Identification of the Catalytic Residues of α-Amino Acid Ester Hydrolase from *Acetobacter turbidans* by Labeling and Site-directed Mutagenesis*

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The α-amino acid ester hydrolase from *Acetobacter turbidans* ATCC 9325 is capable of hydrolyzing and synthesizing the side chain peptide bond in β-lactam antibiotics. Data base searches revealed that the enzyme contains an active site serine consensus sequence Gly-X-Ser-Tyr-X-Gly that is also found in X-prolyl dipeptidyl aminopeptidase. The serine hydrolase inhibitor p-nitrophenyl-p'-guanidino-benzoate appeared to be an active site titrant and was used to label the α-amino acid ester hydrolase. Electrospray mass spectrometry and tandem mass spectrometry analysis of peptides from a CNBr digest of the labeled protein showed that Ser\(^{205}\) situated in the consensus sequence, becomes covalently modified by reaction with the inhibitor. Extended sequence analysis showed alignment of this Ser\(^{205}\) with the catalytic nucleophile of some α/β-hydrolase fold enzymes, which posses a catalytic triad composed of a nucleophile, an acid, and a base. Based on the alignments, 10 amino acids were selected for site-directed mutagenesis (Arg\(^{85}\), Asp\(^{86}\), Tyr\(^{143}\), Ser\(^{156}\), Ser\(^{205}\), Tyr\(^{206}\), Asp\(^{338}\), His\(^{370}\), Asp\(^{509}\), and His\(^{510}\)). Mutation of Ser\(^{205}\) to Asp\(^{338}\), or His\(^{370}\) to an alanine almost fully inactivated the enzyme, whereas mutation of the other residues did not seriously affect the enzyme activity. Circular dichroism measurements showed that the inactivation was not caused by drastic changes in the tertiary structure. Therefore, we conclude that the catalytic domain of the α-amino acid ester hydrolase has an α/β-hydrolase fold structure with a catalytic triad of Ser\(^{205}\), Asp\(^{338}\), and His\(^{370}\). This distinguishes the α-amino acid ester hydrolase from the Ntn-hydrolase family of β-lactam antibiotic acylases.

The α-amino acid ester hydrolases have been known for their applicability in the biocatalytic synthesis of semisynthetic β-lactam antibiotics since 1972 (1). These enzymes can hydrolyze the amide bond that connects the acyl side chain to the β-lactam nucleus. Starting from esterified acyl precursors, they can also catalyze the reverse reaction. Remarkable features of these enzymes are the ability to accept charged substrates such as α-amino acid esters, the preference for esters over amides, and the low pH optimum (pH 6.2) (2, 3). Despite these attractive properties, a gene encoding an α-amino acid ester hydrolase (AEH)\(^1\) was only recently cloned and characterized (4). Thus far, all the known β-lactam antibiotic acylases, such as penicillin G acylase (5), penicillin V acylase (6), and cephalosporin acylase (7), belong to the Ntn-hydrolase family. However, protein data base searches showed no homology of the AEH of *Acetobacter turbidans* with known β-lactam antibiotic acylases. The N-terminal amino acid sequence of the AEH was determined; it revealed a signal sequence, but no N-terminally located Thr, Ser, or Cys, characteristic for members of the Ntn-hydrolase family, was found. It was therefore postulated that the AEHs belong to a new class of β-lactam antibiotic acylases (4).

An alignment of the AEH sequence with those of homologous proteins showed the presence of the active site serine consen-sus motif GXSXYG (4), which is described for the X-prolyl dipeptidyl aminopeptidases (8). No x-ray structure of the aminopeptidases is known, but they are members of a group of proteins that belong to the prolyl oligopeptidase family. Of this family two structures have been solved, which both contain an α/β-hydrolase fold (9, 10) and have a catalytic triad of Ser, Asp, and His. Therefore, it is possible that the X-prolyl dipeptidyl aminopeptidases and hence AEH also have a catalytic triad. This assumption is further supported by the identification of a catalytic triad in the recently solved crystal structure of a cocaine esterase (11) that is also related to AEH. Earlier experiments with inhibitors already suggested the importance of a histidine for the catalytic activity of AEH (12). However, common serine hydrolase inhibitors such as phenylmethylsulfonyl fluoride, diisopropylfluorophosphate, or Pefabloc SC showed no inhibition of AEH activity (4, 12). On the other hand, inhibition was observed with the serine hydrolase inhibitor p-nitrophenyl-p'-guanidino-benzoate (p-NPGB), but the inhibition was incomplete, which left uncertainty about the catalytic role of a serine in AEH (4). In this study, we used active site labeling, site-directed mutagenesis, and sequence analysis to demonstrate that AEH is a member of a class of β-lactam antibiotic acylases that belongs to the α/β-hydrolase fold family and possesses a classical catalytic triad of Ser, Asp, and His.

