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The Crystal Structure of a Penicilloyl-serine Transferase of Intermediate Penicillin Sensitivity

THE DD-TRANSPEPTIDASE OF *STREPTOMYCES* K15*

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Eveline Fonzé‡§, Marcel Vermeire¶, Martine Nguyen-Distèche‖, Robert Brasseur, and Paulette Charlier‡§**

*From the ‡Centre d'Ingénierie des Protéines and the ¶Service de Cristallographie, Université de Liège, Institut de Physique, B5 and the ‖Centre d'Ingénierie des Protéines, Université de Liège, Institut de Chimie, B6, Sart Tilman B-4000, Belgium and the **Centre de Biophysique Moléculaire Numérique, Faculté Universitaire des Sciences Agronomiques, Passage des Déportés, 2, Gembloux B-5030, Belgium*

The serine DD-transpeptidase/penicillin-binding protein of *Streptomyces* K15 catalyzes peptide bond formation in a way that mimics the penicillin-sensitive peptide cross-linking reaction involved in bacterial cell wall peptidoglycan assembly. The *Streptomyces* K15 enzyme is peculiar in that it can be considered as an intermediate between classical penicillin-binding proteins, for which benzylpenicillin is a very efficient inactivator, and the resistant penicillin-binding proteins that have a low penicillin affinity. With its moderate penicillin sensitivity, the *Streptomyces* K15 DD-transpeptidase would be helpful in the understanding of the structure-activity relationship of this penicillin-recognizing protein superfamily. The structure of the *Streptomyces* K15 enzyme has been determined by x-ray crystallography at 2.0-Å resolution and refined to an *R*-factor of 18.6%. The fold adopted by this 262-amino acid polypeptide generates a two-domain structure that is close to those of class A β-lactamases. However, the *Streptomyces* K15 enzyme has two particular structural features. It lacks the amino-terminal α-helix found in the other penicilloyl-serine transferases, and it exhibits, at its surface, an additional four-stranded β-sheet. These two characteristics might serve to anchor the enzyme in the plasma membrane. The overall topology of the catalytic pocket of the *Streptomyces* K15 enzyme is also comparable to that of the class A β-lactamases, except that the Ω-loop, which bears the essential catalytic Glu^{166} residue in the class A β-lactamases, is entirely modified. This loop adopts a conformation similar to those found in the *Streptomyces* R61 DD-carboxypeptidase and class C β-lactamases, with no equivalent acidic residue.

The bacterial cell wall peptidoglycan assembly machinery comprises, among other components, multimodular (transglycosylase/transpeptidase) membrane enzymes that catalyze the polymerization of lipid-transported disaccharide-pentapeptide units. Cross-linking of peptidyl moieties of adjacent glycan strands is a two-step reaction. The first step involves the rupture of the D-alanyl-D-alanine bond of a peptide unit precursor acting as carbonyl donor, the release of the carboxyl-terminal D-alanine, and the formation of a serine ester-linked peptidyl enzyme. The second step involves the breakdown of the peptidyl enzyme and the formation of a new peptide bond between the carbonyl of the D-alanyl moiety and the ω-amino group of another peptide unit acting as amino acceptor. The enzyme is classified as a DD-transpeptidase because the susceptible peptide bond of the carbonyl donor extends between two carbon atoms with the D-configuration.

Penicillins, the most familiar antibacterial agents, exert their effects by inactivating the serine DD-transpeptidase modules of the peptidoglycan-synthesizing enzymes. Because penicillin is a cyclic analogue of the D-alanyl-D-alanine-terminated carbonyl donors, the reaction stops at the level of the serine ester-linked penicilloyl enzyme, and the enzymes behave as penicillin-binding proteins (PBPs).[1]

Resistance to β-lactam antibiotics is PBP- and β-lactamase-mediated. PBPs with a reduced affinity for the drug result from local changes in the amino acid sequences of the wild-type forms. Serine β-lactamases hydrolyze penicillin into penicilloate via the formation of a hydrolytically labile serine ester-linked penicilloyl enzyme. The emergence of an increasing number of resistant bacteria has become a threatening public health problem. It has fostered interest in understanding the biochemical and mechanistic features of the penicillin targets.

The *Streptomyces* K15 serine DD-transpeptidase/PBP (hereafter referred to as the K15 enzyme) is a 262-amino acid monomodular protein with a molecular mass of 27,474 Da (1). Although lacking transmembrane segments, the K15 enzyme is associated with the plasma membrane of the wild-type strain. Overexpression of the encoding gene in *Streptomyces lividans* results in the secretion of ~30% of the synthesized enzyme in the culture medium. The cloned enzyme has the same enzymatic properties as the membrane-associated form and requires the presence of 0.5 M NaCl to remain water-soluble (2).

The fate and rate of the two-step transpeptidation reaction catalyzed by the K15 enzyme depend on both the nature of the scissile bond (peptide, ester, thiol ester) of the carbonyl donor and the acceptor activity of an exogenous nucleophile (3). With (*R*)-D-alanyl-D-alanine-terminated peptide donors and in aqueous media, the released D-alanine is reutilized as an amino

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The atomic coordinates and structure factors (codes 1skf and r1skfsf) have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

§ To whom correspondence should be addressed. Tel.: 32-43-66-36-19; Fax: 32-43-66-47-41; E-mail: Eveline.Fonze@ulg.ac.be or Paulette.Charlier@ulg.ac.be.

[1] The abbreviations used are: PBPs, penicillin-binding proteins; MIRAS, multiple isomorphous replacement with anomalous scattering; MHP, molecular hydrophobicity potential; Wat, water.

acceptor in the enzyme deacylation step so that the peptide donor is continuously regenerated and the enzyme is seemingly silent, although it turns over one time every 10 s. In the presence of amino acceptors structurally related to cell wall peptidoglycan, the peptidyl ((*R*)-D-alanyl) enzyme intermediate is aminolyzed much more effectively than it is hydrolyzed, and under certain conditions, the acyltransferase functions exclusively as a DD-transpeptidase (4). In contrast, the DD-carboxypeptidases/PBPs have a much increased preference for water as the attacking nucleophile of the peptidyl enzyme.

