Acetobacter turidans α-Amino Acid Ester Hydrolase
HOW A SINGLE MUTATION IMPROVES AN ANTIBIOTIC-PRODUCING ENZYME*

Received for publication, October 14, 2005, and in revised form, November 21, 2005 Published, JBC Papers in Press, December 23, 2005, DOI 10.1074/jbc.M51187200

Thomas R. M. Barends1, Jolanda J. Polderman-Tijmes2, Peter A. Jekel1, Christopher Williams3, Gjalt Wybenga1, Dick B. Janssen3, and Bauke W. Dijkstra3
From the Laboratories of 8Biophysical Chemistry and 9Biochemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

The α-amino acid ester hydrolase (AEH) from Acetobacter turidans is a bacterial enzyme catalyzing the hydrolysis and synthesis of β-lactam antibiotics. The crystal structures of the native enzyme, both unliganded and in complex with the hydrolysis product D-phenylglycine are reported, as well as the structures of an inactive mutant (S205A) complexed with the substrate ampicillin, and an active site mutant (Y206A) with an increased tendency to catalyze antibiotic production rather than hydrolysis. The structure of the native enzyme shows an acyl binding pocket, in which D-phenylglycine binds, and an additional space that is large enough to accommodate the β-lactam moiety of an antibiotic. In the S205A mutant, ampicillin binds in this pocket in a non-productive manner, making extensive contacts with the side chain of Tyr112, which also participates in oxyanion hole formation. In the Y206A mutant, the Tyr112 side chain has moved with its hydroxyl group toward the catalytic serine. Because this changes the properties of the β-lactam binding site, this could explain the increased β-lactam transferase activity of this mutant.

Thirty years ago, several bacterial strains, such as Acetobacter turidans and Xanthomonas citri, were identified that were able to efficiently produce semi-synthetic β-lactam antibiotics from β-lactam nuclei produced by fermentation, and synthetic acyl compounds with an α-amino group (1). Important antibiotics with such acyl chains include cephalaxin, cephadroxil, ampicillin, and amoxicillin. Given the difficulties in preparing such antibiotics by chemical means (2), much effort has been put into harnessing the β-lactam antibiotic synthesizing activity of these bacteria for application in the industrial production of antibiotics. It appeared that this activity originated from enzymes preferentially hydrolyzing esters of α-amino acids, the α-amino acid ester hydrolases (AEHs) (3).

Because of its potential usefulness in antibiotic synthesis, the AEH from A. turidans has been studied extensively, and it was the first of its family for which the gene was cloned and overexpressed (4). The sequence showed a GXXYG active site motif (4), which is characteristic of serine hydrolases of the X-prolyl dipeptidyl aminopeptidase family (5). Labeling studies with a suicide inhibitor, sequence alignments, and site-directed mutagenesis identified a catalytic triad of Ser205, Asp338, and His370 in what was proposed to be a catalytic domain with an α/β-hydrolase fold (6).

Recently, the crystal structure of the X. citri AEH was solved (7). This enzyme shares 63% sequence identity with the A. turidans AEH. The structure showed a tetrameric arrangement of monomers consisting of three domains each: an α/β-hydrolase domain at the N terminus, a helical cap domain, and a C-terminal jellyroll fold domain. The active site indeed contained a Ser-His-Asp catalytic triad, the constituents of which were found in their canonical positions in the α/β-hydrolase domain. Furthermore, a putative oxyanion hole was found in which a negative charge could be stabilized by a backbone amide and a tyrosine hydroxyl group. Similar folds and arrangements of active site residues were observed for the homologous cocaine esterase CocE (8) and Lactococcus lactis X-prolyl dipeptidyl aminopeptidase PepX (9). A particularly striking feature of the Xanthomonas AEH active site was a cluster of three carboxylate residues, which are conserved among the AEHs and were proposed to bind the positively charged α-amino group of the substrate from which the AEHs draw their name (7).