**EXPERIMENTAL PROCEDURES**

**Materials—** The chromogenic substrate 2-2-nitro-5-(phenylglycyl)aminobenzoic acid (NIPGB) was obtained from Syncom (Gronin-

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\(^1\) The abbreviations used are: AEH, α-amino acid ester hydrolase; p-NPGB, p-nitrophenyl-p'-guanidino-benzoate; p-NP, p-nitrophenol; NIPGB, 2-2-nitro-5-(phenylglycyl)aminobenzoic acid; ES, electro-spray; MS, mass spectrometry; HPLC, high-pressure liquid chroma-tography; DMF, dimethylformamide; pdb, Protein Data Bank.

28474 This paper is available on line at http://www.jbc.org
Identification of Catalytic Residues of AEH

Oligonucleotides used in site-directed mutagenesis. Only the sense primers are shown. Introduced restriction sites are underlined, and sequence differences with wild type are shown in bold.

| Oligonucleotide sequence 5’ → 3’ | Restriction site | Amino acid substitution |
|---------------------------------|-----------------|------------------------|
| C GAG GTT ATG GTA CCC ATG GGG AAC GGC GTG AAG | Rsal | B58A |
| GTT ATG GTA CCC ATG GGG GCC GGC GTG AAG GTG | Rsal | D68A |
| G TTG ATG GCC GGC GCT ATC CGC GTG TTT CAG | HaeII | Y143A |
| GC GGG AAA TAT GGC GCT CAG GGC GAT TAT G | AspI | S205A |
| GGT ATG ACA GGG TCG TCC GAT GCG GGT TTT ACT | AspI | Y206A |
| G GGT ATG GCC TGG TCG GCC GCT GAT GGG GTG AAC | NcoI | D338A |
| GAA CAG GGC TGG TGG GCT CAG GAA GAT ATG TG | NdeI | H370A |
| CA GAA TCC CCG CGG GCT GTG AGT ACA TAT GAA AC | | D509A |
| C CAT GTG TTT GCA AAA GGG GCT CGG ATT ATG GTG | | H610A |

**TABLE II**

Purification of N-terminal His$_6$-tagged AEH from *E. coli*

| Purification step | Total volume | Total protein | Total activity$^a$ | Specific activity$^a$ | Purification | Recovery |
|-------------------|--------------|--------------|------------------|---------------------|--------------|----------|
| Cell-free extract | 72           | 312          | 5550             | 17.6                | 1            | 100      |
| Ni$^{2+}$-agarose | 4.8          | 2.7          | 3350             | 76                  | 65           |          |
| Gel filtration    | 8.0          | 2            | 3350             | 1670                | 95           | 60       |

$^a$ Cephalxin synthesis; cexU, units of cephalixin.

**FIG. 1. SDS-PAGE of AEH with and without C-terminal His$_6$ tag.** The proteins were separated on 12.5% SDS-PAGE and stained with Coomassie Brilliant Blue. Lane M, molecular mass marker (masses are indicated at the left in kDa); lane 1, AEH isolated from *E. coli* BL21(DE3)pLysS(pETAT) with subunits of 70 kDa; lane 2, AEH with the C-terminally attached Myc epitope and His$_6$ tag isolated from *E. coli* TOP10(pBADAT) with subunits of 72 kDa.

**TABLE III**

Kinetic parameters of cephalxin hydrolysis for mutants of AEH

| Enzyme | Mean ± SE of $K_m$ (mM) | Mean ± SE of $k_{cat}$ (s$^{-1}$) | Mean ± SE of $k_{cat}/K_m$ (s$^{-1}$) |
|--------|--------------------------|----------------------------------|----------------------------------|
| AEH-His$_6$ | 0.45 ± 0.06             | 274 ± 7                          | 609                              |
| S205A  | 4.0 ± 0.2                | 120 ± 2                          | 30                               |
| D338A  | 0.3 ± 0.1                | 0.20 ± 0.04                      | 0.6                              |
| H370A  | 0.4 ± 0.1                | 0.20 ± 0.02                      | 0.4                              |
| R85A   | 0.18 ± 0.06              | 184 ± 6                          | 1022                             |
| S156A  | 0.25 ± 0.08              | 132 ± 3                          | 528                              |
| H610A$^b$ | 0.9 ± 0.2               | >69 ± 2                          | >77                              |

$^a$ No conversion was observed at 1, 10, or 25 mM cephalxin; detection limit is given.