The K15 enzyme is a PBP. The value of the second-order rate constant of enzyme acylation by benzylpenicillin is ~150 M^{-1} s^{-1}, and the serine ester-linked penicilloyl enzyme undergoes hydrolytic breakdown at an extremely slow rate via two pathways that give rise to benzylpenicilloate and phenylacetylglycine, respectively. Cefoxitin is a better inactivating agent (rate constant of ~850 M^{-1} s^{-1}), and in this respect, the K15 enzyme differs from many PBPs for which benzylpenicillin is one of the most efficient known inactivators tested (5). Low enzyme acylation rate values confer increased "intrinsic" resistance on the bacterial cell. Thus, for example, the values of the rate constants of enzyme acylation by benzylpenicillin are 10–20 M^{-1} s^{-1} for the low affinity PBP2′ of the methicillin-resistant *Staphylococcus aureus* and PBP5 of *Enterococcus hirae*. In contrast, the rate constant values are 18,000 M^{-1} s^{-1} for the *Streptomyces* R61 DD-carboxypeptidase/PBP, 58,000 M^{-1} s^{-1} for *Streptococcus pneumoniae* PBP2x, and 300,000 M^{-1} s^{-1} for *Bacillus licheniformis* PBP1 (6–9). In comparison with those extreme values, the K15 enzyme may be considered as a PBP of intermediate penicillin sensitivity.

The PBPs and the serine β-lactamases are members of the penicilloyl-serine transferase family (10). The three-dimensional structures are known for the class A β-lactamases of *S. aureus* PC1 (11), *Streptomyces albus* G (12), *B. licheniformis* 749/C (13), and *Escherichia coli* TEM (14–16) and for the two class C β-lactamases of *Citrobacter freundii* (17) and *Enterobacter cloacae* P99 (18). Among the PBPs, only the structure of the monomodular DD-carboxypeptidase of *Streptomyces* R61 has been determined at high resolution (19), and that of the multimodular PBP2x from *S. pneumoniae* has been established at low resolution (20). Understanding the structure-function relationships among members of the penicilloyl-serine transferases requires a detailed knowledge of each type of enzyme of this family at the atomic level. The K15 enzyme is the only serine DD-transpeptidase/PBP that has been characterized in detail in biochemical terms. In this paper, we describe the high resolution x-ray crystallographic structure of this enzyme, compare its structure with that of the class A serine β-lactamases, and emphasize the most significant structural features that distinguish these two types of enzymes.

EXPERIMENTAL PROCEDURES

Crystallization and Data Collection—The K15 enzyme is synthesized in the form of a 291-amino acid precursor possessing a cleavable 29-amino acid signal peptide. The overproduced soluble enzyme was purified to 95% homogeneity, and the mass spectrum revealed a mass corresponding to the theoretical value of 27,474 Da. Large bipyramidal crystals with maximum dimensions of 0.6 × 0.5 × 0.3 mm were obtained at 20 °C within a few days in 10-μl drops containing 1.3 mg/ml protein solution in 50 mM Tris-HCl (pH 7.2), 0.4 M NaCl, 10 mM NaN_3, 0.2 mM dithiothreitol, and 7.5% (w/v) polyethylene glycol 4000 or 8000, equilibrated against a 1-ml reservoir well containing 15% (w/v) polyethylene glycol 4000 or 8000 in the same buffer. The crystals belong to the orthorhombic space group $P2_12_12_1$, with cell dimensions of a = 46.59 Å, b = 54.53 Å, and c = 108.70 Å. They contain 51% solvent and one molecule/asymmetric unit (21).

X-ray diffraction data were collected at 20 °C using a Siemens X1000 area detector. The x-ray source was 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. Indexing, integration, scaling, and merging of the intensity data were carried out using the XENGEN Version 2.0 (Native 1 and 2 and heavy atom derivatives) (22) and the SAINT (Native 3 and 4) (23) software packages. To improve the completeness of the native data, the four native sets were merged with the BIOMOL package provided by the Groningen-CF/BIOMOL Group (Department of Chemistry and Biophysical Chemistry, Crystallography Group, Rijkuniversiteit Groningen) (Table I).

Structure Determination and Refinement—The structure was solved by the method of multiple isomorphous replacement with anomalous scattering (MIRAS) using the PHASES program (24). Of the 30 heavy atom derivatives tested, only *p*-chloromercuribenzoate and mersalyl mercury derivatives showed binding sites. Of the two cysteine residues present in the K15 enzyme at positions 38 and 223, respectively, only one is labeled by *p*-chloromercuribenzoate in aqueous solution, resulting in a drastic decrease in the peptidase activity and penicillin-binding capacity (25). Table I gives the refinement parameters of the heavy atom derivatives at 3.0-Å resolution. The quality of the 3.0-Å MIRAS electron density map was improved by solvent flattening using the SQUASH program (26).

The protein structure was built stepwise using TURBO-FRODO (27) and refined by simulated annealing using X-PLOR (28). Fourteen cycles of refinement (positions and temperature factors) and model refitting were carried out. At each step, the structure was constructed on the basis of $2F_o - F_c$ and $F_o - F_c$ maps computed at a 20-Å lower resolution limit. As long as the model was incomplete, the MIRAS phases were used and combined with those determined by the model in order to improve the quality of the electron density map and to avoid model bias. The structure was refined to 2.0-Å resolution with a conventional *R*-factor of 18.6%, and the statistics of refinement are summarized in Table I.

Calculation of the Molecular Hydrophobicity Potential (MHP)—The hydrophobicity of a molecule is usually defined by a single parameter representing its partition coefficient between water and octanol. For the analysis of complex interactions between lipids and proteins, this parameter was extended to the most elaborate concept of helical hydrophobic moment (29). The calculation of the MHP along the peptide sequence was based upon the concept introduced by Furet *et al.* (30) and Fauchère *et al.* (31) for a small molecule. This approach was extended to a three-dimensional representation of the molecular envelope with isopotential contour lines around an 18-residue helical peptide (32). We assumed that the hydrophobic interaction between two residues decreases exponentially with the distance between the residues according to the following equation: $\mathrm{MHP} = \Sigma\, E_{tri} \exp(r_i - d_i)$, where E_{tri} is the transfer energy of the atom i, r_i is the radius of the atom i, and d_i is the distance between atom i and a point M.

