Chapter 2

Altering the Substrate Specificity of Cephalosporin Acylase by Directed Evolution of the β-Subunit

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Using directed evolution, we have selected an adipyl acylase enzyme that can be used for a one-step bioconversion of adipyl-7-aminodesacetoxycephalosporanic acid (adipyl-7-ADCA) to 7-ADCA, an important compound for the synthesis of semi-synthetic cephalosporins. The starting point for the directed evolution was the glutaryl acylase from *Pseudomonas* SY-77. The gene fragment encoding the β-subunit was divided into 5 overlapping parts that were mutagenised separately using error-prone PCR. Mutants were selected in a leucine-deficient host using adipyl-leucine as the sole leucine source. In total, 24 out of 41 plate-selected mutants were found to have a significantly improved ratio of adipyl-7-ADCA versus glutaryl-7-ACA hydrolysis. Several mutations around the substrate-binding site were isolated especially in two hotspot positions: residue Phe375 and Asn266. Five mutants were further characterised by determination of their Michaelis-Menten parameters. Strikingly, mutant SY-77^{N266H} shows a nearly 10-fold improved catalytic efficiency (k_{cat}/K_m) on adipyl-7-ADCA, resulting from a 50 % increase in k_{cat} and a 6-fold decrease in K_m, without decreasing the catalytic efficiency on glutaryl-7-ACA. In contrast, the improved adipyl/glutaryl activity ratio of mutant SY-77^{F375L} mainly is a consequence of a decreased catalytic efficiency towards glutaryl-7-ACA. These results are discussed in the light of a structural model of SY-77 glutaryl acylase.

Introduction

Semi-synthetic cephalosporins and penicillins are the most widely used antibiotics. All clinically important semi-synthetic cephalosporins are made from 7-amino-cephalosporanic acid (7-ACA) or 7-aminodesacetoxycephalosporanic acid (7-ADCA). 7-ACA is derived from cephalosporin C (aminoadipyl-7-ACA), which is obtained by fermentation of the fungus *Cephalosporium acremonium*. Deacylation is performed either chemically or by a two-step enzymatic process using a D-amino acid oxidase and a glutaryl acylase. The latter enzyme can be found in several *Pseudomonas* and *Acinetobacter* species [13,26,27,100-103] as well as in some Gram-positive bacteria [104,105]. 7-ADCA is produced from penicillin G made by *Penicillium chrysogenum* involving several polluting chemical steps followed by enzymatic deacylation by penicillin acylase [106]. A first step towards the introduction of a simplified, more environmentally friendly production of 7-ADCA was the development of a genetically modified *P. chrysogenum* that produces adipyl-7-ADCA [18]. For the deacylation of this novel β-lactam, an adipyl acylase is needed. Since the presently identified acylases show little or no activity towards adipyl-7-ADCA, it is of interest to investigate whether a glutaryl acylase can be converted into an adipyl acylase.

In the past few years, directed evolution has been successfully implemented in changing the substrate specificity of several enzymes [107,108], resulting in biocatalysts with novel activities. It has become clear that the success of a directed evolution experiment greatly

depends on the availability of a good selective substrate, which unfortunately is absent for most bioconversions [107]. Artificial substrates that mimic one of the desired catalytic steps may be used for selection; however, it is not clear to what extent the resulting mutants will have lost activity on their natural substrate. Here we describe a strategy to evolve the glutaryl acylase of *Pseudomonas* SY-77 into an adipyl acylase with an improved activity toward adipyl-7-ADCA. The glutaryl acylase from *Pseudomonas* SY-77 has proven to be particularly suitable for developing an industrial process for deacylation [14]. The natural action of the enzyme seems to be directed at hydrolysing di-amino acids with a glutaryl side chain as judged from its high activity on glutaryl-7-A(D)CA. It appears that the enzyme also has a low activity on adipyl-7-ADCA but no activity on cephalosporin C [13].

Since deacylation of β-lactam compounds cannot be used for a growth selection, we took advantage of the di-amino hydrolysing capability of the acylase by replacing the β-lactam moiety with leucine, a compound that can be selected for in a leucine-deficient *Escherichia coli* host strain [109]. In this way, only enzymatic hydrolysis of adipyl-leucine allows for growth on minimal medium. To obtain the desired acylase variant, we have constructed five libraries of overlapping gene fragments of the β-subunit of SY-77 by error-prone PCR (epPCR) and used these libraries separately in the selection procedure. Transformants that prevailed in growth were further characterised and tested for their activity on glutaryl-7-ACA and adipyl-7-ADCA. Mutants with an improved growth capability on the selection substrate also showed an improved activity toward the β-lactam substrate. Mutations were found to accumulate in the proximity of the substrate binding pocket. Frequent mutations were identified at positions Asn266 and Phe375. Crystallographic models have pointed at the role of Phe375 in the determination of substrate specificity, whereas Asn266 was not mentioned before. Strikingly, mutation N266H induces an 8-fold improved catalytic efficiency (k_{cat}/K_m) on adipyl-7-ADCA. The results are discussed on the basis of the three-dimensional structure of SY-77 glutaryl acylase.

