Chapter 4

Analysis of a substrate specificity switch residue of cephalosporin acylase

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Residue Phe375 of cephalosporin acylase has been identified as one of the residues that is involved in substrate specificity. A complete mutational analysis was performed by substituting Phe375 with the 19 other amino acids and characterising all purified mutant enzymes. Several mutations cause a substrate specificity shift from the preferred substrate of the enzyme, glutaryl-7-ACA, towards the desired substrate, adipyl-7-ADCA. The catalytic efficiency ($k_{cat}/K_m$) of mutant SY-77$^{F375C}$ towards adipyl-7-ADCA was increased 6-fold with respect to the wild type enzyme, due to a strong decrease of $K_m$. The $k_{cat}$ of mutant SY-77$^{F375H}$ towards adipyl-7-ADCA was increased 2.4-fold. The mutational effects point at two possible mechanisms by which residue 375 accommodates the long side chain of adipyl-7-ADCA, either by a widening of a hydrophobic ring-like structure that positions the aliphatic part of the side chain of the substrate, or by hydrogen bonding to the carboxylate head of the side chain.

Introduction

Cephalosporin acylase is a crucial enzyme for the development of new enzymatic pathways leading to the production of semi-synthetic cephalosporins. 7-aminocephalosporanic acid (7-ACA) and 7-aminodesacetoxycephalosporanic acid (7-ADCA) are important intermediates in these pathways. Large quantities of these compounds are needed, but the traditional production routes are time-consuming, expensive and/or polluting [106]. An alternative route to obtain 7-ADCA or 7-ACA is the one-step enzymatic deacylation of either adipyl-7-ADCA [18] or cephalosporin C (CPC) [102]. However, currently there is no biocatalyst available that is capable of performing this task cost-efficiently [106]. A good starting point for the generation of such an adipyl acylase or CPC acylase could be *Pseudomonas* SY-77 glutaryl acylase [13,25], a cephalosporin acylase that is highly active on a similar compound glutaryl-7-ACA (Figure 1).

Crystallisation studies of cephalosporin acylase with and without glutaryl-7-ACA or glutarate showed that Phe375 is part of the substrate binding site of the enzyme [30,32,115]. In an unbiased approach using directed evolution of *Pseudomonas* SY-77 glutaryl acylase, mutant SY-77$^{F375L}$ was found as one of the mutants that significantly increases the activity of the enzyme on adipyl-7-ADCA [116]. However, directed evolution by the introduction of point mutations in the codon for Phe375 allows for the substitution by only six residues (Leu, Ile, Val, Cys, Tyr and Ser) and consequently explores a limited part of the sequence space at this position. Additionally, the mutants were selected on an improved hydrolysis of adipyl-7-ADCA analogues. Consequently, neutral and negative effects and effects on the hydrolysis of other β-lactam compounds that deepen the knowledge of the cephalosporin acylase-substrate interaction could not be observed. Therefore, it was decided to use a saturated mutagenesis approach to analyse the effects of all possible residues at position 375 on the hydrolysis of three β-lactam compounds.
Mutant enzymes with all natural amino acids at position 375 were overproduced and purified to determine their activities in the hydrolysis of glutaryl-7-ACA, adipyl-7-ADCA and CPC, and autocatalytic processing. Several mutations were shown to improve the kinetic parameters towards adipyl-7-ADCA. The highest increase in $k_{cat}$ was due to the incorporation of a histidine, whereas the largest decrease of the $K_m$ value was found for mutant SY-77F375C that also shows the highest increase in catalytic efficiency. The effects of all mutations on the kinetic parameters are discussed on the basis of the crystal structure of the enzyme, and allowed to elucidate the possible modes by which residues at position 375 can modify substrate specificity.

\[ \text{Compound} \quad n \quad R1 \quad R2 \]
- Glutaryl-7-ACA: 1, -H, -CH$_2$OCOCH$_3$
- Adipyl-7-ADCA: 2, -H, -CH$_3$
- CPC: 2, -NH$_2$, -CH$_2$OCOCH$_3$
- 7-ACA: -, -, -CH$_2$OCOCH$_3$
- 7-ADCA: -, -, -CH$_3$

Figure 1. The production of 7-ACA and 7-ADCA from β-lactam compounds.

Materials and methods

Chemicals
The β-lactam substrates glutaryl-7-ACA, adipyl-7-ADCA and CPC (as sodium salt) were gifts from DSM, The Netherlands. Fluorescamine was from Sigma, BugBuster from Novagen, 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium were from Duchefa, The Netherlands.