The transfer energy E_{tri} represents the energy for individual atoms, calculated from the transfer energy compiled by Tanford (33). Assuming that the experimental molecular energy is the sum of the atomic energies, the E_{tri} values are estimated for the different atoms. The MHP for proteins was calculated by a cross-sectional computation method. A plane was moved every 3 Å, and the MHP was computed for all points in the plane defined by a 3-Å side grid.

RESULTS

Overall Structure—The overall three-dimensional structure of the K15 enzyme consists of a single polypeptide chain organized into two domains. One domain contains mainly α-helices, and the second one is of α/β-type. The K15 enzyme bears the signature fold topology of the penicilloyl-serine transferase superfamily, but it exhibits more overall similarity to the class A β-lactamases (Fig. 1). Using the standard secondary structure numbering of the class A β-lactamases (35), the first domain contains a central helix (α2) that is surrounded by four helices (α4, α5, α6, and α9) and, in addition, a four-stranded antiparallel β-sheet (β2a to β2d) and helix α3. The α/β-domain consists of a five-stranded antiparallel β-sheet (β1 to β5) that is covered on one face by the short helix α10 and the long carboxyl-terminal helix α11 and on the other face by helix α8 and one turn of a 3_{-10} helix (α0).

Comparison of the K15 Enzyme and the Class A β-Lactamases—Optimal superimposition of the K15 enzyme and class A β-lactamases was performed using a maximum distance cutoff of 1.2 Å and, as fitted atoms, the equivalent C-α atoms of the

TABLE I
Diffraction data, phasing, and refinement statistics

Diffraction data statistics		No. of observations	Unique reflections	Multiplicity	Completeness[a]	$\langle I/\sigma I \rangle$	R_{merge}[b]
Data set	Resoluton limit						
	Å				%		%
Native 1	1.94	38,110	16,141	2.4	77 (1.94)	18.0	4.8
pCMB[c]	2.16	57,166	22,898	2.5	81 (2.16)	20.6	5.2
Mersalyl	2.43	25,151	12,873	1.9	66 (2.43)	26.6	4.3
Native 2	2.15	35,607	12,641	2.8	83 (2.15)	18.3	5.2
Native 3	2.07	27,430	11,812	2.3	78 (2.07)	24.3	9.7
Native 4	1.75	67,888	22,874	2.9	79 (1.75)	34.2	8.3
1 + 2 + 3 + 4	1.75		23,063		81 (1.75)		9.9
					94 (2.00)		

Refined parameters of the heavy atom derivatives at 3.0Å resolution *versus* the Native 1 data set

Derivatives	R_{iso}[a]	Site	x[e]	y[e]	z[e]	B	q[f]	Phasing power[g]
	%							
pCMB	21.1	1	0.85	0.76	0.90	12.3	0.87	1.96
Mersalyl	19.2	1	0.36	0.69	0.09	18.2	0.57	1.43
		2	0.50	0.12	0.33	15.4	0.54	

Refined Model			
Spacing (Å)	8.0 to 2.0	B model, isotropic (Å^2)	
No. of reflections (I > 3σ)	17,859	Overall (B_{mean})	20.0
Completeness (%)	93.9	Main Chain	17.8
R-factor (%)	18.6	Side chain	20.2
R_{free} (%)	24.1	Solvent	34.7
No. of residues (of atoms)	262	B_{mean} estimated from Wilson plot	17.1
No. of protein atoms	1927		
No. of solvent atoms	149	rms deviations form ideal geometry of	
Estimated coordinate error		Lengths (Å)	0.015
(low resolution cutoff of 5.0 Å)		Bond angles	3.0°
From Luzatti plot (Å)	0.20	Fixed dihedral angles	23.9°
From Sigma (Å)	0.19	Improper dihedral angles	1.24°

[a] The numbers given in parentheses denote the respective resolution limit.
[b] $R_{merge} = \Sigma\Sigma|\langle I \rangle - I_i|/\Sigma\Sigma I_i$.
[c] pCMB, *p*-chloromercuribenzoate; rms, root mean square.
[d] $R_{iso} = \Sigma |F^2_{PH} - F^2_P| / \Sigma |F^2_P|$, where PH and P are related to the heavy atom derivative and the native enzyme, respectively.
[e] x, y, and z are positions of heavy atoms in fractional atomic coordinates.
[f] B is the isotropic thermal factor, and q is the absolute occupancy.
[g] Phasing power = $|\langle F_{H(calc)} \rangle| / E$, where $\langle F_{H(calc)} \rangle$ and E are average structure factor and lack of closure error, respectively.

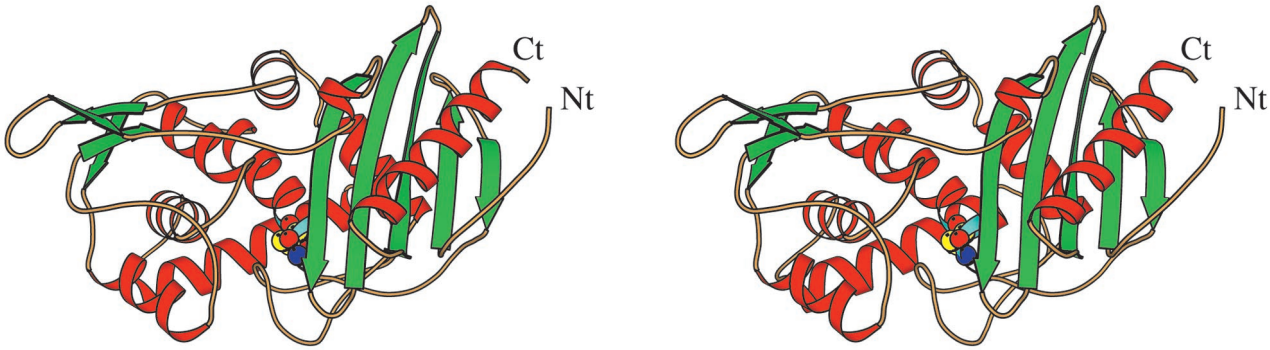

FIG. 1. **Stereo view of the ribbon diagram of the *Streptomyces* K15 DD-transpeptidase structure.** The helices are colored *red*, the 3_{10} helix α0 is *blue*, and the β-sheets are *green*. The active Ser[35] residue of the K15 enzyme is identified in Corey-Pauling-Koltun. The figure was made with MolScript Version 1.4 (34). *Ct*, COOH terminus; *Nt*, NH_2 terminus.

