**Staphylococcus aureus Aminopeptidase S Is a Founding Member of a New Peptidase Clan***

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Biochemical data for the AmpS homologue from *Bacillus stearothermophilus*, known as AmpT or aminopeptidase II, indicate that the enzyme can be inactivated by metal-chelating agents such as EDTA or 1,10-orthophenanthroline. The activity can be restored by the addition of exogenous cobalt ions, or, to a lesser extent, the addition of zinc ions. Equilibrium dialysis indicated there are 2.2 bound cobalt ions per subunit of the enzyme, and an affinity for cobalt ions of ~5 μM (9). Functional data for the AmpS homologue from *Thermus aquaticus* provide the strongest evidence to date for an aminopeptidase activity in this group of enzymes. These data indicate a rather broad substrate specificity, with a preference for hydrophobic residues such as Leu, Val, Phe, or Tyr at the amino terminus (8).

The lack of structural data for AmpS or any other sequence related enzymes has invited speculations about possible similarities to other dimetal-dependent aminopeptidases. The aminopeptidase activity of the enzymes and the maximal activity in the presence of cobalt ions are reminiscent of the clan MG methionine aminopeptidases. However, the pattern of con-

Staphylococcus aureus aminopeptidase S (AmpS) has been named for its predicted, but experimentally untested, aminopeptidase activity. The enzyme is homologous to biochemically characterized aminopeptidases that contain two cobalt or zinc ions in their active centers, but it is unrelated to all structurally characterized metallopeptidases. Here, we demonstrate AmpS aminopeptidase activity experimentally, and we present the 1.8-Å crystal structure of the enzyme. Two metal ions, with full occupancy and a third metal ion with low occupancy are present in the active site. A water molecule and Glu-319 serve as bridging ligands to the two metals with full occupancy. One of these metal ions is additionally coordinated by Glu-253 and His-348 and the other by His-381 and Asp-383. In addition, the metals are involved in weak metal-donor interactions to a water molecule and to Tyr-355. In the crystal, AmpS forms a dimer with a large internal cavity. The active sites are located at opposite ends of this internal cavity and are essentially inaccessible from the outside, suggesting that an in-active conformation was crystallized. Because gel filtration and analytical ultracentrifugation data also suggest dimer formation, the problem of substrate access to the active site cavity remains unresolved.

Aminopeptidases with at least two metal ions in the active site are a structurally heterogeneous group. Based on their folds, their active site architectures, and the identity of the metal ions, they have been divided into clans MF, MG, and MH in the MEROPS data base (1). Clan MF metallopeptidases, exemplified by the well characterized bovine lens leucine aminopeptidase II, have the pita-bread fold and contain two zinc ions in their active centers (2, 3). Clan MG metallopeptidases, such as the methionine aminopeptidases, have the pita-bread fold and contain two cobalt or two manganese ions in their active centers (4). Clan MH enzymes, typically with two zinc ions in their catalytic sites, form the most heterogeneous group. Only some enzymes in this clan are aminopeptidases; others have carboxypeptidase or dipeptidase activities. Very recently, some aminopeptidases in this group have been shown to form very large molecular assemblies (5, 6).

Aminopeptidase S (AmpS) from *Staphylococcus aureus* and its homologues (MEROPS family M29) attracted our interest, because biochemical data for this family of enzymes indicated the presence of an active site with two metal ions, in the absence of any sequence similarity, even at position-specific iterated-BLAST level, to enzymes in the well characterized clans of metallopeptidases with two metal ions in the active site. Moreover, AmpS was interesting to us, because it was one of only a few peptidase genes that were picked up in a genome wide screen for their importance for cell growth in culture conditions. Production of ampS antisense RNA was not lethal to cells, but resulted in an impaired growth phenotype (7). The detailed reasons for the growth defect of the mutant are not known, and physiological substrates for AmpS have not been identified. It also appears that the enzyme has never been studied biochemically, in contrast to the AmpS homologues from thermophilic organisms that have attracted interest because of possible applications of heat-stable peptidases in the biotechnology industry (8–10).

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Data collection and refinement statistics

<table>
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Refinement statistics

| Reflections work/test | 38,644/2,034 |
| Protein atoms (excluding hydrogen) | 3,296 |
| Metal ions | 3 (1 with low occupancy, taken 0.3) |
| Water molecules | 380 |
| R-factor (%) | 17.2 |
| R-free (%) | 21.2 |
| r.m.s.d. bond distance (Å) | 0.02 |
| r.m.s.d. angles (°) | 2.1 |
| Average B-factor (Å²) | 20.8 |
| Ramachandran core region (%) | 92.3 |
| Ramachandran allowed region(%) | 7.5 |
| Ramachandran additionally allowed region (%) | 0.3 |
| Ramachandran disallowed region (%) | 0.0 |

* r.m.s.d., root mean square deviation.

erved metal ligands in clan MG methionine aminopeptidases is not compatible with the pattern of conserved residues in the AmpS family. Based on the presence of a short, conserved motif in AmpS-like peptidases, Motoshima and Kaminogawa (11) have tentatively suggested a similarity between AmpT-like (and thus AmpS-like) enzymes and cattle lens leucyl aminopeptidase, a typical representative of clan MF aminopeptidases. Very recently, another prediction about the possible metal ligands in M29 peptidases has appeared in the Web-based version of the MEROPS data base (merops.sanger.ac.uk/) (12), but not yet in print.