The coupling of acyl side chains to β-lactams by AEHs most likely proceeds through enzyme acylation at the catalytic serine residue by an acyl compound, followed by transfer of this acyl group to the amino group of an amino-β-lactam, through “aminolysis” of the acyl enzyme (3, 4, 10, 11). However, the acyl enzyme can also be hydrolyzed, and the synthesized antibiotic can also serve as substrate and be cleaved, as indicated in Fig. 1. Thus, the hydrolysis of the ester and the product occur as undesired side reactions (3, 11–13). Therefore, the AEH-catalyzed synthesis of antibiotics requires a kinetically controlled scheme. In such a scheme, the enzyme is added to a mixture of the acyl donor, e.g. D-phenylglycine methyl ester, and the acyl acceptor, e.g. 6-aminopenicillanic acid. All three reactions (synthesis of the antibiotic, hydrolysis of the ester, and hydrolysis of the antibiotic) will occur simultaneously, but after a certain period of time, a maximum in the antibiotic concentration is reached, at which time the reaction is stopped and the product harvested. The kinetic parameters of the enzyme govern the maximum level of antibiotic accumulation $P_{\text{max}}$ in such a process (14). Of crucial importance is the ratio of the specificity constants of the enzyme toward the acyl donor and the antibiotic, α, which is defined as $(k_{\text{cat}}/K_m)_{\text{product}}/(k_{\text{cat}}/K_m)_{\text{acyl donor}}$. For favorable antibiotic yield, α needs to be as small as possible. Another important factor is the ratio of the rates of synthesis and hydrolysis, measured as the ratio of the initial rates, $V_s/V_i$. At a certain concentration of nucleophile, this ratio indicates the tendency of the acyl enzyme to undergo aminolysis rather than hydrolysis. For optimal antibiotic yield, $V_s/V_i$ needs to be maximal (14, 15). Kinetic work has shown that mutation of Tyr206 to alanine decreases the unwanted hydrolytic activity toward cephalaxin in the Acetobacter enzyme (6). The corresponding residue in the Xanthomonas AEH was proposed to contribute to transition state stabilization with its main chain atoms (7).
The Y206A mutant of Acetobacter AEH was further investigated in terms of its usefulness as a biocatalyst for antibiotic production (16).

The present work reports the structure of the AEH from A. turbidans, aimed at understanding the precise mode of action of this enzyme. In addition to the native structure, the structures of the complex with d-phenylglycine, the Y206A mutant, and the inactive S205A mutant complexed with the antibiotic ampicillin are reported, as are the effects of the Y206A mutation on the kinetic parameters.

The structures give insight into the catalytic residues, substrate binding, and the effects of mutations. In particular, the structure of the Y206A mutant helps to explain the higher ratio of the rates of synthesis and hydrolysis that this mutant displays.

**EXPERIMENTAL PROCEDURES**

**Protein Production**—Plasmids, bacterial strains, growth conditions, and purification methods for the production of native, S205A, and Y206A A. turbidans AEH with a C-terminal myc epitope and His tag were as described in Ref. 6. Briefly, proteins were produced in Escherichia coli TOP10 cells carrying constructs derived from pBAD. Cells were harvested at 14 °C for 4 days in LB medium with 100 μg/ml ampicillin and 0.01% (w/v) arabinose for induction.

**Crystal Preparation**—His-tagged native and mutant AEHs were purified from this lysate by metal ion affinity chromatography using nickel-nitrilotriacetic acid–agarose (Qiagen). A stepwise gradient of 50–200 mM imidazole in 150 mM NaCl, 50 mM sodium phosphate, pH 7.4, was used for elution. Pure protein eluted around 75–100 mM imidazole. Subsequently, the protein was desalted using gel filtration. Determination of the oligomeric state was carried out by gel filtration on a Superdex 200 column equilibrated with 50 mM sodium phosphate buffer, pH 6.2, containing 200 mM sodium chloride. Elution volumes were calibrated using Bio-Rad gel filtration markers.

**Determination of Kinetic Parameters**—For the determination of enzyme behavior in a cephalixin synthesis reaction, the enzymes were incubated with 15 mM d-phenylglycine methyl ester (d-PGM) and 30 mM β-lactam nucleus 7-amino-desacetoxycephalosporanic acid in 50 mM Na-phosphate buffer, pH 6.2, at 30 °C. Kinetic parameters for cephalixin hydrolysis were previously reported in Ref. 6 and are given here for reference. The synthesis of cephalixin and the hydrolysis of d-PGM were followed by high performance liquid chromatography as described before (4). Enzymes were used at a concentration that catalyzed the hydrolysis of d-PGM with an initial rate of 0.20 to 0.30 mm/min. To obtain the rate of enzymatic d-phenylglycine production, the observed rate of d-phenylglycine production was corrected for the first-order chemical hydrolysis of d-PGM. The relative rate of antibiotic formation versus hydrolysis of d-PGM (V/Vm) was determined from the initial slope of antibiotic formation divided by the rate of enzymatic formation of the hydrolysis product (d-phenylglycine). The rates were measured at less than 10% conversion of d-PGM to minimize the influence of product hydrolysis. The kinetic parameters K_m and k_cat were calculated using nonlinear regression fitting (Scientist, Micromath) with Michaelis-Menten and substrate inhibition kinetics. The calculated parameters are given with their standard deviations. The maximum product concentration (P_m) was determined by following the concentrations of cephalixin and d-phenylglycine over time, until the product concentration started to decrease. All measurements were at least performed in duplicate.