$^b$ Partially purified, approximately 30% pure.

5’-CCGGCCACACACATTGGGAGCATGATA-3’ (start codon shown in *bold*), was based on the N-terminal sequence including the signal sequence, and an NcoI site (*underlined*) was introduced. The reverse primer, 5’-CATACTGCGCAAGCTCTCTGTTTTCACACCGGGAG-3’, (the HindIII site is *underlined*), lacked the stop codon to allow the C-terminal attachment of the myc epitope followed by a polyhistidine region of six histidine residues (His$_6$ tag), which are encoded on pBAD/Myc-HisA.

**Protein Purification**—Wild-type and mutated AEHs were expressed in *E. coli* TOP10 from the pBAD/Myc-HisA-derived constructs. To obtain soluble protein, two 2.5-liter cultures supplemented with 1-arginine (0.01%, w/v) were inoculated with 1 ml of culture grown overnight at 30 °C and incubated for 64 h at 14 °C. Induced cells were harvested from the cultures by centrifugation at 5000 × g and suspended in 50 mM sodium phosphate buffer, pH 6.2. All further steps were carried out at 4 °C. The cytoplasmic content was released by sonication, and the remaining cell debris was removed by centrifugation at 13,000 × g for 40 min. The supernatant was added to 1 ml of nickel-agarose (Qiagen...
GmbH, Hilden, Germany) equilibrated with wash buffer (25 mM imidazole, 500 mM NaCl, and 50 mM sodium phosphate buffer, pH 7.4). After mixing by inversion for 90 min at 4 °C, the bed was allowed to form (20/11003 8-mm bed in a polyprep chromatography column (Bio-Rad)). The unbound protein was washed from the column with 30 column volumes of wash buffer. The bound protein eluted from the column at 75–100 mM imidazole in a stepwise gradient from 50 to 200 mM imidazole, 150 mM NaCl, 50 mM sodium phosphate, pH 7.4, in 20 column volumes. The protein was brought to 50 mM sodium phosphate buffer, pH 6.2, with the use of an Econo-Pac gel filtration column (Bio-Rad). All purification steps were monitored by SDS-PAGE, and the enzymatic activity was measured with NIPGB (4). The protein concentrations were determined using the Bradford method with bovine serum albumin as the standard.

Analysis of Conformation by Circular Dichroism Spectroscopy—Far-ultraviolet CD spectra from 250 to 190 nm were recorded on an AVIV circular dichroism spectrometer model 62A DS (AVIV Associates, Lakewood, NJ) at 25 °C using a quartz cuvette with a path length of 0.1 cm. The concentration of wild-type and mutant enzymes was 0.2 mg/ml in 50 mM sodium phosphate buffer, pH 6.2. Three separate spectra were collected per sample and averaged using a step interval of 0.5 nm/min and an averaging time of 5 s. The phosphate buffer was used as a blank and subtracted from each recording. The data were converted to mean residue ellipticity (θ, deg·cm²·dmol⁻²). From the CD spectra, the percentage of secondary structure elements was calculated using CD spectra deconvolution (CDNN Version 2.1, available on the World Wide Web). These values were standardized to 100% total structure elements.

Activity Assays—The hydrolysis and synthesis of cephalexin at 30 °C were followed by high-pressure liquid chromatography (HPLC) as described previously (4). The hydrolysis of p-NPGB was measured at concentrations varying from 0.1 to 1 mM with 1.5 μM enzyme. The release of p-NP was followed.

![Figure 2](image_url)

**FIG. 2.** A, reactivation of AEH after preincubation with p-NPGB. *Solid line,* untreated enzyme; *dotted line,* enzyme preincubated for 15 min with 1 mM p-NPGB in 1% DMF. The release of p-NP was followed. B, time course of reaction of AEH with of 1 mM p-NPGB. *Dotted line,* chemical hydrolysis at 30 °C p-NPGB; *solid line,* conversion by 0.68 μM AEH.