In this communication, we report the expression, purification, biochemical characterization, and 1.8-Å crystal structure of AmpS from *S. aureus*. We demonstrate aminopeptidase activity for the enzyme and show that this activity can be abolished by the metal chelator EDTA and restored by the addition of cobalt or zinc ions. From the crystal structure, we identify the set of metal ligands and show that the MEROPS prediction, in contrast to the other predictions, was essentially correct, although it misses subtle features that are apparent from the crystal structure. Although the AmpS fold is unrelated to the folds of other dimetal-dependent peptidases, we find an unexpected similarity of the active site with the active site of clan MH metalloproteases, which likely results from convergent rather than divergent evolution.

**EXPERIMENTAL PROCEDURES**

**Cloning and Expression**—The *ampS* gene was amplified from genomic DNA of *Staphylococcus aureus* strain NCTC8325–4 by PCR and cloned into a pET14 vector (Novagen) using standard cloning techniques to generate pET14ampS. In this construct, the ampS open reading frame is fused to a vector-encoded, N-terminal histidine tag, and thrombin cleavage site and placed under the control of the T7 promoter. For expression, *Escherichia coli* BL21(DE3) cells were transformed with pET14ampS and grown in LB medium containing 100 mg/liter ampicillin at 37 °C until the *A* 600 reached 0.7. Then isopropyl 1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM, and cells were incubated for the next 5–6 h at 30 °C. Cells were harvested by centrifugation (10 min at 4,000 rpm), resuspended in buffer A (10 mM Tris, 100 mM NaCl, pH 7.5), and stored frozen at −20 °C for later use. After thawing, cells were disintegrated by sonication, and the soluble fraction was clarified by ultracentrifugation at 50,000 × g for 30 min.

**Protein Purification**—The cell lysate was loaded on nickel(II)-nitrioltriacetic acid-agarose resin (Qiagen) equilibrated in buffer A, and washed with 30 column volumes of buffer A, containing 40 mM imidazole. The histidine tag was cleaved on the column (50 units of thrombin per ml of bed volume) in an overnight cleavage step at 4 °C. The last purification step was a preparative gel filtration run on Sephacryl S-300 HR in 5 mM Tris (pH 7.5). The AmpS association state was tested by sizing chromatography on a Superose 12 HR column (Amersham Biosciences) and by analytical ultracentrifugation.

**Sedimentation Velocity Experiments**—The sedimentation velocity profiles were collected by monitoring the absorbance signal at 280 and 230 nm as the samples were centrifuged at 55,000 and 58,000 rpm and 4 °C in a Beckman Optima XL-I centrifuge fitted with a four-hole AN-60 rotor and double-sector aluminum centerpieces. Sedimentation coefficient distributions were determined by the C(s) method implemented in the Sedfit software package (13). Observed, apparent molecular masses at different protein concentrations were extrapolated to zero concentration to obtain the molecular mass of AmpS.

**Sedimentation Equilibrium Experiments**—Sedimentation equilibrium experiments were performed with a Beckman Optima XL-I centrifuge at 12,900, 20,800, and 26,200 rpm, 4 °C, and three different protein concentrations (A 200 0.6, 0.4, and 0.2). Six-sector charcoal-Epon centerpieces were used. Data were analyzed by Global Fitting, as implemented in the UltraScan 7.1 software package. Buffer density and viscosity corrections were made according to data published by Laue et al. (14) as implemented in UltraScan. The partial specific volume of AmpS was estimated from the protein sequence according to Cohn and Edsall (15).

**AmpS Crystallization**—AmpS crystals were grown by vapor diffusion in sitting drops. With reservoir buffer C (0.1 HEPES/NaOH, pH 7.6, 2.0 mM (NH₄)₂SO₄, 2% polyethylene glycol 4000), crystals in space group P6(3)22 with cell constants 102.44 × 102.44 × 231.12 Å and two molecules in the asymmetric unit appeared after about 4 weeks. Crystals could be frozen in 18 μl of buffer C mixed with 2 μl of 1-(-)-2,3-butanediol, but diffracted only to ~6 Å. Another crystal form was obtained with reservoir buffer D (0.1 HEPES/NaOH, pH 7.2, 0.2 mM (NH₄)₂SO₄, 30% polyethylene glycol 6000), but looked intergrown and were unsuitable for crystallographic analysis. Therefore, crystals were harvested, redissolved, and recrystallized from the same buffer. Much better crystals in space group C22(2)1 with cell constants 70.18 × 81.86 × 152.89 Å were obtained, which could be flash-cryocooled from the mother liquor. At BW6, DESY, these crystals diffracted to 1.8-Å resolution (Table I).