catalytic residues Ser[35], Lys[38], Ser[96], Lys[213], and Gly[215] for the K15 enzyme and Ser[70], Lys[73], Ser[130], Lys[234], and Gly[236] for the class A β-lactamases. The structurally superimposable regions comprise 170 amino acids, and the root mean square deviation for the 170 pairs of C-α atoms varies from 2.1 to 2.5 Å depending on the β-lactamase being compared. As shown in Fig. 2, one may note that pairwise comparison between the amino acid sequences reveals only ~15% of strict identity between the K15 enzyme and each of the class A β-lactamases.

In comparison with the class A β-lactamases, the K15 enzyme exhibits four main structural differences (Fig. 3*A*): (i) the absence of the NH_2-terminal helix α1; (ii) the insertion of 11 residues after helix α5, resulting in the extended helix α6; (iii) the absence of helix α7, which contains the class A β-lactamase-specific E*X*ELN motif; and (iv) the occurrence of a large insert between helices α9 and α10. This insert contains strands β2c and β2d of the four-stranded β-sheet, and the stability of this β-sheet on the surface of the α-domain is reinforced by two salt bridges involving Asp[54]–Arg[86] and Asp[81]–Arg[191].

The MHP analysis (32) of the K15 enzyme and class A β-lactamases (Fig. 4) reveals an increase in the hydrophobic isopotential areas in the K15 enzyme, generated by the N- and C-terminal regions that cover the five-stranded β-sheet and the loop corresponding to the 20-amino acid insert. This marked