**Crystal Preparation**—Plasmids, bacterial strains, growth conditions, and purification methods for the production of native, S205A, and Y206A A. turbidans AEH with a C-terminal myc epitope and His tag were as described in Ref. 6. Briefly, proteins were produced in Escherichia coli TOP10 cells carrying constructs derived from pBAD. Cells were harvested at 14 °C for 4 days in LB medium with 100 μg/ml ampicillin and 0.01% (w/v) arabinose for induction. After harvesting and washing, cells were either sonicated or passed through a French press and a clear lysate was prepared by centrifugation. AEH and mutant AEHs were purified from this lysate by metal ion affinity chromatography using nickel-nitrilotriacetic acid–agarose (Qiagen). A stepwise gradient of 50–200 mM imidazole in 150 mM NaCl, 50 mM sodium phosphate, pH 7.4, was used for elution. Pure protein eluted around 75–100 mM imidazole. Subsequently, the protein was desalted using gel filtration. Determination of the oligomeric state was carried out by gel filtration on a Superdex 200 column equilibrated with 50 mM sodium phosphate buffer, pH 6.2, containing 200 mM sodium chloride. Elution volumes were calibrated using Bio-Rad gel filtration markers.

**Determination of Kinetic Parameters**—For the determination of enzyme behavior in a cephalixin synthesis reaction, the enzymes were incubated with 15 mM d-phenylglycine methyl ester (d-PGM) and 30 mM β-lactam nucleus 7-amino-desacetoxycephalosporanic acid in 50 mM Na-phosphate buffer, pH 6.2, at 30 °C. Kinetic parameters for cephalixin hydrolysis were previously reported in Ref. 6 and are given here for reference. The synthesis of cephalixin and the hydrolysis of d-PGM were followed by high performance liquid chromatography as described before (4). Enzymes were used at a concentration that catalyzed the hydrolysis of d-PGM with an initial rate of 0.20 to 0.30 mm/min. To obtain the rate of enzymatic d-phenylglycine production, the observed rate of d-phenylglycine production was corrected for the first-order chemical hydrolysis of d-PGM. The relative rate of antibiotic formation versus hydrolysis of d-PGM (V/Vm) was determined from the initial slope of antibiotic formation divided by the rate of enzymatic formation of the hydrolysis product (d-phenylglycine). The rates were measured at less than 10% conversion of d-PGM to minimize the influence of product hydrolysis. The kinetic parameters K_m and k_cat were calculated using nonlinear regression fitting (Scientist, Micromath) with Michaelis-Menten and substrate inhibition kinetics. The calculated parameters are given with their standard deviations. The maximum product concentration (P_m) was determined by following the concentrations of cephalixin and d-phenylglycine over time, until the product concentration started to decrease. All measurements were at least performed in duplicate.
TABLE 1
Data collection and refinement statistics
Where applicable, values for the highest resolution shell are given in parentheses.

Table 1

| Statistics                                      | WT   | WT-D-phenylglycine | S205A-ampicillin | Y206A |
|------------------------------------------------|------|--------------------|------------------|-------|
| Protein Data Bank code                         | 2B9V | 2B4K               | 1NX9             | 1RYY  |
| Wavelength (Å)                                 | 0.933| 0.934              | 0.8463           | 0.933 |
| Space group and cell dimensions                | $P_2_1$, $a = 98.4$, $b = 275.6$, $c = 197.0$ Å, $\beta = 90.1^\circ$ | $I23$, $a = b = c = 341.0$ Å, $\alpha = \beta = \gamma = 90^\circ$ | $I23$, $a = b = c = 341.8$ Å, $\alpha = \beta = \gamma = 90^\circ$ | $P_2_1$, $a = 91.6$, $b = 177.5$, $c = 170.0$ Å, $\beta = 91.0^\circ$ |
| Resolution range (Å)                           | 40–2.0| 40–3.0$^a$         | 99–2.2           | 40–2.8 |
| No. of unique reflections                      | 598,744 | 121,384           | 322,911          | 134,932 |
| Completeness (%)                               | 85.5 (67.8) | 93.2 (91.5)       | 97.2 (84.3)      | 99.4 (99.6) |
| Redundancy                                     | 2.8   | 2.6                | 4.5              | 3.2    |
| $R_{crys}$                                     | 0.091 (0.341) | 0.186 (0.917)     | 0.081 (0.322)    | 0.066 (0.184) |
| $I/\sigma$                                     | 6.6 (1.5) | 7.1 (2.8)         | 14.3 (3.7)       | 17.2 (6.9) |