**AmpS Structure Determination**—The anticipated presence of a dimetal center in the AmpS crystals and the strong diffraction from the ortho-

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rhombic crystal form should have allowed us to solve the AmpS structure easily. Unfortunately, this was not the case. Because the recrystallization protocol to obtain good diffractions could not be repeated, we instead used the molecular modeling program O (17), until phases were of sufficient quality for the ARPKWARP software (18) to deliver a nearly complete interpretation of the electron density, which essentially only lacked one stretch of residues around a cis-proline residue. Waters were picked with the ARP software (19), and the model was refined with the REFERENCE software (20). Final refinement statistics and model quality indicators appear satisfactory (Table I).

AmpS Activity—To determine metal ion identity, x-ray fluorescence spectra and excitation scans through the candidate absorption edges were recorded, the imaginary parts of the scattering factors of the metals were refined, and atomic absorption spectroscopy was performed. X-ray fluorescence spectra were recorded with an incident energy of 11,807 eV using a Si-PIN diode detector and multichannel analyzer (AmpTek). To correct for wavelength-dependent detector-efficiency and absorbance, the spectra of a non-diffracting crystal were compared with the spectra of a standard solution that contained equimolar amounts of cobalt, nickel, copper, and zinc. The zinc to cobalt ratio of the tested specimen was 2:1 and varied over time as the crystal equilibrated with harvesting buffer. Substantial amounts of copper and nickel, likely from the affinity purification step, were also detected. Excitation scans (variable incident energy, fluorescence readout) supported the conclusion that a mixture of metals was present.

The result was also consistent with the refined values for the imaginary part of the scattering factor of the metals. The final model without the metal ions was used to bring the synchrotron dataset at 1.05 Å and an in-house dataset at 1.54 Å to an absolute scale. Imaginary scattering factors of the two fully occupied metal ions were refined with the SHARE program, using external phases from the model. We found that \( f' = 1.65 < 0.05 \) for the 1.05-Å data and \( f' = 2.4 > 0.2 \) for the 1.54-Å in-house data. If all metal ions were cobalt, one would expect \( f' \) (cobalt, 1.05 Å) = 1.9 and \( f' \) (cobalt, 1.54 Å) = 3.6, whereas if all metal ions were zinc, the values should be \( f' \) (zinc, 1.05 Å) = 2.8 and \( f' \) (zinc, 1.54 Å) = 0.7 according to the crossec program (21). Metal peaks, but no sulfur peaks, were present in the anomalous 1.54-Å difference Fourier map and also in the dispersive difference Fourier map. As tabulated values \( |f'| \) (zinc, 1.54 Å versus 1.05 Å) = 0.9 and \( f' \) (zinc, 1.54 Å) = 0.7 are only marginally larger than \( f' \) (sulfur, 1.54 Å) = 0.5, this should not be the case if all metal ions were zinc.

Finally, atomic absorption spectroscopy was performed as a commercial service by the Laboratory of Environmental Pollution Analysis, Warsaw University, using a Milestone ETHOS PLUS microwave mineralizer and a Salam M6 atomic absorption spectrometer. For the batch of protein solution that was checked by this method, an approximate 2:1 ratio of cobalt to zinc was found.

### RESULTS

**AmpS Is a Metal-dependent Aminopeptidase**—The annotation of AmpS as an aminopeptidase in databases appears to be entirely based on its similarity to the biochemically characterized aminopeptidases from hyperthermophilic organisms. Therefore, we set out as a first step to confirm experimentally that AmpS is indeed a metal-dependent aminopeptidase. Because natural substrates for AmpS have not been identified, we instead tried Met-enkephalin, a pentapeptide with sequence Tyr-Gly-Gly-Phe-Met that had previously been used successfully for the characterization of the thermophilic homologues. Although Met-enkephalin is a physiologically irrelevant substrate, it turned out to be well suited for the characterization of AmpS activity by TLC.

The time-course Met-enkephalin degradation at 37 °C was monitored by TLC (Fig. 1A) and is consistent with a stepwise cleavage of single amino acids from the amino terminus. The cleavage of the N-terminal tyrosine residue is particularly efficient, as would be expected if the substrate specificity of the enzymes from hyperthermophilic sources carried over to the enzyme from *S. aureus*.

