metabolic labelling. NIH3T3 cells were stably transfected with pRSV-E1A 12s (E1A) or a neovincent control vector and assayed for E1A expression (data not shown). For metabolic labelling, 107 NIH3T3 cells were incubated for 4 h with 0.5 mM m-132 inorganic phosphate (Amersham) in phosphate-free medium (Sigma) before immunoprecipitation of CBP.

Immunoprecipitation and western blotting. Stringent immunoprecipitations were performed using standard procedures; cells were lysed in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% N-P40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA). SRC-1 and P/C/AF were undetectable in the immunoprecipitates (data not shown). Mild immunoprecipitation was performed as described. All buffers contained protease (Boehringer) and phosphatase (Sigma) inhibitors. Western blotting was done using standard procedures and visualized using an ECL + kit (Amersham). Anti-CBP antibodies used were the A22 antibody (Santa Cruz) for immunoprecipitation and the NM11 antibody (Pharmingen) for western blotting.

In vitro phosphorylation and phosphatase treatment. Cyclin E/Cdk2 protein kinase, expressed in baculovirus-infected Sf9 cells, was purified by standard chromatographic procedures. GST proteins were purified as described and dialysed against TBS-G (20 mM Tris, pH 8.0, 150 mM NaCl, 1% N-P40, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA). SRC-1 and P/C/AF were undetectable in the immunoprecipitates (data not shown). His-Rb was prepared using the Qiagen protocol. Recombinant proteins were in vitro phosphorylated by incubation with 50 ng cyclin E–Cdk2, 100 μM ATP and γ-32P-ATP (100 μCi mmol−1 final specific activity) for 45 min at 30°C in 30 μl buffer containing 25 mM Tris, pH 7.5, 0.1 mM NaVO4, 0.1 mM EGTA, 10 mM magnesium acetate, 0.04 mM DTT, 0.1 mM ZnSO4 and protease inhibitors. Phosphatase treatment was performed using 400 U of lambda protein phosphatase (Biolabs) for 30 min at 30°C.

HAT assay. HAT assays were done using a synthetic peptide (Chiron) corresponding to the first 24 amino acids of histone H4 coupled through a linker sequence to a biotin molecule.


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Crystal structure of a bacterial signal peptide in complex with a β-lactam inhibitor

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The signal peptide (Spase) from Escherichia coli is a membrane-bound endopeptidase with two amino-terminal transmembrane segments and a carboxy-terminal catalytic region which resides in the periplasmic space. Spase functions to release proteins that have been translocated into the inner membrane from the cell...
interior, by cleaving off their signal peptides. We report here the X-ray crystal structure of a catalytically active soluble fragment of E. coli SPase (SPase Δ2–75). We have determined this structure at 1.9 Å resolution in a complex with an inhibitor, a β-lactam (5S,6S penem), which is covalently bound as an acyl-enzyme intermediate to the γ-oxygen of a serine residue at position 90, demonstrating that this residue acts as the nucleophile in the hydrolytic mechanism of signal-peptide cleavage. The structure is consistent with the use by SPase of Lys 145 as a general base in the activation of the nucleophilic Ser 90, explaining the specificity requirement at the signal-peptide cleavage site, and reveals a large exposed hydrophobic surface which could be a site for an intimate association with the membrane. As enzymes that are essential for cell viability, bacterial SPases provide a feasible antibacterial target: our determination of the SPase structure therefore provides a template for the rational design of antibiotic compounds.

The E. coli SPase Δ2–75 structure has a mainly β-sheet protein fold, consisting of two large antiparallel β-sheet domains (termed I and II and coloured green and blue, respectively, in Fig. 1), two small 3₁₀-helices (consisting of residues 246–250 and 315–319), and one small α-helix (residues 280–285). There is one disulphide bond, as was found in earlier biochemical studies, between Cys 170 and Cys 176. This bond is located immediately before a β-turn in the domain II β-sheet (Fig. 1). In addition, an extended β-ribbon (residues 107–122, coloured purple in Fig. 1) protrudes from domain I, together with the N-terminal strand, giving the SPase Δ2–75 molecule an overall conical shape with rough dimensions of 60 × 40 × 70 Å (Figs 1, 2).

Sequence alignments indicate that highly conserved regions of primary sequence within the prokaryotic and eukaryotic SPases reside within domain I of the E. coli SPase structure, whereas the two insertions representing the extended β-ribbon (residues 107–122) and domain II are variably present from species to species. In addition, domain I shares structural similarities with UmuD’ protease, the proteolytic domain of a self-cleaving repressor protein involved in the ‘SOS’ DNA-repair response in E. coli. Although the overall mainchain connectivity in UmuD’ and domain I of SPase differs in some regions, 68 common Cα atoms can be superimposed with a root mean square (r.m.s.) deviation of 1.6 Å. Domain II and the extended β-ribbon of SPase have no structural counterparts in UmuD’. Domain I, containing all of the essential and conserved catalytic elements, represents a new protease structural motif that is likely to be conserved from bacteria to man.