Refinement statistics

| Residue range in refinement (Å)                 | 40–2.0$^a$ | 15–3.3            | 15–2.2           | 40–2.8 |
| No. reflections in refinement                   | 504,379    | 91,758            | 304,675          | 125,967 |
| Protein atoms                                   | 77,072     | 19,536            | 19,532           | 39,016 |
| Water molecules                                 | 3,654      | 6                 | 2,229            | 0      |
| Ligand atoms                                    | d-Phenylglycine, glycerol, 18 (3 molecules); Ampicillin, 96 (4 molecules); glycerol, 24 (4 molecules) |
| $R$-factor                                      | 0.199      | 0.258             | 0.166            | 0.253  |
| $R_{free}$                                      | 0.235      | 0.289             | 0.184            | 0.272  |

Ramachandran plot

| Bonds (Å)                                       | 0.008      | 0.009             | 0.007            | 0.009  |
| Angles (˚)                                       | 1.7        | 1.0               | 1.1              | 0.8    |

$^a$ Due to the high merging $R$-factor at 3.0-Å resolution, only data to 3.3-Å resolution were used for refinement.

same solution containing 25% glycerol, and cryocondensed in liquid nitrogen. Y206A crystals were subjected to the same procedure, substituting D-phenylglycine methyl ester for ampicillin. Crystals of a S205A mutant-ampicillin complex were grown by mixing 3 μL of protein solution with 3 μL of a reservoir solution containing 0.3 M sodium citrate buffer, pH 5.6, 13–14% PEG 4000, and 10 mM sodium ampicillin, followed by equilibration in hanging drops against 500 μL of this latter solution for 3–4 days. Rhombic dodecahedra with a size of 0.3 × 0.3 × 0.3 mm were harvested and cryocondensed after soaking for 30 s in reservoir solution containing 26% glycerol. WT-D-phenylglycine complex crystals were grown in the same way, substituting WT protein for the mutant and D-phenylglycine for ampicillin. All crystallization and soaking experiments were carried out at room temperature.

Data Collection and Structure Solution—Useful diffraction data from WT, WT-D-phenylglycine, and Y206A crystals were collected to 2.0-, 3.3-, and 2.8-Å resolution, respectively, on beam line ID14-4 at the ESRF in Grenoble, France. Diffraction data from crystals of the S205A mutant co-crystallized with ampicillin were collected to 2.2-Å resolution on the BW7B beam line of the EMBL outstation at the DESY synchrotron in Hamburg, Germany. All data were processed with DENZO and SCALEPACK (17). The structure of the A. turbidans AEH was solved by molecular replacement, using the program AMoRe (18) and the previously determined tetrameric structure of the X. citri AEH as a search model. The structure determination of the WT protein was hampered by twinning in combination with pseudo-crystallographic translation symmetry, and the structure elucidation process followed with this protein is further described in Ref. 19. Briefly, a high twinning fraction was observed, which made it impossible to detwin the data mathematically. Therefore, the twinning was idealized by averaging intensities of twin-related reflections (20) and the structure of the one twin domain, solved by molecular replacement, was used to obtain detwinned intensities for refinement. It should be noted, that because the measured data are relatively incomplete (85.5% completeness), not all twin-related reflection pairs could be averaged for lack of availability of one of the reflections. After averaging and detwining, this leads to the availability of only 75% of all possible reflections for refinement. However, this number of available reflections corresponds to the number of reflections that a 100% complete dataset of 2.2-Å resolution from this crystal would have, thus effectively limiting the resolution to ~2.2 Å. The twinning also results in the loss of independence of twin-related reflections, lowering the observation/parameter ratio further. However, there are 16 AEH monomers in the asymmetric unit (four AEH tetramers), enabling the use of tight 16-fold non-crystallographic symmetry restraints, which ensures that the refinement problem is sufficiently well determined.