The aminopeptidase activity is metal-dependent (Fig. 1B): removal of the metal with metal-chelating agents abolished the activity (lane 1). After removal of the chelating agent by a gel filtration run, the AmpS activity could be restored by the addition of cobalt or zinc ions, with no obvious advantage for cobalt over zinc. Activity was recovered with metal concentrations in the range from 1 to 100 μM (lanes 2–4 and 6–8). If the concentration of metal ions was increased further to 1 mM, the activity was reduced or abolished (lanes 5 and 9). Similar inhibition was observed when AmpS samples that were not pretreated with either metals or metal-chelating agents (lane 10) were treated with 1 mM cobalt or zinc ions (lanes 11 and 12). We note that inhibition of metallopeptidases by metals in unphysiologically large concentrations is not uncommon and has been reported before for other metallopeptidases (22, 23).

**AmpS Structure Determination**—AmpS crystals were grown by the vapor diffusion method in sitting drops at 19 °C, but diffraction patterns showed that these crystals were unsuitable for crystallographic analysis. Much better crystals were obtained when the original crystals were dissolved, rebuffered, and recrystallized under the original conditions, suggesting that the first crystallization step afforded a degree of purification not matched by the chromatographic procedures. Crystals belonged to space group C222 (1) and contained one monomer in the asymmetric unit.

**AmpS Fold**—The description of the AmpS fold can be simplified by the division of the molecule into an N-terminal domain, termed the “N-domain” (drawn in gold in Fig. 2), and a C-terminal domain, termed the “C-domain” (drawn in green in Fig. 2), and a "N-domain" (drawn in gold in Fig. 2), and a C-terminal domain, termed the “C-domain” (drawn in green in Fig. 2).

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**Fig. 1. AmpS activity assay with the model-peptide Met-enkephalin (Tyr-Gly-Gly-Phe-Met).** 0.5 pmol of AmpS was incubated with 5 μg (8.5 nmol) of Met-enkephalin at 37 °C in 5 mM Tris, pH 7.5. A, time course of Met-enkephalin degradation. Note that Met-enkephalin itself stains poorly. B, metal dependence of Met-enkephalin degradation. Copurified metal was chelated out with 10 mM EDTA, the chelating agent was removed by gel-filtration, and the protein was incubated overnight with the indicated concentrations of either zinc or cobalt ions. Met-enkephalin degradation was run for 20 min, and the reaction was stopped with 10 mM EDTA.
The animation formation of the N-domain consists of at least seven helices that are organized around a central, parallel ß-sheet with topology 2134. The ß-α-ß motif that involves stands 1 and 2 is right-handed and canonical. The connection between strands 3 and 4 is right-handed as well, but more unusual, because the ß-stands are connected by two consecutive helices that are almost perpendicular to each other. The N-domain mediates protomer contacts in the crystal but does not anchor any of the metal chelating residues.

The C-domain is organized around two ß-sheets, which stack against each other in such a way that the ß-strand directions in the two sheets are almost perpendicular to each other. Therefore, the resulting strand arrangement is somewhat similar to the arrangement in a barrel, although a very imperfect one with a large, sheet-like extension. The larger sheet is antiparallel, with strand order 7612345. In this strand arrangement, five hairpins occur. Three among them, those that connect strands 1 to 2, 4 to 5, and 6 to 7 are tight. The other two hairpins are quite long. The one between strands 2 and 3 contains a ß-strand, labeled "A" in Fig. 2, that is part of the smaller ß-sheet. Like the larger sheet, the small sheet is strictly antiparallel, with strand order I, II, III, then IV. All three hairpins that result from this arrangement comprise over 25 residues and are therefore quite complicated in shape. The small antiparallel ß-sheet with its immediate surrounding serves as the principle anchorage site for residues that are involved in metal chelation in the active site. Strand II (residues 317–322) anchors Glu-319, strand III (residues 348–352) and its surroundings anchor Glu-343, His-348, Ser-353, and Tyr-355, and strand IV (residues 381–385) anchors His-381 and Asp-383 (Figs. 2 and 3).

The AmpS-fold is not strongly similar to other peptidase folds. A DALI quantitative structure comparison identified an alcohol dehydrogenase (Protein Data Bank (PDB) accession code 2ohx), a quinone oxidoreductase (PDB accession code 1qor), and a phosphoglycerate mutase (PDB accession code 1eji) as the most similar proteins in the PDB. The Z-scores that express the similarity in standard deviations above the random score are surprisingly high, 5.9, 5.3, and 5.2, but superposition shows that the similarities merely reflect the very canonical architecture of the N-domain with its recurrent ß-α-ß motifs. The active site of AmpS is outside the superposition region in all three cases.