![Figure 3](image_url)

**FIG. 3.** Reaction scheme showing the labeling of AEH by p-NPGB. The inhibitor p-NPGB reacts with the catalytic serine of AEH, resulting in p-NP and a labeled enzyme (AEH-GB).

![Figure 4](image_url)

**FIG. 4.** HPLC elution pattern of CNBr-peptide fragments of labeled (*solid line*) and unlabeled (*dotted line*) AEH. The peak indicated as control and peaks 1–3 were analyzed by mass spectrometry.
state reactions were performed in 50 mM 4-morpholinepropanesulfonic acid buffer at pH 7, with 1 mM p-NPGB. The enzyme concentration used was 1.32 or 0.86 μM (α/β, 144 kDa). Progress curves (absorbance, P) were fit to Eq. 1 to obtain the amplitude (B), the first order rate constant (k_ab) for the burst phase, and the velocity of the steady-state reaction (A), using the program Scientist.

\[
[P] = A \times t + B(1 - e^{-k_{ab}t})
\]  

Eq. 1

Inactivation and Reactivation of AEH-His—The enzyme (2.4 μM, 144 kDa) was inactivated by incubation with p-NPGB (1 mM; 1% DMF) for 15 min at 30 °C. Control experiments involved incubation under the same conditions of enzyme only and enzyme with 1% DMF. To study reactivation, the inactivated enzyme was diluted 76-fold in 15 mM NIPGB dissolved in 50 mM sodium phosphate buffer, pH 6.2. The time course of reactivation was monitored by following the hydrolysis of NIPGB at 30 °C and 405 nm.

Labeling of the Enzyme—The enzyme (15.4 μM) was incubated with 0.5 mM p-NPGB in 50 mM sodium phosphate buffer, pH 6.2, 0.5% dimethylformamide, for 15 min at 30 °C. The excess of p-NPGB was removed by dialysis against 70% formic acid. To reduce any disulfide bonds, the enzyme solution was dialyzed against 70% formic acid with 1 mM dithiothreitol for 10 min at 30 °C. The dialyzed enzyme was treated with a 100-fold molar excess of CNBr over the Met content. The reaction was allowed to proceed for 24 h at room temperature under N2 in the dark and was stopped by the addition of 10 volumes of water. The reaction mixture was freeze-dried and dissolved in HPLC eluents. The generated peptides were separated by reversed-phase HPLC using a Nucleosil-5 C18 column (4.6 × 300 mm; Alltech Associates, Inc.) at 1 mL/min in a linear gradient of 0 to 67% acetonitrile in 0.1% trifluoroacetic acid. The peptide profile was monitored at 280 nm. The control experiment involved the same conditions as described above, except that no p-NPGB was added. The peaks that were different from the control experiment were collected and rechromatographed on the same column in a linear gradient from 0 to 67% acetonitrile in 0.1% ammonium acetate, pH 5.0. The individual peaks were collected, concentrated, and injected directly into the mass spectrometer.

Mass Spectrometry—Electrospray (ES) mass spectrometry (MS) was performed on an API3000 mass spectrometer (Applied Biosystems/MDS-SCIEX, Toronto, Canada), a triple quadrupole mass spectrometer supplied with an atmospheric pressure ionization source, and an ion-spray interface (13). The spectra were scanned in the range between m/z 400 and 1600. Tandem mass spectrometry product ion spectra were recorded on the same instrument by selectively introducing the m/z 1229.5 (singly charged unlabeled peptide) and m/z 695.9 (doubly charged labeled peptide) precursor ions from the first quadrupole into the collision cell (second quadrupole). The collision gas was nitrogen with 30 eV collision energy. The product ions resulting from the collision were scanned over a range of m/z 10 to 1395 with a step size of 0.1 atomic mass unit and a dwell time of 2 ms.

Sequence Analysis—PSI-Blast (14) and a homology-based fold prediction program (15) were used to predict the catalytic residues and the fold of AEH. The secondary structure elements of AEH were predicted using the consensus of the following programs: PSIPred (17), Jpred (18), and SAM-T99sec (19).