RESULTS AND DISCUSSION

Native Structure: Oligomeric State—The structures of the WT, WT-D-phenylglycine, Y206A, and S205A AEH were successfully determined by x-ray crystallography. Despite different space groups and crystallization conditions, all structures showed the same tetrameric arrangement of monomers (Fig. 2a), which was also observed for the X.
The native structure of the *Acetobacter* AEH monomer (Fig. 2b) resembles that of the *Xanthomonas* AEH monomer. It can be superimposed with *Xanthomonas* AEH to within a root mean square difference of 4.5 Å. Like the *Xanthomonas* enzyme, it includes an N-terminal arm, an α/β-hydrolase domain with a helical cap domain, and a C-terminal jellyroll fold domain. The N-terminal arm consists of 24 residues that form an elongated polypeptide that interacts with the surface of another monomer. Residues 62–73, in the middle of the arm, could not be identified in the electron density map. These residues span the gap between the two monomers held together by the arm, and may thus be expected to be mobile, because they make no non-bonded contacts with either monomer. However, their approximate positions can be inferred from the short distance between the beginning and end of this gap and their better defined positions in the S205A mutant structure.

The arm is followed by a typical α/β-hydrolase fold domain, consisting of a highly twisted β-sheet flanked on both sides by α-helices (23–25). Apart from the second one, all strands in the sheet are parallel. Adopting the nomenclature in Ref. 23, the catalytic residues Ser205, His370, and Asp338 are found at their canonical positions on the loops between strand β5 and helix αC, between β6 and αD, and between β7 and αE, respectively. Compared with the classical α/β-hydrolase fold, which has eight β-strands in the central sheet, AEH displays two additional β-strands at the C terminus of the domain. Furthermore, several insertions are seen, most notably a predominantly helical cap domain between β6 and αD. Another insertion between β3 and αA contains the conserved Tyr112, which is in a position to contribute with its hydroxyl group to oxyanion stabilization during the reaction. The C-terminal domain adopts a jellyroll fold with several insertions, one of which contains a helix that approaches the active site as in the *X. citri* AEH.

**Active Site Structure**—The active site is found at the interface of the α/β-hydrolase, cap, and jellyroll domains. The catalytic Ser205 is located on the so-called nucleophile elbow between β5 and αC and has unfavorable main chain dihedrals as is usual in α/β-hydrolase family members (25). It is closely approached by His370, which in turn is hydrogen bonded to Asp338. Thus, a canonical Ser-His-Asp catalytic triad is formed. The backbone amide of Tyr206, which directly follows the catalytic serine in the sequence, and the phenolic OH of the Tyr112 side chain form an oxyanion hole similar to that observed in *X. citri* AEH, CocE, and PepX (7–9). As in these proteins, Tyr112 makes stacking-like interactions with the Pro111 ring, as does Tyr206 on the other side of the Pro111 side chain (figure 3a). The Tyr112 side chain is further restrained by a hydrogen bond with the Tyr206 side chain (not shown).

Close to the active serine, a pocket is present, delineated by residues stemming mostly from the cap domain. It is found in the position were the acyl chain would be expected in an acyl-enzyme compound. In a
Structures of A. turidans α-Amino Acid Ester Hydrolase

FIGURE 3. a, stereo figure showing the active site residues of A. turidans AEH. The catalytic triad and the residues forming the oxyanion hole are shown in stick representation. An asterisk indicates the expected position of the oxyanion hole. The $2F_o - F_i$ electron density map is overlaid on the structure at a 1.0-$σ$ contour level. b, stereo figure, showing $(F_o - F_i)$ difference electron density calculated with D-phenylglycine co-crystal data prior to including α-phenylglycine in the model, overlaid on the refined α-phenylglycine complex structure. The ammonium group of α-phenylglycine is in close proximity to the carboxylate cluster. c, stereo figure comparing the active site residues of WT (gray) and Y206A (blue) AEH. Transparent spheres indicate the van der Waals radii of the atoms of the interacting Tyr112 and Asn257 residues in the Y206A structure. The changes upon the mutation of Tyr to Ala are indicated with numbers: 1, mutation of Tyr to Ala; 2, loss of the hydrogen bond with Tyr; 3, movement of the Glu257 side chain; 4, van der Waals interactions of Glu257 side chain with Tyr112 side chain; 5, movement of Tyr112 side chain toward oxyanion hole; 6, possible hydrogen bond between Tyr112 Oγ and Ser205 Oγ. The figure was prepared using Xfit (22), BobScript (31), and Raster3D (32).

difference Fourier synthesis using data collected from an AEH crystal grown in the presence of D-phenylglycine, strong difference density was observed in three of four active sites of the tetramer, consistent with D-phenylglycine binding to this site, again pointing to this pocket being the acyl chain binding site (Fig. 3b). In the complex, the aromatic ring of phenylglycine makes stacking interactions with Tyr. On the other side of the catalytic serine, a much larger space is observed, which could accommodate a bulky leaving group during the enzyme acylation step of the reaction, and could bind a large acyl acceptor during the enzyme deacylation step.