Fig. 2. Stereo ribbon representation of AmpS. The N-domain is shown in gold, and the C-domain in green. Metal ligands are presented in all atom representation, and metal ions are pink spheres for the metals with full occupancy and a yellow sphere for the metal with low occupancy. ß-Strands I–IV in the N-domain correspond to residues 28–33, 55–60, 99–104, and 144–148. ß-Strands I–V in the C-domain represent residues 211–216, 219–225, 258–268, 278–287, 291–299, 392–396, and 402–407. ß-Strands I–IV are built from residues 254–256, 317–322, 348–352, and 381–385 according to the dssp assignment.

AmpS Ion Cofactors—The AmpS structure reveals the presence of two fully occupied metal ions in the active site of the enzyme (labeled "1" and "2" in Fig. 3A). In addition to the two strong peaks in the 2Fo − Fc density map, several additional peaks are present in the active site that are not accounted for by ligand atoms and can be interpreted either as water molecules or as ions with partial occupancy (Fig. 3B). To distinguish between these two possibilities, an anomalous difference Fourier map was calculated for the 1.05-Å data with the phases of the final model omitting the metal ions (Fig. 3C). A strong signal was obtained for metal ions 1 and 2. A much weaker, but still significant signal was obtained for a third peak in the active site (labeled "3" in Fig. 3A). We therefore conclude that the atom labeled 3 probably represents a metal ion with partial occupancy, but we cannot exclude that this position is filled with a water molecule in some AmpS molecules in the crystal. We note that our finding of two fully occupied sites and of a third, partially occupied metal site in AmpS is in excellent agreement with the value of 2.2 metal ions per molecule that was determined by equilibrium dialysis for a homologue of AmpS from a hyperthermophilic source (9).

At the time of data collection, we failed to detect clear metal peaks in the x-ray fluorescence spectra, concluded that the metal ions had been lost during purification, and collected only a single wavelength dataset. Therefore, the identity of the metals had to be checked on different protein batches and crystal specimens. X-ray fluorescence spectra (fixed incidence wavelength and variable readout wavelength), excitation scans (varying incident energy and fluorescence readout), refined imaginary scattering factors, and atomic absorption spectroscopy all consistent with a mixture of cobalt and zinc in the crystals. The fluorescence spectra also indicated the presence of copper and nickel, presumably from a mixture of cobalt and zinc in the crystals. The fluorescence spectra also indicated the presence of copper and nickel, presumably from the affinity purification step. There is no evidence in our data that different sites would bind different metals. Because the probe for the present study was purified from a recombinant source and subjected to extensive purification, the detection of a mixture of metals need not be relevant for the enzyme from the native source.

AmpS Metal Coordination—The AmpS active site contains two metal ions with full occupancy, plus a third metal ion that is present only in a small minority of the molecules in the crystal. The two metal ions with full occupancy, metal ions 1 and 2 in Fig. 3A, are bridged by a solvent molecule (probably a hydroxide ion) and by the terminal carboxylate group of Glu-319. Metal ion 1 is additionally coordinated by Glu-253Oe and by His-348Nε, which is oriented for this interaction by a hydrogen bond from His-348Nε to Ala-346O. Only one of the oxygen atoms of Glu-253 acts as a true coordinating ligand, the other oxygen atom is slightly further away and behaves as a
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FIG. 3. A, view of the active site along a direction that places the plane through metal ions 1–3, shown in purple and green, in the plane of the paper. The map around the metal ions is an anomalous difference Fourier map that was calculated with phases from the final model omitting the metal ions. Contacts between metal ions and ligand atoms that are shorter than 2.3 Å are presented as thin orange lines. Thick orange lines are also used to show the hydrogen bonds from His-381Nε to Ser-353O and from His-348Nε to Ala-346O. B, contour map of the 2Fo−Fo electron density in the plane of the metals. C, contour map of the anomalous difference Fourier map. D, arrangement of the AmpS metal ligands in the amino acid sequence of the C-domain of AmpS.

“weak donor.” The same is true for a water molecule near metal ion 1 that is too far away to act as a true ligand. Metal ion 2 is coordinated by the bridging ligands described above, and additionally by His-381Ne and Asp-383Oε. The imidazole ring of His-381 is preoriented by a hydrogen bond from His-381Nε to Ser-353O, and Asp-383 coordinates the metal ion in a way that is intermediate between monodentate and bidentate coordination. The conserved Tyr-355OH is too far away from the metal ion for direct coordination, but still closer to the metal ion than the sum of the van-der-Waals radii would allow and can therefore be classified as a “weak donor” ligand. Metal ion 3, where present, is liganded by Asp-383, Glu-343, and a solvent molecule. Two additional oxygen atoms, one from a water molecule and one from the terminal carboxylate group of Glu-343, act as additional weak donors. If the site of the third partially occupied metal atom was filled by a water molecule, most of the coordinating interactions to site 3 would be replaced by hydrogen bonds. As even short hydrogen bonds are longer than canonical distances between a donor ligand and a zinc or cobalt atom, this would require a slight change of geometry, which would be hard to detect crystallographically if mixtures were present in the crystal.