A large, unusually exposed hydrophobic surface extends across the SPase Δ2–75 molecule and includes the substrate-binding site and catalytic centre (labelled S1, S3 and Ser 90 in Fig. 2). The residues contributing to the hydrophobic character of this surface...
protrude from the main β-sheet of domain I and include Phe79, Ile80, Tyr81, Phe100, Leu102, Trp300, Met301, Phe303, Trp310, Leu314, Leu316, and Ile319. On the basis of our observations of the structure we suggest that, in vivo, the membrane-anchored N-terminal strand and the associated β-ribbon, from residues 106 to 124 (Fig. 1), would bend in the appropriate manner to allow the exposed hydrophobic surface of SPase to insert into the membrane lipid bilayer, presumably optimizing contact with the signal-peptide cleavage site. This proposal is consistent with earlier experiments that showed that the detergent Triton-X100 is essential for optimal activity of SPase Δ2–75 (ref. 3), as well as for optimal growth of the SPase Δ2–75 crystals. Recent biophysical studies have revealed that SPase Δ2–75 inserts into the outer leafllet of the E. coli inner membrane. In addition, it has been suggested that Trp300 and Trp310 (Figs 1, 2) are essential for the catalytic activity of E. coli SPase. This result is intriguing given the distance (>20 Å) between these residues and the active site and their location on the hydrophobic surface, the proposed membrane-association surface (Figs 1, 2). Tryptophans and other aromatic residues are commonly found at membrane–protein interfaces.

Although bacterial SPases are not inhibited by standard protease inhibitors, they are inhibited by β-lactam compounds with 55 stereoechemistry. We have determined the structure of SPase Δ2–75 in the presence of a 55,65 β-lactam (penem), an SPase inhibitor (Fig. 3). The electron density shows a covalent bond between SPase Ser90 Oy and the carbonyl carbon (C7) of the inhibitor, with the four-membered β-lactam ring being cleaved between C7 and N4 (Fig. 4). This is, to our knowledge, the first direct evidence for the role of Ser90 Oy as the acylating nucleophile in catalysis. The structure shows that the Ser90 Oy attacks the si-face of the β-lactam amide bond, a peptide-bond analogue. This indicates that SPase may be unique among serine-dependent hydrolases, including the serine proteinases and the group 2b β-lactamases, which prefer a re-face attack. A si-face nucleophilic attack by E. coli SPase was predicted previously on the basis of stereoechemical requirements of several inhibitory compounds.

The main-chain amide of Ser90 forms a strong hydrogen bond (of length 2.9 Å) with the carbonyl oxygen (O8) of the cleaved β-lactam ring (Fig. 4). This indicates that the Ser90 amide might contribute to the formation of an ‘oxyanion hole’, lending electrophilic assistance by stabilizing the tetrahedral transition-state intermediate. There appears to be no suitably positioned second main-chain or sidechain amide that could contribute to the oxyanion hole (as is found in the group 2b β-lactamases and the serine proteinases). However, the Ser88 side chain could potentially participate in such an interaction by a simple rotation from the observed χ1 of −54° (Fig. 4) to a value of +60° (Fig. 5). This interaction is prevented in the inhibitor complex by an unfavourable van der Waals contact between the Ser88 Oy in the +60° conformation and the S1 and C15 atoms of the inhibitor (Fig. 4). The Ser88 side chain has the highest temperature factors in the active-site region, indicating that it is not in an optimal environment in the inhibitor complex. The contribution of a serine hydroxyl to an oxyanion hole has been seen previously in lipolytic enzymes such as cutinase.

The Lys143 Nπ position is fixed relative to Ser90 Oy by hydrogen bonds to Ser278 Oy (bond length 2.9 Å) and to the carbonyl oxygen (O10) of the inhibitor side chain (bond length 2.9 Å) (Fig. 4). The Nπ of Lys145 is 2.9 Å away from the Ser90 Oy and is the only titratable group in the vicinity of the active-site nucleophile (Fig. 4). The next closest ionizable group, 7.5 Å away from Ser90 Oy, is Asp280 which is held in place by a strong salt bridge to Arg282 (Fig. 4). Thus, the e-amino group of Lys145 is suitably positioned to act as the general base in both acylation and deacylation steps of catalysis. It appears as though the inhibitor has displaced the deacylating water, as no water molecules are found within 5.5 Å of the covalent inhibitor link. As the co-crystals containing enzyme and inhibitor were grown weeks before the data collection, the acyl-enzyme must be extremely stable, supporting the idea that a deacylating water molecule is displaced. The side chain of Lys145 is completely buried in this inhibitor complex (Fig. 4), in which it makes van der Waals contacts with the sidechain atoms of Tyr143, Phe133 and Met270, and with the main-chain atoms of Met270, Met271, Gly272 and Ala279, all of which come from domain I.