Substrate Specificity—On one side of the putative acyl binding pocket, a cluster of acidic residues was observed (Fig. 3b). These residues (Asp239, Glu340, and Asp341) form the conserved cluster proposed to recognize the α-amino group of the substrate (7) and can be superimposed with the corresponding residues of the X. citri AEH to a root mean square deviation of 0.88 Å. In the difference electron density observed in the AEH-D-phenylglycine complex, D-phenylglycine could be placed with its ammonium group close to the acidic residues (Fig. 3b).

Interestingly, despite the presence of a canonical catalytic triad, compounds that lack an α-amino group are not normally converted. Also, AEH is not inhibited by the neutral reagent phenylmethanesulfonyl fluoride, but it is inhibited by p-nitrophenyl-p'-guanidinobenzoate (6), which carries a positive charge in solution at neutral pH. These observations can be explained in two ways. First, the binding of a neutral substrate would bury the three carboxylate groups of the cluster without neutralizing their negative charge, which would be energetically unfavorable. Second, the distance between the Asp239 Oδ and the catalytic Ser205 Oγ is only 3.5 Å. The close proximity of the negatively charged Asp239 Oδ would severely decrease the nucleophilicity of Ser205 Oγ by inhibiting its deprotonation by the catalytic His/Asp combination, thereby precluding the acylation reaction unless the charge of Asp239 is compensated by a positive amino group on the substrate. These effects would also explain why AEHs convert esters of phenylglycine, but are not inhibited by phenyl acetic acid, which differs from phenylglycine only in the absence of the α-amino group.

Effects of Mutations—Previously, several important residues in AEH were identified by site-directed mutagenesis. Specifically, the influence of mutation of the GXSXG consensus motif was investigated, which helped to identify the catalytic serine 205. Mutation of this residue almost completely abolished activity (4, 6). The crystal structure of the S205A mutant shows no appreciable differences in side or main chain conformations in the active site compared with the native enzyme. Thus, its inactivity is solely because of the loss of the Ser205 hydroxyl group.

As mentioned above, previous work identified Tyr206 as a candidate for mutation in the development of AEH mutants with improved biocatalytic properties. Kinetic parameters for the wild-type enzyme and the Y206A mutant were determined and are reported in Table 2. Compared with wild-type enzyme, the Y206A mutant shows a reduced $k_{cat}$ toward both cephalaxin and α-phenylglycine methyl ester. Furthermore, it displays a 3-fold increase in the ratio of the initial rates of
TABLE 2
Kinetic properties of WT and Y206A AEH

|                      | D-PGM hydrolysis | Cephalaxin hydrolysis (from Ref. 6) | Cephalaxin synthesis from d-PGM/7-ADCA* |
|----------------------|-------------------|-------------------------------------|----------------------------------------|
|                      | $k_{cat}$         | $K_m$                               | $k_{cat}/K_m$                          | $\alpha$ | $V/V_{max}^{ini}$ | $p_{max}$ |
|                      | $s^{-1}$          | $mM$                                | $s^{-1}$                                |           |                  |           |
| WT                   | 1067 ± 56         | 1 ± 0.1                             | 1067 ± 121                             | 0.6       | 2.4 ± 0.8        | 5.4 ± 0.1 |
| Y206A                | 655 ± 39          | 5.3 ± 0.9                           | 124 ± 22                               | 0.24      | 9 ± 2            | 8.6 ± 0.6 |

*7-ADCA, 7-amino-desacetoxycephalosporanic acid.

FIGURE 4. Stereo picture of the 2Fo - Fo electron density of ampicillin in the active site of the S205A mutant, after refinement of the initial molecular replacement solution, prior to inclusion of ampicillin in the model. The density was contoured at 1.0 $\sigma$, and overlaid on the final refined structure. The figure was prepared using Xfit (22) and Raster3D (32).