RESULTS AND DISCUSSION

Expression of AEH in E. coli with N-terminal Histag—To achieve a higher expression level and an easier purification of AEH than that obtained with a previous construct (4) the achA gene was cloned in pBAD/Myc-HisA (pBADAT), coupling both the myc epitope and the His6 tag C-terminally to the protein. The use of the arabinose promoter in the pBADAT plasmid resulted in an overproduction of 5-fold (1% of the total protein in cell-free extract) compared with the expression in the wild-type A. turbinata strain (4). Furthermore, with the resulting construct, the number of necessary purification steps was reduced from four to two by use of a nickel-agarose column (Table II). Two mg of >90% pure protein could be obtained from a 5-liter culture and was stable at 4 °C for at least 60 days. The attachment of the tag resulted in a 2-kDa increase in the molecular mass of each subunit of the homodimeric AEH, as is clearly visible on an SDS-PAGE gel (Fig. 1). To check whether
the properties of AEH had changed upon the addition of the myc epitope and the His tag, the kinetic parameters of the purified enzyme for cephalexin hydrolysis were measured (Table III) and compared with those of untagged recombinant protein (4). The $K_m$ values of both proteins appeared to be similar (0.45 and 0.34 mM, respectively). The $k_{cat}$ of the fusion protein is somewhat lower than that for the untagged recombinant protein (347 s⁻¹¹⁰⁰⁰₂), but the values are in the same order of magnitude. This indicates that proper folding of the recombinant protein occurs and shows that there is no dramatic influence of the additional C-terminal amino acids.

**Conversion of p-NPGB by AEH**—To check whether the previously observed inhibition by p-NPGB (4) is irreversible, the enzyme was preincubated with p-NPGB and then mixed with substrate solution. Upon dilution into the NIPGB solution, the inactivated enzyme gradually reverted to the active form (Fig. 2A). After 20 min, the enzyme recovered a major part of its activity, indicating that the inactivation by p-NPGB involves a reversible modification at the active site. To further test the conversion of p-NPGB, AEH was incubated with p-NPGB, and the formation of p-NP was followed by stopped-flow spectroscopy. The reaction with p-NPGB followed a biphasic time course (Fig. 2B), consisting of an initial burst followed by a phase that corresponds to the steady-state hydrolysis. The formation of the acyl-enzyme intermediate was faster than its hydrolysis, resulting in an accumulation of the acyl-enzyme and the burst of p-NP, which is in agreement with what is expected for an active site-directed covalent inhibitor. Subsequently, in the steady-state phase, the acyl-enzyme complex was slowly hydrolyzed with a $k_{cat}$ of $1.3 \pm 0.6 \times 10^{-3}$ s⁻¹.

**Identification of Catalytic Residues of AEH**

| 202 | Gly | 204 | Ser | 205 | Tyr | Glu | Phe | Thr | Val | Val | hsl |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| b<sup>+</sup> | 102.1 | 159.1 | 246.1 | 333.1 | 496.2 | 625.3 | 682.3 | 829.3 | 930.4 | 1029.5 | 1128.5 | 1211.6 |
| b<sup>+</sup> label | 102.1 | 159.1 | 407.2 | 494.2 | 657.3 | 789.3 | 843.3 | 990.4 | 1091.4 | 1190.5 | 1289.6 | 1372.6 |
| b<sup>+</sup>205 | 102.1 | 159.1 | 246.1 | 404.2 | 494.2 | 657.3 | 789.3 | 843.3 | 990.4 | 1091.4 | 1190.5 | 1289.6 | 1372.6 |

**FIG. 6.** ES/tandem mass spectrometry analysis of peptide Thr<sup>202</sup>-Met<sup>213</sup>. A, the peptide sequence and its calculated monoisotopic singly charged masses for the product ions of type b of the unlabeled (b<sup>+</sup>) and labeled peptide at either position 204 (b<sup>+</sup>204) or position 205 (b<sup>+</sup>205). Met<sup>213</sup> is modified to a homoserine lactone (hsl). The m/z values observed in the spectra are shown in **bold**. B, the product ion scan spectrum of the precursor ion m/z 1229.5 obtained with peptide Thr<sup>202</sup>-Met<sup>213</sup> from the unlabeled enzyme. C, the product ion scan spectrum of the precursor ion m/z 695.9 obtained with the same peptide from the labeled enzyme.
The steady-state rate of the conversion of p-NPGB within the concentration range of 0.1 to 1 mM p-NPGB was constant (data not shown), indicating that the $K_{m}$ for p-NPGB is <0.1 mM. Therefore, the burst at 1 mM p-NPGB can be directly related to the number of active sites. The burst was measured in duplicate with two different enzyme concentrations and was found to correspond to $2.7 \pm 0.7 \mu M$ released product with 1.32 $\mu M$ enzyme and $1.1 \pm 0.2 \mu M$ released product with 0.66 $\mu M$ enzyme. In view of the subunit composition, this indicates that each subunit has one active site.