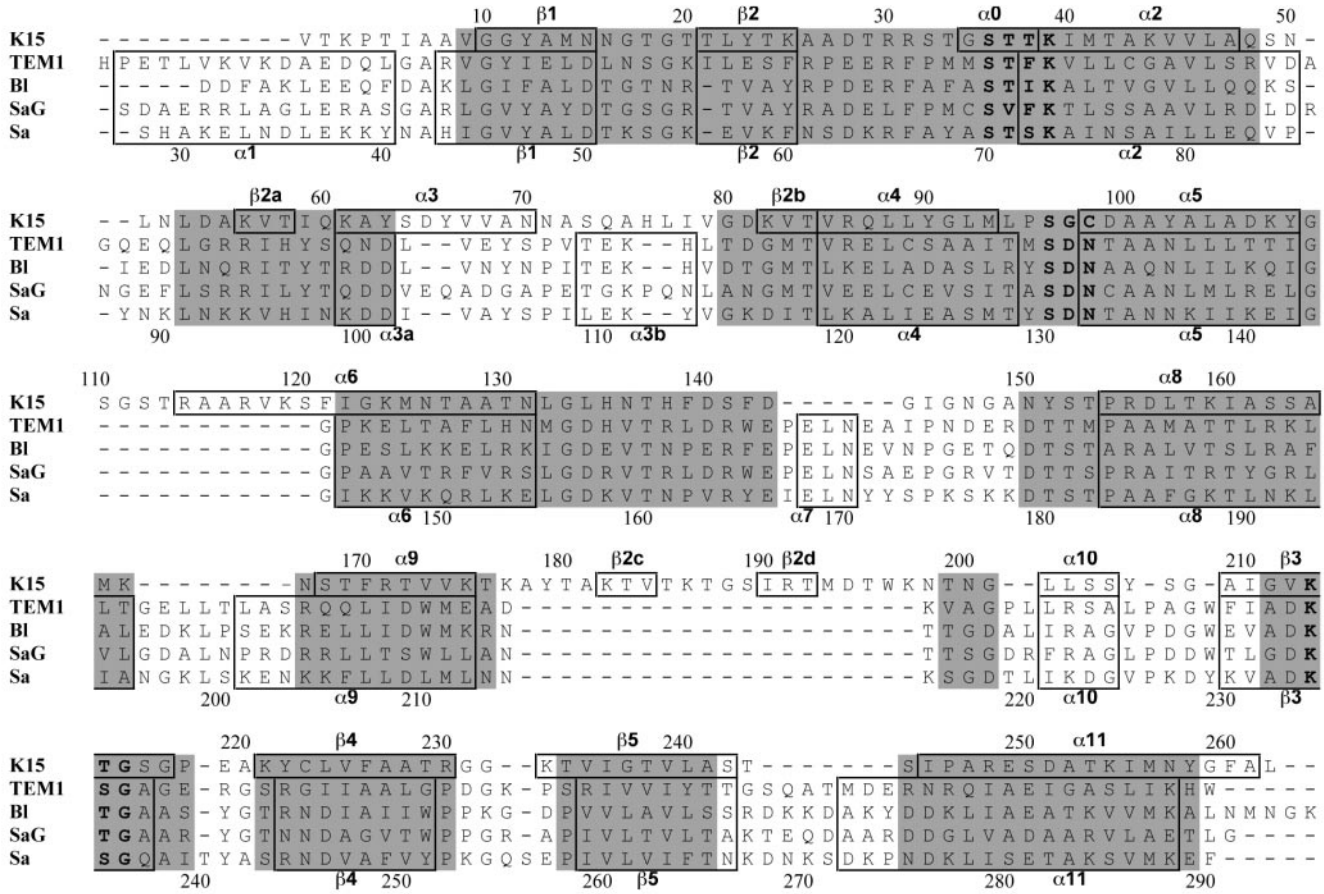

FIG. 2. **Amino acid sequence and secondary structure alignments of the *Streptomyces* K15 DD-transpeptidase and four class A *β*-lactamases.** The *β*-lactamases of *E. coli* (Protein Data Bank code 1XPB), *B. licheniformis* (Protein Data Bank code 4BLM), *S. albus* G (Protein Data Bank code 1BSG), and *S. aureus* (Protein Data Bank code 3BLM) are labeled TEM1, Bl, SaG, and Sa, respectively. The amino acid numbering adopted for the K15 enzyme and class A *β*-lactamases is shown above and below the sequences, respectively. The secondary structures of the K15 enzyme and TEM1 *β*-lactamase, as defined according to the DSSP algorithm (36), are *boxed* and denoted above and below the sequences, respectively. The three main active-site defining motifs are in *boldface*, and the structurally superimposable 170-amino acid regions between the K15 enzyme and class A *β*-lactamases are shown in *shaded boxes*.

hydrophobic characteristic, which is very different from the homogeneous distribution of the hydrophilic/hydrophobic isopotential areas observed in soluble exocellular proteins, could lead to the interaction of the K15 enzyme with the plasma membrane.

In comparison with the *Streptomyces* R61 carboxypeptidase/PBP (Protein Data Bank code 3PTE) and the class C *β*-lactamase of *E. cloacae* P99 (Protein Data Bank code 1BLS), the K15 enzyme shows a much higher extent of divergence (Fig. 3*B*). The only common secondary structures are the central helix *α*2 and the five-stranded *β*-sheet. Helices *α*5, *α*6, *α*8, and *α*11 are also present, but they have different lengths and orientations.

Catalytic Pocket—As observed with the other penicilloyl-serine transferases, the active site of the K15 enzyme is located at the interface between the two domains and is mainly defined by three conserved structural elements (Fig. 5). The center of the catalytic cleft is occupied by the Ser^{35}-Thr^{36}-Thr^{37}-Lys^{38} tetrad, which includes the nucleophilic Ser^{35} residue at the amino-terminal end of helix *α*2. One side of the cavity is defined by the Ser^{96}-Gly^{97}-Cys^{98} loop connecting helices *α*4 and *α*5. The Lys^{213}-Thr^{214}-Gly^{215} triad lies on strand *β*3 on the opposite side of the cavity. The backbone NH group of the essential Ser^{35} residue and that of Ser^{216} downstream from the motif Lys^{213}-Thr^{214}-Gly^{215} occupy positions that are compatible with the oxyanion hole function required for catalysis.

With *X* denoting a variable amino acid, the amino acid sequence signature of the penicilloyl-serine transferases is defined by the three active-site structural elements: the invariant S**XX*K tetrad, the S*X*(N/C/S) or Y*X*N triad, and the (K/H/R)(T/S)G triad. Divergence at the level of the second element might have given rise to the "tyrosine" and "serine" enzyme subgroups, respectively (37). All known PBPs, including the K15 enzyme and the class A *β*-lactamases, belong to the so-called serine subgroup, whereas the *Streptomyces* R61 DD-carboxypeptidase/PBP and the class C *β*-lactamases belong to the tyrosine subgroup. Superimposition of the active sites reveals that the *γ*-OH of the serine residue of the first subgroup and the phenolic OH of the tyrosine residue of the second subgroup occupy equivalent positions with respect to the nucleophilic serine and strand *β*3, respectively (Fig. 6*A*). As the side chain of a tyrosine residue is bulkier than that of a serine residue, this spatial equivalence must be the result of a reorganization of both the corresponding loops and the interconnecting secondary structures of all the *α*-domains. Accordingly, the serine and tyrosine subgroups show a larger extent of divergence at the level of the *α*-domains than at the level of the *α*/*β*-domains.

The top of the catalytic cleft of the K15 enzyme is defined mainly by the Thr^{199}-Asn^{200}-Gly^{201} triad (immediately upstream from helix *α*10), and the bottom of the cleft by the backbone of the Phe^{142}-Asp^{143}-Gly^{144} triad. The Thr^{199}-Asn^{200}-Gly^{201} triad adopts the same topology as in the class A *β*-lactamases (Fig. 6*B*) despite large structural differences between the helix *α*9/strand *β*3-connecting polypeptide chains.