The explanation of the increase in the ratio of the initial rates of synthesis and hydrolysis ($V/V_{max}^{ini}$) during cephalaxin synthesis is less straightforward. Apparently, the mutation affects the interaction of the acyl enzyme with the competing nucleophilic acyl acceptors, water and the $\beta$-lactam nucleus, by altering the way in which the acyl enzyme is presented to an acyl acceptor, or the way in which the acceptor is bound and/or activated. Possibly, the geometry of the atoms around the scissile bond between enzyme and acyl group is changed to favor attack by a $\beta$-lactam rather than by water (26). An alternative explanation is that Tyr$^{112}$ participates directly in binding the acceptor, in which case a change in the position of Tyr$^{112}$ could affect both binding affinity and binding mode.

Indeed, the Tyr$^{112}$ phenol ring is part of the surface of the site where the $\beta$-lactam part of the product would be expected to bind. The involvement of Tyr$^{112}$ in $\beta$-lactam binding has been proposed for the X. citri AEH (7). Interestingly, in the homologous PepX (9), the corresponding residue is in the middle of a large hydrophobic patch on the surface of the active site, and could thus play a role in substrate binding in this case as well. The corresponding tyrosine in the homologous CocE has been proposed to interact with the leaving group part of the substrate in cocaine hydrolysis, too (27). Participation of this tyrosine residue in substrate binding would mean that it plays a dual role in that apart from oxanion stabilization through the $\beta$-lactam binding has been proposed for the.

The higher maximum product accumulation in antibiotic synthesis by the Y206A mutant has been attributed to a decrease in the affinity of the enzyme for the antibiotic (6, 16). This, too, could be caused by an altered positioning of Tyr$^{112}$ influencing its affinity for a $\beta$-lactam.
Structures of A. turbidans α-Amino Acid Ester Hydrolase

mer. The antibiotic predominantly makes hydrophobic contacts with Tyr112, both with its acyl chain and its β-lactam moiety (Fig. 4). Additional interactions are made with Tyr257, Ser371, Arg117, and Glu207. The carbon atom of the scissile amide bond is ~10 Å away from the Ala205 Cα, indicating that in the native protein, attack by Ser205 would not be possible in this binding mode. Perhaps, the non-productive binding mode of ampicillin in the AEH active site is caused by the high glycerol concentration used to cryoprotect the crystal. Electron density compatible with a glycerol molecule is observed in the putative acyl binding pocket, suggesting that the glycerol has displaced the ampicillin from its productive binding site into the position observed. Although ampicillin does not bind in a productive mode in the S205A structure, this result does show the propensity of Tyr112 for ligand binding.

Conclusions and Outlook—The structure determination of the A. turbidans α-amino acid ester hydrolase and of two mutants has resulted in a view of an active site that combines a classical catalytic triad with unusual elements like a cluster of acidic residues and an oxyanion hole with a tyrosine side chain contribution. In detail, the structures show unusual elements like a cluster of acidic residues and an oxyanion hole close to Ser371. In five of the 16 monomers in the wild-type structure, a platform for such a water molecule in the AEH structures yields a position to the acyl enzyme ester bond and the catalytic histidine. It is then activated by which it could be activated for attack. In the A. turbidans AEH S205A-ampicillin complex, this position is occupied by a water molecule is observed hydrogen bonded to the Ser371 side chain, close to the catalytic histidine Nε-2, by which it could be activated for attack. In the A. turbidans AEH S205A-ampicillin complex, this position is occupied by an oxygen atom of ampicillin. In the X. citri AEH structure, a water molecule was observed in the corresponding position, too. Identifying a hydrolytic water molecule in AEH might allow additional mutations to be made that reduce the hydrolysis rate and lead to even better catalytic properties.

Acknowledgments—We thank Dr. Jan-Metske van der Laan (DSM food specialties) for a fruitful cooperation and the ESRF in Grenoble and the EMBL outstation in Hamburg for synchrotron beam time. Drs. Ehrike Polli and Cris-tofer Enroth are gratefully acknowledged for assistance with data collection.