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Identification of Active Site Ser205 by Labeling with p-NPGB—The slow conversion of the acyl-enzyme intermediate

**Fig. 7. Conserved regions of AEH and structure-based alignment with homologous α/β-hydrolase fold enzymes.** The conserved residues resulted from an alignment with proteins 20% identical to AEH identified by BLAST (14). The alignments were done using ClustalW 1.8 with blosum weight matrix and default parameters. Bold residues are identical among the proteins used for the alignment. The secondary structure of AEH, without its signal sequence, was predicted using PSIPred (17), Jpred (18), and SAM-T99sec (19). The consensus is shown. The alignments shown in the figure were based on a homology-based fold prediction (15). Gray-shaded residues are located in a β-sheet, and black-shaded residues are located in a helix. $\text{Coc}$, cocaine esterase from *Rhodococcus* sp., pdb 1JU4; *Pro*, proline iminopeptidase from *X. campestris*, pdb 1AZW; *Chl*, chloroperoxidase T from *Streptomyces aureofaciens*, pdb 1A7U; *Oli*, prolyl oligopeptidase from porcine muscle, pdb 1QFM.
during reaction of p-NPGB made it possible to covalently label the enzyme (Fig. 3). AEH was incubated with excess p-NPGB, and the covalent form was trapped by the addition of acid and subsequently fragmented with CNBr. Twenty peptide fragments in which the methionines had been modified to homoserine lactone were generated, varying in mass from 0.102 to 20.9 kDa. The elution pattern of the peptide mixture obtained from labeled AEH showed a few different peaks compared with the control (Fig. 4). These peaks were individually collected and analyzed by ES/MS. The peak indicated as the control in the HPLC elution pattern (Fig. 4) corresponded to the fragment 562GGYELPVSM570 (903.4 Da), indicated by its singly, (M + H)+, and doubly, (M + 2H)2+, charged peak in the mass spectrum, m/z 904.4 and 452.6, respectively. This fragment had the same mass when isolated from unlabeled or p-NPGB-labeled proteins (Fig. 5, A and B). Peak 1 could not be assigned to an expected CNBr fragment and is likely the result of incomplete digestion. ES/MS analysis of peak 3 showed a mixture of peptides, and the major component of the mixture did not change upon labeling. The peptide eluting in peak 2 was identified as CNBr fragment 202TGSSYEFGTVVM213 (1228.6 Da), of which m/z 1229.5, (M + H)+, and m/z 615.6, (M + 2H)2+, were present in the ES/MS analysis of the unlabeled protein (Fig. 5C). When isolated from protein that was preincubated with p-NPGB, a peptide was found at this position with a mass of 1390 Da, indicated by the peaks with m/z 1390.8, (M + H)+, and m/z 696.0, (M + 2H)2+ (Fig. 5D). This mass is in agreement with the fragment of 1228.6 Da plus the guanidino benzoate label (161 Da; Fig. 3), indicating that the fragment that harbors the potential active site serine was labeled by p-NPGB. The increase in absorbance of the peptide after labeling is in agreement with the attachment of an aromatic group. The presence of the (M + H)+ ion at m/z 1229.5 in the spectrum of the labeled peptide fragment is probably due to some fragmentation in the orifice skimmer region of the mass spectrometer, resulting in loss of the charged label.

To determine which serine (204 or 205) of fragment 202TGSSYEFGTVVM213 was modified by p-NPGB, the labeled peptide was analyzed by ES/Tandem mass spectrometry using product ion scan to obtain the significant fragments. The expected b+ fragments for the peptides labeled at either 204 or 205 were calculated (Fig. 6A) and compared with the data. The product ion scan of the precursor ion m/z 1229.5, (M + H)+, of the unlabeled peptide displayed most of the possible b+ fragments, together with the precursor ion itself (Fig. 6, A and B). The product ion scan of the (M + 2H)2+ ion at m/z 695.9 of the labeled peptide showed an increase in the masses by 161 Da of the b+ fragments starting at b13, compared with the unlabeled protein (Fig. 6, A and C). The same increase in mass was found only for the detected y fragments2 y9−(hsl-Ser205) to y11−(hsl-Gly203) of the labeled peptide compared with the unlabeled peptide (data not shown). Both the b and complementing y fragments that were found are in agreement with the label positioned on Ser205 and exclude labeling at Ser 204.