In the class A *β*-lactamases, the E*XX*LN motif of the Ω-loop

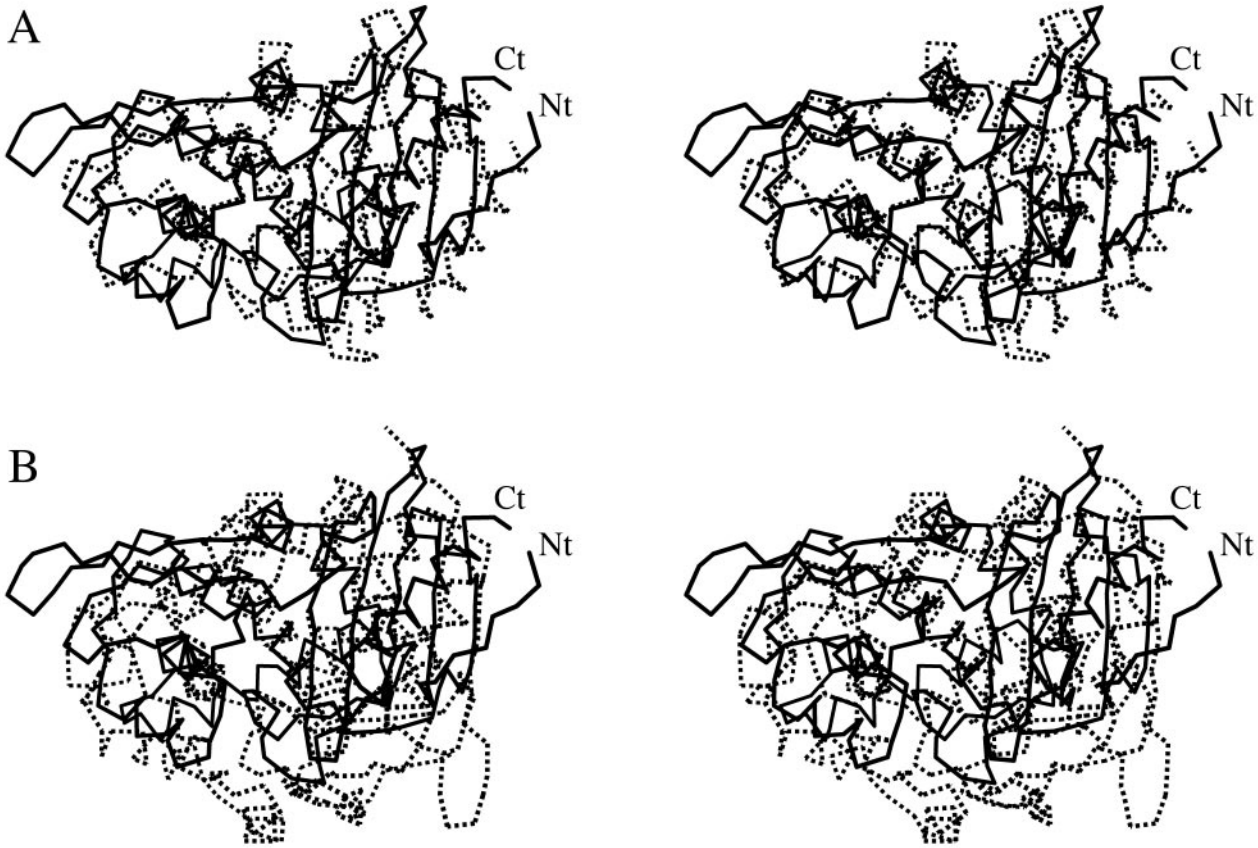

FIG. 3. **Stereo views of the superimposed C-α traces of serine transferase enzymes.** *A*, the K15 enzyme (*continuous line*) and the TEM1 class A β-lactamase of *E. coli* (*dashed line*); *B*, the K15 enzyme (*continuous line*) and the *Streptomyces* R61 DD-carboxypeptidase (*dashed line*). *Ct*, COOH terminus; *Nt*, NH_2 terminus.

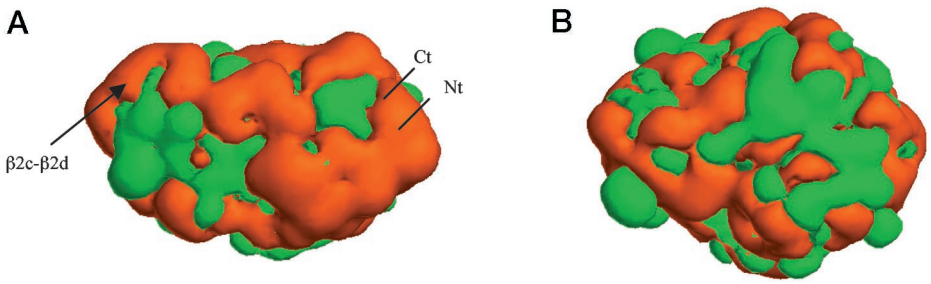

FIG. 4. **Visualization of hydrophobic (*brown*) and hydrophilic (*green*) isopotential areas surrounding molecules.** *A*, the K15 enzyme; *B*, the TEM1 class A β-lactamase of *E. coli*. Both molecules are oriented as in Fig. 3*A*. *Ct*, COOH terminus; *Nt*, NH_2 terminus.

(which connects helices α6 and α7) lies at the bottom of the active site, and the side chain of Glu^{166} plays an important role in the catalyzed hydrolysis of penicillin. The K15 enzyme has no motif similar to the Ω-loop, and the fold defined at this position by the Phe^{142}-Asp^{143}-Gly^{144} triad is structurally equivalent to that defined by the Gln^{219}-Ala^{220}-Tyr^{221} triad in the *E. cloacae* class C β-lactamase and by the Ser^{236}-Ala^{237}-Gly^{238} triad in the *Streptomyces* R61 DD-carboxypeptidase (Fig. 6*A*).

Solvent Structure—In the crystal structure, the K15 enzyme is solvated by 149 ordered water molecules mainly located in the first coordination shell, directly hydrogen-bonded to protein atoms. In the active site, where a dense hydrogen bond network prevails, the solvent molecules that interact with the essential amino acids (Table II) are the most buried ones and, accordingly, exhibit the lowest temperature factor values. As the charge distribution induced by the side chains varies among the different classes of enzymes, it is obviously difficult to make a rigorous comparison. The unique water contact with the hydroxyl group of the active Ser^{35} residue is made with Wat^{336}. This water molecule could be compared with the one found in the oxyanion hole of the class A (Wat^{295} for the TEM1 enzyme) and class C (Wat^{1087} for the P99 enzyme) β-lactamases. However, Wat^{336} is not buried so deeply in the catalytic pocket and does not make any interaction with the backbone amide groups of Ser^{35} and Thr^{214}. In comparison with the other penicilloyl-serine transferases, the side chain of the nucleophilic serine of the K15 enzyme is displaced through the χ_1-dihedral angle rotation. The values of χ_1 are 52.3°, −71.5°, −16.3°, and 56.6° in the K15, TEM1, R61, and P99 structures, respectively. The shift of the hydroxyl group of Ser^{35} toward strand β3 and away from the bottom of the active site can certainly be related to the displacement of Wat^{336}.