Mutagenesis of residue Phe375 of Pseudomonas SY-77 glutaryl acylase
Mutagenesis was performed on plasmid pMcSY-2 [116] harbouring the gene encoding Pseudomonas SY-77 glutaryl acylase under control of the tac-promoter. The random primer 5′-atg cag gtg ccg acc nng/c aac aac gtc rac gec g was used to mutate the codon for Phe375 in a megaprimer reaction with Pfu DNA polymerase (Stratagene) [42]. The resulting PCR product was ligated into pMcSY-2 by digestion using the BgII and SsrI restriction sites. After transformation into Escherichia coli DH10B, cells were plated on LB-agar plates containing 50 µg/ml chloramphenicol. Single colonies were picked for DNA sequence determination. Nine different mutants were obtained using this method, the remaining ten were produced by PCR using the site directed version of the same primer, in which nng/c was replaced with the codon for the desired residue.

A test was conducted to confirm that the transformants were still able to produce acylase. 10 ml 2*YT medium [41] supplemented with 50 µg/ml chloramphenicol and 0.1% glycerol was
inoculated with 0.1 ml of an overnight culture, and grown at 30ºC for 24 h. Cells were harvested from 5 ml of this culture and lysed with the non-ionic detergent BugBuster \[116\]. The soluble fraction was spotted on a nitrocellulose membrane, which was subsequently incubated with a polyclonal rabbit antibody against purified \textit{Pseudomonas} SY-77 glutaryl acylase (Eurogentec S.A.) and an alkaline phosphatase-conjugated goat anti-rabbit antibody. The appearance of purple spots after incubation with 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium confirmed the presence of acylase enzyme in the sample. Transformants that did not produce acylase were discarded.

\section*{Characterisation of mutant enzymes

Mutant and wild type enzymes were produced in \textit{E. coli} DH10B in 100 ml cultures and purified by anion exchange and hydrophobic interaction column chromatography as described earlier \[116\]. The protein concentration in the purified samples was determined using the DC protein assay (Bio-Rad) with BSA as the reference protein. Analysis of the samples by SDS-PAGE \[110\] was done using a 12.5% gel, which was stained with Coomassie Brilliant Blue. The kinetic parameters $k_{\text{cat}}$, $K_m$ and $k_{\text{cat}}/K_m$ of mutant and wild type enzyme towards glutaryl-7-ACA and adipyl-7-ADCA were determined using the automated fluorescamine assay in 96-well format as described earlier \[116\]. A concentration range of the $\beta$-lactam substrates was incubated with a fixed amount of enzyme in 20 mM phosphate buffer pH 7.5 at 37ºC. The reaction was stopped by the addition of acetate buffer pH 4.5, and the amount of product 7-A(D)CA formed was determined by incubation with fluorescamine and measuring the absorption at 380 nm. The kinetic parameters $k_{\text{cat}}$ and $K_m$ were calculated from the rates of reaction at different concentrations of substrate. Activity towards CPC was determined by the fluorescamine assay at a high concentration of substrate \[116\], using approx. 20 µg of purified enzyme and 5 mM CPC in 200 µl of reaction mixture. Aliquots were taken after 24 and 48 h incubation at 37ºC and incubated with fluorescamine to determine whether CPC had been deacylated.

\section*{Results

\subsection*{Production and autocatalytic processing of mutant enzymes

All 19 mutant enzymes and wild type could be expressed and purified from \textit{E. coli} DH10B at a yield of more than 10 mg/l culture. On SDS-PAGE (Figure 2), all purified mutants displayed the bands corresponding to the mature $\alpha$- and $\beta$-subunits, with the exception of mutant SY-77$^{F375P}$, which showed only the band corresponding to the unprocessed propeptide consisting of the $\alpha$-subunit, spacer peptide and the $\beta$-subunit. Mutants SY-77$^{F375I}$, SY-77$^{F375R}$, SY-77$^{F375T}$ and SY-77$^{F375V}$ showed a band corresponding to the unprocessed propeptide in addition to the $\alpha$ and $\beta$-subunits, indicating that they underwent partial processing. An impaired processing could also have led to the accumulation of the polypeptide consisting of $\alpha$-subunit plus spacer peptide, but no band corresponding to this polypeptide was seen in any of the samples. Remarkably, the elution profiles of all mutants were similar to that of wild type enzyme in all column chromatography steps, indicating that the folding of the matured and the non-matured enzymes is very similar, which has also been observed for the S199A mutant of cephalosporin acylase \[118\].
A substrate specificity switch of cephalosporin acylase

Figure 2. Maturation of wild type and mutant cephalosporin acylases.
Cephalosporin acylase is produced as a propeptide consisting of a signal sequence, α-subunit, spacer peptide and β-subunit. Impairment of the first maturation step leads to the accumulation of propeptide. Impairment of the second maturation step would lead to accumulation of α-subunit + spacer peptide, visible as a band just above the band corresponding to the α-subunit. Samples were incubated at 100°C for 2 min; each lane contains 3 µg purified protein. The residues in which Phe375 has been mutated are indicated by the one letter code.
Ma = Marker proteins (Bio-Rad); α = α-subunit; β = β-subunit; pp = propeptide.