Active Site Topology and Site-directed Mutagenesis—An alignment of AEH with homologous proteins that were identified with BLAST (14) revealed a number of conserved residues (Fig. 7). Extended homology searches using PSI BLAST (14) and fold prediction (15) showed that the catalytic residues of the α/β-hydrolase fold enzyme cocaine esterase (11) aligned with Ser205, Asp338, and His370 in AEH. Furthermore, the N-terminal part (residues 67–374) of AEH aligned with 11% identity to proline iminopeptidase (34 kDa) from Xanthomonas campestris pv. citri (pdb 1AZW). Its catalytic domain also exhibits an α/β-hydrolase fold and is considered to be a suitable model for the catalytic domain of the prolyl oligopeptidase family (10, 20). The catalytic Ser, Asp, and His of this protein align with the same residues from AEH as indicated above. In a smaller region (residues 94–370), 11% identity was found with a chloroperoxidase and bromoperoxidase (pdb 1A7U, 30.3 kDa (alignment shown in Fig. 7) and IBRT, 30.2 kDa, respectively). The catalytic nucleophile and acid of these α/β-hydrolase fold enzymes (21) align with AEH at position 205 and 338, respectively. Additionally, the active site serine of prolyl oligopeptidase from porcine muscle (pdb 1QFM, 80.2 kDa) aligned with Ser205. Extending the alignment of chloroperoxidase and prolyl oligopeptidase with AEH manually on basis of the predicted structural elements resulted in the conservation of the other catalytic residues as well (Fig. 7).

Based on these alignments, a catalytic triad of Ser205, Asp338, and His370 in AEH is predicted to be conserved among the α/β-hydrolase fold enzymes. The catalytic nucleophile and acid of these α/β-hydrolase fold enzymes (21) align with AEH at position 205 and 338, respectively. Additionally, the active site serine of prolyl oligopeptidase from porcine muscle (pdb 1QFM, 80.2 kDa) aligned with Ser205. Extending the alignment of chloroperoxidase and prolyl oligopeptidase with AEH manually on basis of the predicted structural elements resulted in the conservation of the other catalytic residues as well (Fig. 7).

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and His\textsuperscript{370} is expected, which is supported by the identification of Ser\textsuperscript{205} as the catalytic nucleophile by the labeling experiments. To support this, Asp\textsuperscript{338} and His\textsuperscript{370}, together with Ser\textsuperscript{205} as a control, were mutated to an alanine. Other conserved residues from AEH, specifically Arg\textsuperscript{85}, Asp\textsuperscript{36}, Tyr\textsuperscript{143}, Ser\textsuperscript{156}, Tyr\textsuperscript{206}, Asp\textsuperscript{338} and His\textsuperscript{370}, were also mutated to an alanine. All mutants were properly expressed in the pBAD vector, except for the mutants D86A, Y143A, and D509A. The expression of mutants were properly expressed in the pBAD vector, except for the mutants D86A, Y143A, and D509A. The expression of these mutants was tested at different arabinose concentrations (0.1, 0.01, 0.001, and 0.0001%) and at different temperatures (14 °C, 18 °C, and 30 °C), but no sufficient expression of these mutants for purification could be achieved. Therefore, these residues were assigned an important structural role. Slight variations in expression levels were observed for the other mutants, but they were similar to wild-type AEH according to their behavior in the standard purification procedure. The effects of the mutations on the ability to hydrolyze cephalexin were determined (Table III). Replacement of Ser\textsuperscript{205}, Asp\textsuperscript{338}, or His\textsuperscript{370} by an alanine drastically reduced the activity. These radical changes were not observed for the other purified mutants. The effects of the inactivating mutations on the secondary structure were evaluated with circular dichroism. The spectra obtained with the purified wild-type and mutant enzymes were superimposable (Fig. 8), and the calculated percentages of the secondary structure elements were essentially the same as those calculated from the wild-type data. According to the data, the wild-type enzyme had 25.4% α-helices, 43.2% β-sheets (β-turns, antiparallel and parallel sheets), and 31.4% random coil. Therefore, from the CD spectra, we conclude that the inactivating caused by the mutations of Ser\textsuperscript{205}, Asp\textsuperscript{338}, or His\textsuperscript{370} did not result from drastic changes in the secondary structure of the enzyme.