REFERENCES
1. Takahashi, T., Yamazaki, Y., Kato, K., and Isona, M. (1972) J. Am. Chem. Soc. 94, 4035–4037
2. Bruggink, A., Roos, E. C., and de Vroom, E. (1998) Org. Process Res. Dev. 2, 128–133
3. Takahashi, T., Yamazaki, Y., and Kato, K. (1974) Biochem. J. 137, 497–503
4. Polderman-Tijmes, J. J., Jekel, P. A., de Vries, E. J., van Merode, A. E. J., Floris, R., van der Laan, J. M., Sonke, T., and Janssen, D. B. (2002) Appl. Env. Microbiol. 68, 211–218
5. Chich, J.-F., Chapot-Chartier, M.-P., Ribadeau-Dumas, B., and Grépon, J.-C. (1992) FEMS Lett. 134, 139–142
6. Polderman-Tijmes, J. J., Jekel, P. A., Jeronimus-Stratingh, C. M., Bruins, A. P., van der Laan, J. M., Sonke, T., and Janssen, D. B. (2002) J. Biol. Chem. 277, 28474–28482
7. Barends, T. R. M., Polderman-Tijmes, J. J., Jekel, P. A., Hensgens, C. M. H., de Vries, E. J., Janssen, D. B., and Dijkstra, B. W. (2003) J. Biol. Chem. 278, 23076–23084
8. Larsen, N. A., Turner, J. M., Stevens, J., Rosser, S. J., Basran, A., Lerner, R. A., Bruce, N. C., and Wilson, I. A. (2001) Nat. Struct. Biol. 9, 17–21
9. Rigole, P., Mechin, I., Delage, M.-M., and Chich, J.-F. (2002) Struct. Fold. Des. 10, 1383–1394
10. Kato, K. (1980) Agric. Biol. Chem. 44, 1083–1088
11. Nam, D. H., Kim, C., and Ryu, D. D. Y. (1985) Biotech. Bioeng. 27, 953–960
12. Blinkovsky, A. M., and Markaryan, A. N. (1993) Enzyme Microb. Technol. 15, 965–973
13. Fernandez-Lafuente, R., Hernández-Jistiz, O., Mateo, C., Terreni, M., Alonso, J., García-López, J. L., Moreno, M. A., and Guisan, J. M. (2001) J. Mol. Catal. B. Enz. 11, 633–638
14. Youshko, M. I., Chilov, G. G., Schierbakova, T. A., and Svedas, V. K. (2002) Biochim. Biophys. Acta 1599, 134–140
15. Alkema, W. B. L. (2002) Faculty of Mathematics and Natural Sciences, Ph.D. thesis, pp. 142, University of Groningen, Groningen
16. van der Laan, J. M., Polderman-Tijmes, J. J., and Barends, T. R. M. (2002) International Patent Application WO 02/08611 A2
17. Otwinowski, Z., and Minor, W. (1997) Methods Enzymol. 276, 307–326
18. Navaza, J. (1994) Acta Crystallogr. Sect. A Crystallogr. 50, 157–163
19. Barends, T. R. M., and Dijkstra, B. W. (2003) Acta Crystallogr. Sect. D Biol. Crystallogr. 59, 2237–2241
20. Yeates, T. O. (1997) Methods Enzymol. 276, 344–358
21. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) Acta Crystallogr. Sect. D Biol. Crystallogr. 53, 240–255
22. McRee, D. E. (1999) J. Struct. Biol. 125, 156–165
23. Ollis, D. L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S. M., Harel, M., Remington, S. I., Silman, L., Schrag, I., Sussman, J. L., Vershueren, K. H. G., and Goldman, A. (1992) Protein Eng. 5, 197–211
24. Heinkheim, P., Goldman, A., Jeffries, C., and Ollis, D. L. (1999) Struct. Fold. Des. 7, R141–R146
25. Nardini, M., and Dijkstra, B. W. (1999) Curr. Opin. Struct. Biol. 9, 732–737
26. Alkema, W. B. L., Dijkhuis, A. J., de Vries, E., and Janssen, D. B. (2002) Eur. J. Biochem. 269, 2093–2100
27. Turner, J. M., Larsen, N. A., Bashan, A., Barbas, C. F., Bruce, N. C., Wilson, I. A., and Lerner, R. A. (2002) Biochemistry 41, 12297–12307
28. Singh, P. T., Smalas, A., Carty, R. P., Mangel, F. W., and Sweet, R. M. (1993) Science 261, 620–622
29. Topl, M., Värnai, P., Schofield, C., and Richards, W. G. (2002) Protein Struct. Funct. Genet. 47, 357–369
30. Kraulis, P. (1991) J. Appl. Crystallogr. 24, 946–950
31. Esnouf, R. M. (1997) J. Mol. Graph. 15, 133–138
32. Merritt, E. A., and Bacon, D. J. (1997) Methods Enzymol. 277, 505–524