Hydrolysis of glutaryl-7-ACA
None of the mutations of residue 375 improved the kinetic parameters towards the preferred substrate of the enzyme, glutaryl-7-ACA (Table 1 and Figure 3). The best mutation was F375C, which lowered the catalytic efficiency to about 50% of the value of wild type due to a decrease of $k_{cat}$. SY-77F375C was the only mutant that did not significantly increase the $K_m$ towards glutaryl-7-ACA. The other sulphur residue Met caused a comparable decrease of $k_{cat}$ accompanied by an 5-fold increase of $K_m$. The substitution of Phe by Tyr lowered $k_{cat}$ only marginally, but it did cause a 2.5-fold increase of $K_m$. A similar effect was caused by the F375S mutation, but the other hydroxyl residue Thr had a stronger negative effect on the kinetic parameters. With regard to the aliphatic residues, the smaller residues Gly and Ala caused a less drastic decline of kinetic parameters than the larger residues Val, Leu and Ile. The introduction of all other residues resulted in a drastic decrease of catalytic efficiency.
Hydrolysis of adipyl-7-ADCA

All mutants showed unique kinetic parameters on the desired substrate adipyl-7-ADCA. Five mutants showed an increased catalytic efficiency, three a catalytic efficiency similar to wild type, and eleven a decreased catalytic efficiency (Table 1 and Figure 3). The catalytic efficiency towards adipyl-7-ADCA was increased 6-fold by the introduction of a Cys at position 375, mainly due to a strong reduction of $K_m$. The other sulphur-containing residue, Met, increased the catalytic efficiency by a factor of 2 by slightly increasing $k_{cat}$ and decreasing $K_m$. The hydrophilic residues Asn, Gln and His all increased $k_{cat}$. The catalytic efficiency was increased more than 2-fold by the introduction of Asn and His but not of Gln. In contrast to Asn, Gln and His, the hydrophilic hydroxyl residues Ser and Thr significantly lowered $k_{cat}$ and did not affect $K_m$. The aliphatic residue Leu increased the catalytic efficiency almost 2-fold by increasing $k_{cat}$ and decreasing $K_m$. The other aliphatic residues Gly, Ala, Val and Ile all decreased $k_{cat}$, and only the smallest residues, Gly and Ala, resulted in a lower $K_m$. The aromatic residue Tyr did not change the kinetic parameters significantly, whereas Trp caused a drastic decrease of $k_{cat}$ accompanied by an increase of $K_m$. Finally, the introduction of a charged residue, e.g. Arg, Asp, Glu or Lys, lowered the catalytic efficiency to less than 3% of wild type due to both a strong decrease of $k_{cat}$ and a significant increase of $K_m$.

Table 1. Kinetic parameters of wild type and mutant cephalosporin acylases.
The kinetic parameters were calculated from Eadie-Hofstee plots. Values given are mean ± S.D. of at least three independent measurements. N.D., not detectable.

<table>
<thead>
<tr>
<th>Residue</th>
<th>adipyl-7-ADCA</th>
<th>glutaryl-7-ACA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>$K_m$ (mM)</td>
</tr>
<tr>
<td>Phe (WT)</td>
<td>0.49 ± 0.04</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Ala</td>
<td>0.20 ± 0.01</td>
<td>0.51 ± 0.08</td>
</tr>
<tr>
<td>Arg</td>
<td>0.018 ± 0.002</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>Asn</td>
<td>0.9 ± 0.1</td>
<td>0.83 ± 0.09</td>
</tr>
<tr>
<td>Asp</td>
<td>0.015 ± 0.004</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>Cys</td>
<td>0.55 ± 0.03</td>
<td>0.17 ± 0.03</td>
</tr>
<tr>
<td>Glu</td>
<td>0.08 ± 0.02</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>Gln</td>
<td>0.68 ± 0.05</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>Gly</td>
<td>0.073 ± 0.004</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>His</td>
<td>1.16 ± 0.08</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>Ile</td>
<td>0.097 ± 0.003</td>
<td>2.07 ± 0.03</td>
</tr>
<tr>
<td>Leu</td>
<td>0.67 ± 0.04</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>Lys</td>
<td>0.036 ± 0.001</td>
<td>2.16 ± 0.07</td>
</tr>
<tr>
<td>Met</td>
<td>0.65 ± 0.02</td>
<td>0.65 ± 0.04</td>
</tr>
<tr>
<td>Pro</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Ser</td>
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<td>0.99 ± 0.09</td>
</tr>
<tr>
<td>Thr</td>
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</tr>
<tr>
<td>Trp</td>
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</tr>
<tr>
<td>Tyr</td>
<td>0.43 ± 0.05</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>Val</td>
<td>0.090 ± 0.003</td>
<td>1.37 ± 0.07</td>
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</table>
Figure 3. Catalytic efficiency of wild type and mutant cephalosporin acylases. The kinetic parameters were calculated from Eadie-Hofstee plots. Values given are mean ± S.D. of at least three independent measurements.