**AmpS Is a Dimer in the Crystal—**AmpS crystals contain a single monomer in the asymmetric unit, but there are extensive interfaces between monomers that are related by 2-fold crystallographic symmetry (Fig. 4, A and B). Salt bridges between Asp-54 and Lys-83, and between Arg-56 and Asp-86, occur in duplicate at the interface. The interface area extends over 0.5 × 2460 Å² = 1230 Å² and is therefore in the range of contact areas for biologically relevant complexes (24). The DFIRE (distance-scaled, finite, ideal-gas reference state) server, which applies statistical potentials to distinguish biologically relevant from purely crystallographic protein-protein interactions with the column and, therefore, repeated the experiment under high salt conditions. Indeed, in the presence of 200 mM sodium chloride, AmpS migrated like a protein with molecular mass 91 ± 10 kDa, in agreement with the calculated molecular mass of the dimer (Fig. 5A).

**AmpS Is a Dimer in Analytical Ultracentrifugation—**To test the biological relevance of the gel filtration data, the association state of AmpS in 20 mM Tris, pH 7.5, 200 mM NaCl was investigated by sedimentation equilibrium and sedimentation velocity ultracentrifugation. The C(s) analysis of sedimentation velocity with Sedfit software package (13) indicated that AmpS sedimented mainly as a single peak with a sedimentation coefficient s20,w of ~6.9 S even at the low protein concentration 5 μM. The calculated molecular mass was 92.0 kDa in agreement with gel filtration data and the calculated molecular mass for the AmpS dimer. The sedimentation equilibrium data supported this observation. We got the best fit for the “two independent component” model. The predominant component was 93.2 kDa, and the minor component was ~80 kDa, probably a degradation product. If a “single component” model was used for the analysis, a good fit and a mass of 89 kDa, very close to the mass of the dimer, were obtained. Unfortunately, limited sensitivity of the optical system precluded the determination of a precise dimerization dissociation constant, but based on the available ultracentrifugation data, we can conclude that the value is below 5 μM (Fig. 5B).

**AmpS Active Sites Are within a Large Internal Cavity—**The active sites of the AmpS dimer are located on the inner walls of a large internal cavity (Fig. 4B). According to the program VOIDOO (26), the total volume of this cavity is 2470 Å³ (probe radius 1.4 Å), equivalent to the volume taken by about 20 residues in a tightly packed protein (assuming a specific volume of 0.74 cm³/g for protein) (27). As most peptides with a length in this range would not be tightly packed, the size restrictions on peptides that could pack into this cavity are probably even more stringent. The distance between the active sites is 51–55 Å (distance between the metal ions), the length spanned by a peptide of ~15 residues in extended conformation. As only aminopeptidase activity has been demonstrated for AmpS, there is no indication so far that the
distance between the active sites in the cavity would be functionally relevant.

**AmpS Active Site Accessibility**—The most puzzling feature of the AmpS structure is the almost complete sequestration of the active site from the environment, which is likely a crystallization artifact. In the crystallized form of AmpS, but probably not in the physiologically relevant state of the molecule, there is only one channel per protomer into the AmpS cavity, which leads almost straight to the active site. The tunnel is lined by Asn-140 and the strongly conserved Trp-144 of the N-domain, and the arrow denotes the location of the highly constricted channel near the active site.

**FIG. 4.** A, Ca trace of the AmpS dimer in the crystals. Color coding is as in Fig. 2. B, cutaway representation of the large internal cavity in the AmpS dimer. Residues in red have been excluded from the surface calculation to allow a view into the cavity. The metals with full occupancy are shown as pink balls, and the arrow denotes the location of the highly constricted channel near the active site. C, enlarged view of this channel. Only residues within 6.5 Å from the region of tightest constriction in the channel are presented. Electron density from a $2F_o - F$ map is shown only for water molecules in the channel. For orientation, the fully occupied metal ions in the active site are shown as pink spheres. D, view toward the active site in the C-domain from the N-domain. The black residues are from a neighboring molecule in the crystal and are present only in the AmpS dimer, but not in the AmpS monomer. The inset is a surface representation of the AmpS monomer to indicate the orientation of the pore.