In the same way, the so-called hydrolytic water molecule found in all class A β-lactamase structures is not really conserved in the K15 enzyme. This water molecule (Wat^{292} in the TEM1 enzyme) forms a bridge between the nucleophilic serine hydroxyl group and the Glu^{166} carboxylate side chain. Without the Ω-loop, the K15, R61, and P99 enzymes cannot exhibit such

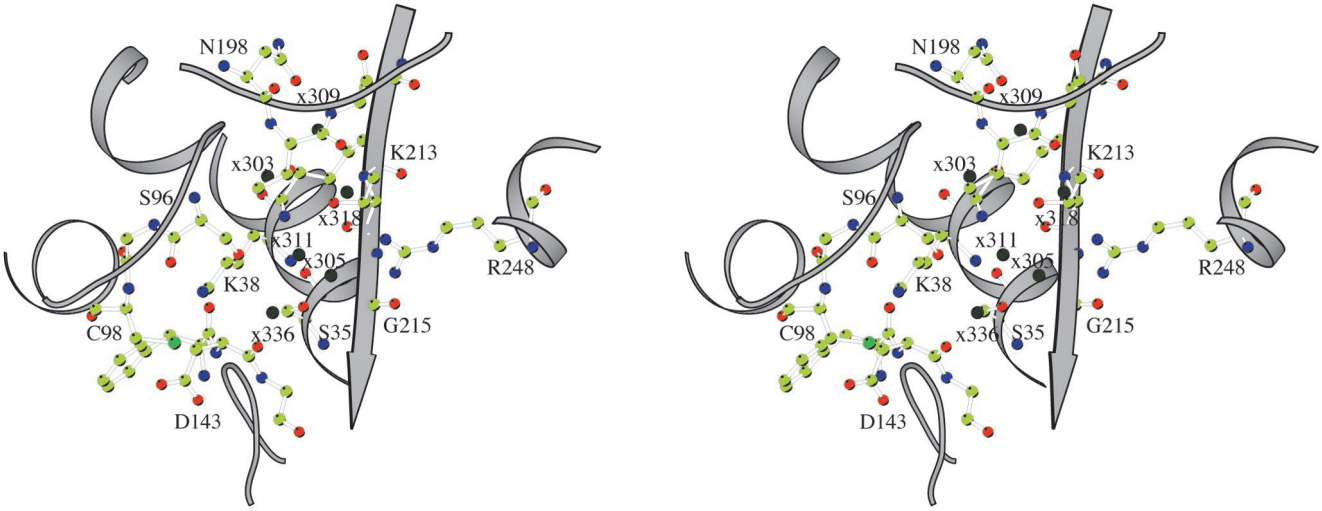

FIG. 5. **Stereo view of the catalytic cleft of the K15 enzyme.** The water molecules are in *black* and denoted by *x*.

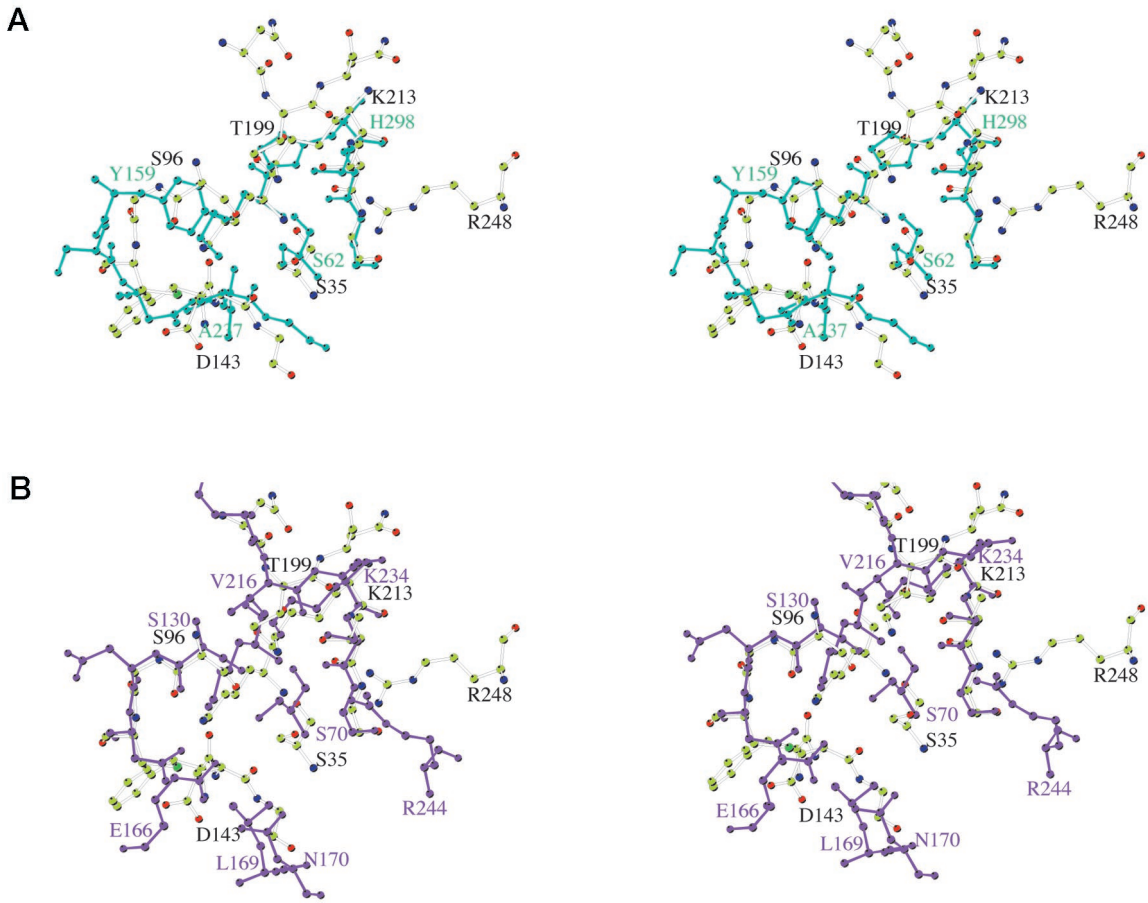

FIG. 6. **Stereo views of the superimposed active sites of serine transferase enzymes.** *A*, the K15 enzyme (colored by atom type) and the DD-carboxypeptidase/PBP of *Streptomyces* R61 (*cyan*); *B*, the K15 enzyme (colored by atom type) and the TEM1 class A β-lactamase of *E. coli* (*magenta*).

a water molecule that is tightly bound and *a fortiori* for the K15 enzyme, which has a different conformation for the active serine. One can only remark that as in the R61 and P99 enzymes, there is in the K15 structure a water molecule (Wat^{381}) that neighbors the class A β-lactamase hydrolytic water molecule, but it does not interact with the essential amino acids and has a high temperature factor (40.3 $Å^2$).