The K\textsubscript{m} for cephalexin was in the same order of magnitude as that found for wild-type enzyme for all the active mutants, except for the Tyr\textsuperscript{206} mutant, for which the K\textsubscript{m} increased significantly (Table III). A possible role for this residue will be given below. Although a significant decrease in K\textsubscript{m} was observed for the alanine mutants of residues Ser\textsuperscript{156}, His\textsuperscript{370} and Arg\textsuperscript{85}, their K\textsubscript{m} and specificity constants are in the same order of magnitude as those found for the wild-type enzyme. Therefore, these residues do not seem to play a crucial role in the hydrolysis of cephalexin. Based on the kinetic characterization and the CD spectra of the inactive mutants, we conclude that AEH is a serine hydrolase and contains a classical catalytic triad of Ser\textsuperscript{205}, Asp\textsuperscript{338}, and His\textsuperscript{370}.

Structural Analysis—The alignments and the conservation of the catalytic triad residues suggest an α/β-hydrolase fold for AEH. This should also be evident from the arrangement of the secondary structure elements. Secondary structure predictions yielded 16 β-strands and 7 α-helices (3 or more residues predicted as strand or helix), excluding the signal sequence (Fig. 7). As found in α/β-hydrolase fold enzymes, the catalytic residues in AEH are preceded by a strand and followed by a helix. Furthermore, the order of the structural elements in the N-terminal part of AEH is similar to that of the α/β-hydrolase fold enzymes, as is evident from a secondary structure-driven alignment with chloroperoxidase, proline iminopeptidase, and prolyl oligopeptidase (Fig. 7). A superimposition of the structural elements of AEH on the topology diagram of the α/β-hydrolase fold visualizes this very clearly (Fig. 9). An additional helix is predicted between strand 6 and 7, which is in agreement with the position of the additional domain in proline iminopeptidase. In other α/β-hydrolase fold enzymes, additional helices are also found at this position and form a cap domain (22). The positions of the structural elements relative to the catalytic residues further indicate that the catalytic domain of AEH, which encompasses the N-terminal part of the protein sequence excluding the signal sequence (residues 41–416), has an α/β-hydrolase fold.

The function of the C-terminal part of AEH (417–667), which is predicted to harbor 8 or more β-strands, remains unclear. In cocaine esterase, this domain adopts a jelly roll-like β-domain and is expected to be important for the arrangement of the overall tertiary structure (11). A role in substrate specificity can also be expected, similar to the role of the N-terminally located β-propeller in prolyl oligopeptidase (9).

In α/β-hydrolase fold enzymes, the main chain NH group of the residue following the nucleophile is usually involved in the formation of the oxyanion hole, donating one of the two hydrogen bonds to the oxygen of the tetrahedral intermediate (22). The second group stabilizing the oxyanion hole is usually located between β-strand 3 and helix A. Gly\textsuperscript{433} and Trp\textsuperscript{111} in proline iminopeptidase (10) and Phe\textsuperscript{32} and Ser\textsuperscript{99} in chloroperoxidase T (21) are suggested to constitute the oxyanion-binding site through their backbone NH groups. In oligopeptidase of porcine muscle and cocaine esterase, the hydrogen bonds are provided by the main chain NH group of the residue following the catalytic serine and by the OH of a Tyr residue (Tyr\textsuperscript{44} and Tyr\textsuperscript{173} respectively) (9, 11). Comparing the structural alignment and the conserved residues, the most likely candidate for the hydrogen donors in AEH are Tyr\textsuperscript{112}, which aligns with the second hydrogen donor of cocaine esterase and prolyl oligopeptidase, and Tyr\textsuperscript{206} (Fig. 7). In agreement with the expected backbone NH involvement, the removal of the hydroxy-phenyl group in the Y206A mutant did not abolish activity. No mutations were made at position Tyr\textsuperscript{112}, but this residue probably donates the second hydrogen bond through its hydroxyl group, as found for cocaine esterase and prolyl oligopeptidase (9, 11).

In conclusion, the results presented in this study indicate that AEH is an α/β-hydrolase fold enzyme and has a classical catalytic triad with Ser\textsuperscript{205} as the nucleophile. The enzyme has a small cap domain and an extensive C-terminal domain that is largely β-stranded. The enzyme is homologous to one other β-lactam antibiotic acylase, glutaryl 7-aminoccephalosporanic acid acylase from Bacillus laterosporus (16), which is expected to have a similar structure. These results define a class of β-lactam antibiotic acylases that is clearly different from other known β-lactam antibiotic acylases that belong to the Ntn-hydrolase family.

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Identification of Catalytic Residues of AEH

28482

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