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**Figure 4** A ball-and-stick representation of the active-site residues of SPase Δ2–75. The β-lactam (55,65 penem) inhibitor shown in Fig. 3 and in this figure (purple) is covalently bound to the Oy of Ser90, with the carbonyl oxygen (O8) of the cleaved β-lactam (the bond between C7 and N4 has been cleaved) sitting in the oxyanion hole formed by the main-chain nitrogen of Ser90 (S90). The methyl group (C16) of the inhibitor, labelled P1, sits in the S1 substrate-binding site.

**Figure 5** A ball-and-stick representation of the active-site residues of SPase Δ2–75 with the P1–P4 residues of an acylated peptide substrate [Ala-Ala-Ala-Ala] modelled into the bindings sites S1–S4. The observed positions of the methyl group (C16) and the carbonyl oxygen (O8) of the inhibitor (Fig. 4) were used as a guide.
hydrophobic environment surrounding the Lys 145 e-amino group is probably essential for lowering its pKₐ so that it can stay in the deprotonated state required for its function as the general base. 

A typical E. coli signal peptide consists of a positively charged N terminus, an inner hydrophobic core, and a C-terminal cleavage-recognition sequence containing small uncharged residues at the P₁ (−1) and P₃ (−3) sites. Alanine residues are the most common residues at the −1 and −3 positions, giving rise to the so-called −1,−3 or Ala-X-Ala, rule. The sidechain methyl group (C16) of the penem is located in the SPase substrate-binding pocket (S1) (Figs 2, 4). This methyl group is essential for the effectiveness of the inhibitor and probably mimics the P₁ (−1) (Ala) side chain of the substrate. The residues making direct van der Waals contacts with the P1 methyl group in the S1 specificity pocket are Met91, Ile 144, Leu 95 and Ile 86 (Fig. 4).

Using the position of the inhibitor methyl group (C16) in the S1 site and of the inhibitor carboxyl group (C7, O8) in the oxyanion hole as a guide, we have modelled a tetrapeptide (poly-Ala) into the active site of SPase (Fig. 5). We needed an extended, β-strand conformation of the peptide substrate to provide both a favourable fit and β-sheet-type hydrogen bonds with the conserved β-strand containing Lys 145, supporting earlier studies which indicated that the C-terminal five to six residues of the signal peptide would adopt a β-sheet conformation. This model helps to explain the cleavage-specificity of SPase. The side chain of the P1 Ala occupies the same site as the inhibitor methyl group and the side chain of the P3 Ala points into a shallow hydrophobic depression formed by Phe 84, Ile 86, Ile 101, Val 132, Ile 144 and the Cβ of Asp 142 (the proposed substrate-specificity site S3; Figs 2, 5). Although alanine is the most common residue at the P3 site of signal peptides, larger aliphatic residues such as Val, Leu and Ile can also occur at this position. Our model building into the electron-density map was done with the program O. 

Future modelling studies aimed at an understanding of the structure and function of the eukaryotic SPases will proceed on the basis of the conservation of primary sequence within the E. coli SPase domain 1, the catalytic core of type 1 SPases. Important issues, such as the reasons behind unique substrate specificity of mitochondrial SPases and the substitution of the catalytic lysine by the more typical histidine in the endoplasmic reticulum SPases, can now be addressed from this first structure of an SPase.

Table 1 Crystallographic data

| Data set | d_max<sup>+</sup> (Å) |afflections | R(|F|) | Rmerge<sup>+</sup> (%) |
|----------|-----------------|-------------|-------|---------------------|
| Native   | 1.9             | 28,379      | 391,951| 21.9                |
| Ethylmercury phosphate | 2.9            | 28,379      | 98,274 | 7.9                 |
| Methylmercury acetate | 2.9            | 21,851      | 91,567 | 6.8                 |

Phasing statistics

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<th>Rmerge&lt;sup&gt;+&lt;/sup&gt; (Acentric/centric)</th>
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Current refinement statistics

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<sup>1</sup> d_max is the maximum resolution of measured X-ray intensities.

<sup>2</sup> R = Σ|Fobs| - |Fcalc|/Σ|Fobs|, where |Fobs| is the observed structure-factor amplitude of reflection i and |Fcalc| is the calculated structure-factor amplitude of reflection i.

<sup>3</sup> Rmerge<sup>+</sup> = Σ|Fobs| - |Fcalc|/Σ|Fobs|, where |Fobs| and |Fcalc| are the observed and calculated structure-factor amplitudes, respectively.

<sup>4</sup> |Fcalc| = Fcalc(Dcalc, |Fcalc|) - Fcalc(Dcalc, |Fcalc|), where Dcalc and |Fcalc| are the observed and calculated structure-factor amplitudes, respectively.

<sup>5</sup> |Fcalc| = Fcalc(Dcalc, |Fcalc|) - Fcalc(Dcalc, |Fcalc|), where Dcalc and |Fcalc| are the observed and calculated structure-factor amplitudes, respectively.

<sup>6</sup> Rmerge<sup>+</sup> = Σ|Fobs| - |Fcalc|/Σ|Fobs|, where |Fobs| and |Fcalc| are the observed and calculated structure-factor amplitudes, respectively.
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