	Distance (Å)
O	2 I		N∂2	218	A	2.77
N	4 I		O	218	A	2.95
Oϵ2	4 I		N∂2	155	A	2.65
Oϵ2	4 I		Oγ	221	A	2.85
Oϵ2	4 I		N	221	A	3.29
Oϵ1	4 I		Nϵ2	64	A	2.79
Oϵ1	4 I		Oγ	221	A	2.62
O	5 I	$_{2.58}$-OW317 A-$_{3.15}$	N∂2	62	A	
O	5 I	$_{3.27}$-OW525 B-$_{3.06}$	O	99	A	
O	5 I	$_{3.27}$-OW525 B-$_{3.23}$	N∂2	62	A	
N	8 I		O	100	B	2.73
O	8 I		N	127	B	2.97
O	9 I	$_{2.57}$-OW561 B-$_{3.21}$	N	102	B	
O	9 I	$_{2.57}$-OW561 B-$_{2.84}$	O	102	B	
N	10 I		O	127	B	2.92
N∂2	12 I	$_{2.84}$-OW315 B-$_{2.91}$	Oγ	221	B	
O∂1	12 I	$_{2.85}$-OW384 B-$_{2.81}$	Oγ	166	B	
O∂1	12 I	$_{2.85}$-OW384 B-$_{2.57}$	Nϵ2	191	B	
O∂1	12 I		N	155	B	2.89
O	2 II		N∂2	218	B	2.60
O	2 II	$_{3.29}$-OW348 B-$_{2.97}$	N	218	B	
N	4 II	$_{3.22}$-OW348 B-$_{2.97}$	N	218	B	
N	4 II		O	218	B	3.04
Oϵ2	4 II		Oγ	221	B	2.64
Oϵ2	4 II		N∂2	155	B	2.67
Oϵ2	4 II		N	221	B	3.29
Oϵ1	4 II		Nϵ2	64	B	2.64
Oϵ1	4 II		Oγ	221	B	2.99
Nζ^+	6 II		N	216	B	2.99
N	7 II	$_{3.08}$-OW324 B-$_{3.13}$	O	127	A	
N	9 II		O	102	A	2.82
O	9 II	$_{2.96}$-OW359 B-$_{3.08}$	N	104	A	
N∂1	10 II		O	130	A	3.27
O∂2	11 II		Oγ	130	A	2.83
N∂2	12 II		O	214	B	2.85
N∂2	12 II	$_{3.05}$-OW350 B-$_{2.98}$	Nϵ2	206	B	
O∂1	12 II	$_{3.29}$-OW538 A-$_{2.99}$	Oγ1	213	B	

The distances are given in Å. For the interaction via water molecules the distances are given in subscript.

them indeed making contacts with different Savinase molecules. Thus bacitracin A seems to undergo conformational changes on binding to Savinase involving the parallel release of the zinc cation. This is in keeping with the interaction of the negatively charged side chain of D-Glu-4, probably the most important residue in cation binding, with the active site of Savinase in the complex.

Although their electron density is well defined, both bacitracins I and II have relatively high atomic temperature factors, indicating significant flexibility in their structure. The average B value for the bacitracins is 25.4 $Å^2$, that of the Savinase 17.1 $Å^2$. The highest flexibility is observed for amino acids not involved in hydrogen bonds. Flexibility in the structure is also seen in the difference in conformation between bacitracin molecules I and II, and the loss of zinc ion, with presumed change in conformation, on complex formation. Bacitracin A is clearly able to take up a number of different conformations depending on the local environment and the dynamic nature of the structure is certainly related to the multifunctional properties of this fascinating molecule. The crystal analysis at least permits two allowed conformations to be identified. The three-dimensional structure will serve as an essential basis for the understanding of the mechanism and action of bacitracin.

Acknowledgement: This study was supported by a FEBS Stipendium for Sabine Pfeffer.

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