**FIG. 5.** A, analytical gel filtration profiles of AmpS. UV profiles were recorded after injection of 10 µg of AmpS. The black (1) and gray (3) continuous UV traces were obtained in low salt (10 mM Tris, pH 7.5, 50 mM NaCl) and high salt (10 mM Tris, pH 7.5, 200 mM NaCl) running buffer, respectively. An intermediate trace (black dotted line, 2) was obtained when the sample was first incubated overnight in high salt and then run in low salt conditions. Note that proteins migrate significantly differently in low salt and high salt conditions. Therefore, separate calibration curves were obtained for low salt (left) and high salt (right). The activity profile was determined in low salt conditions, after injecting 0.1 µg of AmpS. 300-µl fractions of the eluate were collected, and 5-µl aliquots were incubated with 5 µg (8.5 nmol) of Met-enkephalin at 37 °C in 5 mM Tris, pH 7.5, for 2 h. The TLC plate is placed so that the location of the spots represents the average retention time of the fractions. B, sedimentation coefficient distribution for the sedimentation velocity experiments run at 5 µM (continuous line) and 16 µM (dotted line) enzyme concentration. Sedimentation coefficients are uncorrected for buffer composition and temperature.
and by many additional residues of the C-domain (Fig. 4C). In the crystallized form of AmpS, the channel appears too narrow even for unfolded peptides. Therefore, it is presently unclear whether the channel has any relevance at all.

A much wider access to the active sites of AmpS would be available if AmpS dimers dissociated to the monomers. There is no experimental evidence at present that this could occur, but if it does, it would expose a pore of ~8 Å (measured between van der Waals surfaces). The pore is lined by many charged polar residues, most of which are poorly conserved. The charges of Glu-81, Asp-86, and Glu-106 are counterbalanced by Arg-31, Arg-56, Lys-65, Lys-68, and Arg-90 (Fig. 4D). Asp-54 and Lys-83 and the conserved Arg-56 and Asp-86 form salt bridges between the protomers.

**DISCUSSION**

**AmpS Metal Coordination Geometry**

In AmpS, it is difficult to count the exact number of metal ligands and to determine the coordination geometry, even for the fully occupied metal sites. The difficulties arise from large variations of the distances between the metal ions and their potential ligands. For both zinc and cobalt ions, the distance to both nitrogen atoms is ~1.8–2.3 Å, if a coordinate uncertainty of 0.1–0.2 Å is allowed for (28). Most AmpS metal ligands are found at this “correct” distance from the metal ions. However, some are further away, even though they are still closer to the sum of the van der Waals radii would allow (Figs. 3A and 6A). This is true for Tyr-355OH and a water molecule, and applies also to one carboxylate oxygen atom each of Glu-253 and Asp-383. In the latter two cases, the carboxylates interact with the metal in a way that is intermediate between monodentate and bidentate coordination. Such interactions, although surprising at first, were commonly found for zinc, but not for other divalent metal ions (coordination to cobalt ions was not studied) in a recent survey of small molecule structures (29). If all “long” contacts to “weak donor” ligands are included in the description of the AmpS active site, the ligand counts for both metals are 6, and the coordination geometry can be described as distorted octahedral. If some or all weak donor ligands are omitted, the ligand counts go down to 5 or 4, and the geometry can be described as distorted trigonal bipyramidal or even distorted tetrahedral. This uncertainty and the presence of weak donor ligands need not be attributed to crystallographic error in the AmpS structure: a survey of accurately determined small molecule structures has recently shown that it is quite common for four- and five-coordinate complexes of zinc and copper to have additional, weakly bound donor ligands, particularly oxygen and nitrogen (30).

**Comparison with Other Dimetal-dependent Peptidases**

There is no overall sequence similarity between AmpS and other clans of dimetal-dependent peptidases. Nevertheless, the presence of two cobalt ions in AmpS homologues could have suggested a similarity to clan MG peptidases, and the presence of short, conserved sequence motifs in the AmpS family prompted Motoshima and Kaminogawa (31) to discuss a possible similarity between the AmpS family and leucyl aminopeptidase, a representative of clan MF peptidases. With hindsight from the determination of the AmpS crystal structure, it is now clear that all these proposals contained some elements of truth, but they made incorrect predictions about the AmpS fold, the AmpS ligands, and the detailed architecture of the active site. DALI quantitative structure comparisons show that the AmpS fold is not significantly similar to the folds of any of the other clans of dimetal-dependent peptidases. Comparisons of the active site environments in AmpS and other dimetal-dependent peptidases show major differences and almost no common features, strongly suggesting that AmpS-like peptidases evolved independently from other dimetal-dependent peptidases and should be placed into an independent MEROPS clan.