As found in all penicilloyl-serine transferases possessing a lysine residue in the conserved (Lys/His/Arg)-(Thr/Ser)-Gly triad, a water molecule (Wat^{303} in the K15 enzyme) is located at the top of the catalytic pocket and plays a structural role in stabilizing the Lys^{213} side chain. One may note that the *Streptomyces* R61 DD-carboxypeptidase is the only known enzyme of the family that has a histidine residue instead of a lysine or arginine residue in this triad.

Accessible Surface Area—The accessible surface area of the K15 enzyme crystal structure has a value of 10,960 $Å^2$ as computed with the DSSP program (36), and its distribution

TABLE II
Hydrogen bonding pattern in the catalytic cleft

Amino acid/water identification		Distance
		Å
Ser^{35} (OG)	$Ser^{216)\ (N)}$	3.04
	Wat^{336} (0)	2.96
Lys^{38} (NZ)	Se^{96} (OG)	2.98
	Cys^{98} (SG)	2.76
Ser^{96} (OG)	Lys^{213} (NZ)	2.91
Thr^{199} (OG1)	Thr^{214} (OG1)	2.77
	Wat^{303} (O)	2.76
	Wat^{318} (O)	2.66
Lys^{213} (NZ)	Thc^{214} (O)	3.03
	Wat^{303} (O)	2.87
Thr^{214} (OG1)	Wat^{311} (O)	2.95
Wat^{303} (O)	Met^{93} (O)	2.97
	Wat^{309} (O)	3.21
Wat^{305} (O)	Arg^{248} (NH2)	3.02
	Wat^{311} (O)	2.62
Wat^{309} (O)	Asn^{198} (OD1)	2.91
Wat^{336} (O)	Wat^{311} (O)	2.98

along the polypeptide chain is quite similar to that observed in the class A β-lactamases (38). The central helix α2 and the five-stranded β-sheet are inaccessible to solvent, except strand β2, which is more exposed. Helices α4 and α5 are also buried, and the other α-helices have an amphiphilic profile. Strands β2a and β2d of the small β-sheet are very well exposed, strand β2c is amphiphilic, and strand β2b is more buried. The more accessible regions (Val^1–Lys^3, Asn^{50}-Leu^{51}, Asn^{70}-Asn^{71}, Ile^{145}–Ala^{149}, and Gly^{231}-Gly^{232}) correspond to the highest temperature factor values (>30 $Å^2$).

The analysis also reveals very low accessibility values for several charged residues of the K15 enzyme. Among these, Lys^{38} and Lys^{213} are directly involved in the dense hydrogen bond network of the catalytic cleft. Asp^{99} interacts with the α-domain and probably stabilizes the position of the Ser^{96}-Gly^{97}-Cys^{98} motif, as does Asp^{131} in the class A β-lactamases (39). Asp^{143} and Asn^{150}, at the bottom of the active pocket, interact with protein core atoms, allowing the positioning of the carbonyl group of Asp^{143} in the catalytic cleft. Arg^{248}, near strand β3, may play the same role as Arg^{220} or Arg^{244} in the class A β-lactamases (40). Two polar residues, Asn^{198} and Asn^{200}, above the catalytic pocket, are also quite buried. They may be compared with the Asp^{214} (Asn^{214}) /Asp^{233} pair in class A β-lactamases and the Glu^{272}/His^{234} pair in class C β-lactamases. These latter residues are involved in specific interactions, in particular with a water molecule equivalent to Wat^{303} in the K15 enzyme (16).

DISCUSSION

Several scenari may be evoked through which the present day penicilloyl-serine transferases of different groups and classes have evolved from a protein ancestor similar to the K15 enzyme in structure and catalytic properties. One evolution pathway is loss of peptidase activity, loss of membrane association site, and acquired catalyzed hydrolysis of the penicilloyl enzyme. The resulting exocellular β-lactamases hydrolyze penicillin into biologically inactive metabolites, providing the bacteria with a defensive mechanism of remarkable efficacy. On good β-lactam substrates, the TEM β-lactamase may turn over 1000 times/s or more. In this respect and in comparison with the K15 enzyme, the TEM β-lactamase has a 10^6-fold or more increased catalytic center activity. In structural terms, the class A β-lactamases have lost the four-stranded β-sheet at the surface of the all-α-domain; they have acquired an additional α-helix at the amino-terminal end of the polypeptide chain and a specifically designed Ω-loop at the entry of the active site. Essentially, however, the overall polypeptide fold has been maintained.

Another possible evolutionary pathway that retains the activity toward peptide substrates would be the acquisition of membrane-associated carboxyl-terminal amphiphilic α-helices, giving rise to the membrane-bound monofunctional DD-carboxypeptidases/PBPs. Finally, fusion of the acylserine transferase polypeptide to the carboxyl-terminal end of other polypeptide chains may have given rise to the membrane-bound multimodular PBPs. The acylserine transferase/penicillin-binding module of PBP2x of *S. pneumoniae* (Protein Data Bank code 1PMD) has the signature fold topology and active site of the K15 enzyme. But it also possesses two inserts that form an additional groove at the surface of the module, and the head of the non-penicillin-binding module, which is shaped like a pair of sugar tongs, fits into the non-catalytic groove of the acylserine transferase (20).

The structurally related K15 DD-transpeptidase/PBP, DD-carboxypeptidases/PBPs, and serine β-lactamases fulfill different functions and catalyze distinct reactions (albeit exhibiting a similar catalytic mechanism). They illustrate the concept according to which proteins unrelated in sequence and function may diverge from a common ancestor and new enzyme functions may evolve by local structural changes while retaining the same basic fold. The question of which structural features determine the different functionalities (peptide bond synthesis *versus* hydrolysis, penicillin binding *versus* hydrolysis) and vastly different kinetic properties of the monofunctional penicilloyl-serine transferases remains open.

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