Hydrolysis of CPC
Deacylation of CPC by neither wild type Pseudomonas SY-77 glutaryl acylase nor any of the mutants could be detected by the fluorescamine assay under the described conditions, in spite of using over three times more enzyme and a 10-fold longer incubation period than in any of the assays using adipyl-7-ADCA and glutaryl-7-ACA (data not shown).
Discussion

In order to investigate the function of position 375 of cephalosporin acylase all 19 mutants of Phe375 were expressed in *E. coli* and purified by column chromatography. Remarkably, all mutants could be produced in high quantities and showed distinctive kinetic parameters on the desired substrate and preferred substrate of the enzyme, adipyl-7-ADCA and glutaryl-7-ACA, respectively. This points at the importance of Phe375 for substrate specificity.

Some mutants did not mature efficiently. The current model for the autocatalytic processing of cephalosporin acylase comprises an intramolecular cleavage followed by an intermolecular cleavage. The side chain binding pocket of the active site is thought to be involved only in the intermolecular cleavage [118,119]. Surprisingly, however, mutagenesis of position 375, which is part of the side chain binding pocket, does not affect the intermolecular cleavage but can affect the intramolecular cleavage. No band corresponding to the polypeptide consisting of α-subunit plus spacer peptide was visible on SDS-PAGE, but five mutants showed a band corresponding to the precursor consisting of α-subunit, spacer peptide and the β-subunit (Figure 2). Additionally, the hydrolysis activity towards glutaryl-7-ACA of the mutants (Table 1) is not related to the capability to perform autocatalytic processing. Our results thus show that intermolecular processing and catalytic activity are not strictly linked.

Until now, the recognised preferred substrate of cephalosporin acylase is glutaryl-7-ACA. It is therefore not surprising that none of the mutations resulted in an improvement of the kinetic parameters using glutaryl-7-ACA, and most mutations resulted in a decline of the kinetic parameters. The hydrolysis of β-lactam substrates by β-lactam acylases proceeds via the formation of an acyl-enzyme intermediate, which is subsequently hydrolysed. In the case of *E. coli* penicillin G acylase, which active site is very similar to that of cephalosporin acylase [30], it has been indicated that the acylation reaction is the rate-limiting step [120]. Assuming this also to be applicable to cephalosporin acylase, $k_{cat}$ would represent the rate of acylation, and $K_m$ the binding of substrate in the active site. In the crystal structure of the enzyme complexed with glutaryl-7-ACA the side chain of the substrate is held in place by various interactions in order to properly position the scissile bond for a nucleophilic attack by Ser199 and form the acyl-enzyme intermediate [32]. The aliphatic part of the side chain protrudes through a ring-like structure of hydrophobic residues consisting of Leu222, Val268 and Phe375, and the carboxylate head of the side chain is positioned in a hydrophilic cavity by electrostatic interactions with Arg255 and hydrogen bonds with Tyr178 and Tyr231. The aromatic ring of Phe375 is positioned alongside the glutaryl side chain (Figure 4).

This may explain why the incorporation of the small hydrophobic residues Gly, Ala and Cys causes the smallest negative effects on the kinetic parameters towards glutaryl-7-ACA. The incorporation of some polarity can be overcome, as is demonstrated by the relatively small effects of the F375Y and F375S mutations, but the incorporation of residues with larger side chains results in a poor hydrolysis activity, probably due to
stERIC hindrance of the side chain.
In order to shift the substrate specificity of the enzyme towards the desired substrate adipyl-7-ADCA, the longer adipyl side chain must be accommodated by the side chain binding pocket while the scissile bond is maintained in a favourable position with respect to Ser199. Two strategies may be used: the hydrophobic ring has to be widened in order to accommodate a twisted aliphatic chain, or the carboxylate head has to be pulled further into the side chain binding pocket, which was proposed to be the mechanism of the improved hydrolysis of adipyl-7-ADCA by mutant SY-77Y178H [28]

The slightly different fit of glutaryl-7-ACA and glutarate in the substrate binding site [32] and the alkylation of Trp202 by substrate analogue[121] indicate that some flexibility in the binding of substrates exists, a prerequisite for these two strategies.