Despite the overall lack of similarity, a comparison of the atoms that directly coordinate the metals in AmpS and in representative members of other dimetal-dependent peptidase clans shows interesting parallels and differences (Fig. 6, A–D): in all four clans, a water molecule and at least one amino acid ligand, a glutamate or aspartate residue depending on clan, serve as the bridging ligands between the metals (the metals with full occupancy in AmpS). The similarities between AmpS (Fig. 6A) and clan MF (Fig. 6C) and clan MG (Fig. 6D) peptidases do not go much further, but there is a striking parallel between the ligand arrangement in AmpS and the ligand arrangement in clan MH peptidases, as exemplified by the dodecameric, tetrahedral-shaped peptidase (Fig. 6B). We note the presence of an acidic residue in both peptidases, Glu-343 in AmpS and Glu-212 in the tetrahedral-shaped peptidase, that is not involved in the coordination of any of the metals with full occupancy in either peptidase. It is tempting to speculate that this residue could serve as an “anchor” for the free α-amino-group in a substrate, explaining aminopeptidase activity. In this interpretation, which remains very tentative in the absence of an AmpS-ligand complex, the metal ion with low occupancy in the AmpS active site could be regarded as a mimic of the positively charged α-amino group of a substrate.

**Can the Structure Account for the Aminopeptidase Activity of AmpS?**

The homology of AmpS to well characterized enzymes from hyperthermophilic sources has led to a name that implies
aminopeptidase activity. The bioinformatic conclusion is consistent with our experimental data for the degradation of the physiologically unrelated model peptide Met-enkephalin, which also suggest that the proteolysis starts from the amino terminus. However, our tests with a single substrate are clearly insufficient to rule out carboxypeptidase or even endopeptidase activity, although we have no indications for such activities at the moment. The lack of an active site that extends to both sides from the metal center is consistent with the enzyme being an exo- rather than an endopeptidase. The presence of the conserved Glu-343 near the active site, without a role in the coordination of the metals with full occupancy, could explain aminopeptidase activity (see above), but in the absence of a peptidase-ligand complex, the explanation is speculative and may be unnecessary, because other activities of AmpS have not yet been fully ruled out.

Active Site Access

If it is difficult to reconcile the activity of AmpS in solution with the lack of access to the AmpS active sites in the crystal. Various explanations for this puzzle can be considered as in the following.

Protein Concentration—The activity assays were performed with 0.05 μM enzyme, which is significantly lower than the limit on the dimer dissociation constant from the sedimentation equilibrium experiments, and much lower than the protein concentration used for the gel filtration runs. Thus, AmpS may be a dimer in our association assays and a monomer in the activity assays. To test this possibility, we used the TLC assay to record activity profiles instead of the UV-absorbance profiles. This allowed us to lower the amount of AmpS, and we detected the active species only. Results of the TLC assay were not quantitative, but they clearly indicate that the activity migrated as a dimer, unless the recorded activity was due to enzyme that dissociated after the sizing chromatography run (Fig. 5A).

Temperature—The activity assays were performed at 37 °C, whereas the association assays were done at room temperature and at 4 °C. To test whether temperature differences could explain the discrepancy between the biochemical and the crystallographic results, we repeated the gel-filtration runs at 37 °C. If changes in retention of the molecular mass standards were taken into account, AmpS again migration as the dimer, at least at the high concentration that was used to record UV-profiles (data not shown). Higher temperatures could not be probed, because the chromatography resin (Superose 12 HR) is only stable up to 40 °C. We also tested the effect of temperature on AmpS activity. The enzyme has some activity at 25 °C, much higher activity at 40 °C. We also tested the effect of temperature on AmpS activity.

Dimerization Mode—The dimer that occurs in solution. This could happen, if the “true” dimer is not present in the crystal, because the molecule adopts an altered conformation under crystallization conditions, or if the true dimer is present in the crystal, but was overlooked because the wrong crystallization contact was chosen as the dimerization interface in the analysis. The first possibility is difficult to exclude, but unlikely, because the described dimer interface is extensive and predicted to mediate a high affinity contact. The second possibility can be checked by comparing the suggested dimer interface with all other crystal contacts. We find that the described interface is not only the most extensive interface, but also the only one that is scored as a “true interface” by the DIFIRE server (25), even if crystal contacts between molecules that are not related by a pure 2-fold symmetry are included in the analysis.

Protein Flexibility—It is possible that the crystallization condition selects for a closed and inactive form of AmpS, because it packs better than a more open, active form. Because there is only one molecule in the asymmetric unit of the AmpS crystals, this structure cannot provide evidence for such flexibility. However, in the crystals of the hyperthermophilic AmpS homologue, there are five molecules in the asymmetric unit, which also dimerize, partly by local and partly by crystallographic symmetry. Superposition of these molecules suggests the relative positions of the N- and C-domains are variable. Although the movement is limited, this raises the possibility that the boundary between the N- and C-domains may act as a hinge. Good conservation of the possible hinge region would be consistent with the idea, but experimental support is lacking. Clearly, more work is required to understand how AmpS can be active.

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Note Added in Proof—We have learned from Alan Barnett that the new metallopeptidase clan will get the identifier MQ in the MEROPS database.

REFERENCES