Depending on the nature of the residue that substitutes Phe375 either mechanism may apply. The substitution of Phe375 by the smaller hydrophobic residues Cys, Leu and Met will expand the hydrophobic ring and generate extra space for the longer side chain. Apparently, this is a very delicate procedure, since Val and Ile have a negative influence on the kinetic parameters. The data suggest that a greater expansion of the ring, e.g. via substituting Phe375 by Gly or Ala, increases the ability of the side chain binding pocket to accommodate the adipyl side chain, as is indicated by a strong decrease of K_m. At the same time, the lower k_cat values suggest that the positioning of the scissile bond for hydrolysis is less optimal in these mutants.

As for the second mechanism, the increase of k_cat upon substituting Phe375 by the hydrogen-bond donors Asn, Gln or His indicates that position 375 can also be used to pull the adipyl side chain further into the side chain binding pocket by means of direct or indirect hydrogen-bonding to the carboxylate head. Again, the structural alterations seem to be very delicate, since the potentially hydrogen bonding Ser and Thr have a negative effect on the k_cat, and substitution by Tyr, which is basically a phenylalanine with a hydrogen bonding hydroxyl group, does not alter the kinetic parameters. The effects of these mutations on the hydrolysis of glutaryl-7-ACA are different and suggest that the carboxylate head of the glutaryl side chain cannot use hydrogen bonding to residue 375 to increase binding and hydrolysis. Apparently, the shorter length of the side chain limits the degrees of freedom and thereby prohibits alternative binding modes.

The introduction of a charged residue (Arg, Lys, Glu, Asp) or the bulky Trp at position 375 seems to block the passage of the charged carboxylate head of the side chain through the hydrophobic ring and results in very poor catalytic parameters towards both adipyl-7-ADCA and glutaryl-7-ACA. Surprisingly, most of these mutations do not impair the autocatalytic processing of the enzyme, another indication that processing and catalytic activity are not as closely linked as has been suggested by others.

This study shows that hydrolysis of adipyl-7-ADCA can be increased by improving the hydrophobic interactions between amino acid 375 and the aliphatic side chain of the substrate, as well as by hydrogen bonding of residue 375 to the carboxylate head of the substrate. Furthermore, residue 375 can function as a gatekeeper by blocking passage
through the hydrophobic ring of the substrate binding site by steric or electrostatic means. These results demonstrate that residue 375 is a key amino acid in the protein engineering of cephalosporin acylase. The saturation mutagenesis, in which the effects of all 20 amino acids at this position were analysed, was essential for the discovery of the three different modes by which residue 375 can dictate substrate specificity. Although the catalytic efficiency for adipyl-7-ADCA can be improved 6-fold by mutagenesis of Phe375, no activity on CPC could be detected. Apparently, the amino moiety in the side chain of CPC still comprises an insurmountable problem for an efficient hydrolysis, and additional mutations are required. Mutation F375C decreases the $K_m$ towards adipyl-7-ADCA by a factor six while the $K_m$ towards glutaryl-7-ACA remains identical, indicating that the substrate specificity of this mutant has been extended rather than shifted. Mutant SY-77$^{F375C}$ may thus be regarded as the template of choice for future mutagenesis studies.

Figure 4. Three-dimensional model of the binding of the side chain of glutaryl-7-ACA by cephalosporin acylase. 
Residues Leu222, Val268 and Phe375 (green) form a hydrophobic ring-like structure, through which the aliphatic part of the side chain of glutaryl-7-ACA (gold) protrudes, placing the carboxylate head of the side chain in a hydrophilic cavity consisting of Tyr178, Tyr231, Gln248 and Arg255 (cyan). Ser 199 (magenta) performs the nucleophilic attack on the scissile bond of the substrate. The Van der Waals radii of the residues forming the hydrophobic ring are shown in dots. Phe375 interacts with C4 of the side chain and pushes the hydrophilic head into the cavity (Figure made with RasTop version 2.0.3, www.geneinfinity.org/rastop/, using the co-ordinates of PDB entry 1JVZ).

Acknowledgements

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