Materials and Methods

Bacterial strains and plasmids and DNA manipulations

The plasmid pMcSY-2 was constructed by the introduction of four silent mutations into pMcSY-77 (GenBankTM accession number AF458663) [28], resulting in four additional restriction sites in the gene encoding the SY-77 acylase. The plasmid pMcSY-2 and the leucine-deficient *E. coli* DH10B (Invitrogen) were used for the cloning of the libraries and the expression of the SY-77 acylase. The plasmid pMcSY-YH [28] was used as a positive control for the selection and screening procedure. This plasmid encodes a mutant acylase, carrying the mutation Y178H in the α-subunit, which has a three times better catalytic efficiency (k_{cat}/K_m) than wild type in the hydrolysis of adipyl-7-ADCA.

Molecular DNA techniques were executed following standard protocols [41]. The enzymes used for DNA manipulations were purchased from New England Biolabs and Invitrogen and applied

according to the instructions of the manufacturer. Plasmid DNA was isolated using the Qiaprep Spin Miniprep Kit (Qiagen). DNA was extracted from agarose gels using the QIAquick Gel Extraction Kit (Qiagen).

Interesting multiple mutants, which were found, were separated in order to pinpoint the mutation responsible for the improved enzymatic activity. In case a suitable restriction site was available, this was used to separate the mutations. Otherwise, single mutants were made by site-directed mutagenesis using PCR. In both cases, the mutated parts of the genes were sequenced on both strands afterwards.

Construction of the random mutant libraries

For the epPCR, the gene fragment encoding the β-subunit of the SY-77 acylase was divided into five partly overlapping regions using seven unique sites in the plasmid (Figure 1). Each region was mutagenised by PCR amplification under error-prone conditions [50]. The concentration of Mg^{2+} and the annealing temperature that gave the highest PCR yield were used. Using these conditions, a pilot experiment on part I was performed in order to determine the mutation rate and distribution under different concentrations of Mn^{2+}.
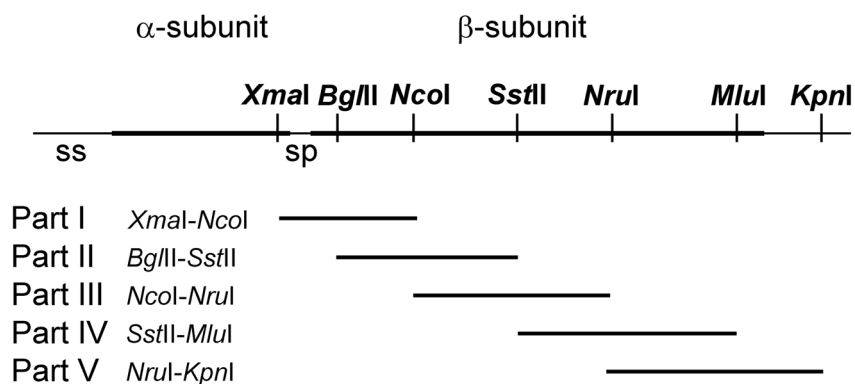

Figure 1. Linear view of the SY-77 acylase gene.
The five parts subjected to epPCR are given below the gene. Unique restriction enzymes used to clone the epPCR parts back into the gene are given above the gene. ss, signal sequence; sp, spacer peptide.

The PCRs were performed in aliquots of 25 μl containing 1x PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 2.5 or 5 mM $MgCl_2$, 0.1–0.5 mM $MnCl_2$, 0.2 mM dATP and dGTP, 1 mM dCTP and dTTP, 1 ng of pMcSY-2, 1.25 U recombinant *Taq* DNA polymerase (Invitrogen), and 0.3 μM of each primer. The following primers (from 5' to 3' including underlined restriction sites) were used: part I, tcgcatcgcccgggcgca and ctcgaagaccgggccatgga; part II, cgccggacttcgagatcta and gattggtgacccgcggca; part III, ccgctccagcgtccatggc and tgtagctggtgaactcgcgat; part IV, ggacgatctgccgcgggt and tcggctcgcgacacgcgtt; part V, gcggcgtgggattcgcgag and cagagcgcccggtaccgaaa.

All PCRs were performed in a Mastercycler® gradient thermocycler (Eppendorf) using the following program: 3 min at 95°C, 20 cycles of 45 s at 95°C, 30 s at the annealing temperature, 45 s at 72°C, and a final step of 10 min at 72°C. PCR product and wild type vector were subjected

to a restriction reaction using the suitable enzymes followed by electrophoresis. After the product of the correct size was excised and purified, ligation was performed overnight at 4°C using T4 DNA polymerase (New England Biolabs). 40 µl of ElectroMAX™ DH10B™ Cells (Invitrogen) were transformed with 1 µl of each mutant library, following the protocol of the manufacturer. A 0.1-cm cuvette was used in a Bio-Rad Gene Pulser unit, which was set at 1.8 kV, 25 µF, 200 ohms. A small aliquot of the transformation mixture was plated onto LB agar containing 50 µg/ml chloramphenicol and 0.4% glucose to determine the transformation efficiency. The rest of the transformation mixture was shock-frozen in liquid nitrogen in 10% glycerol and stored at -80°C. Of each library, 20 clones were picked randomly for the analysis of the mutational efficiency.

Selection on agar plates

Cells were slowly thawed on ice and washed twice with 0.9% NaCl. They were starved by incubation in 0.9% NaCl for 2 h at 37°C and plated onto selective minimal medium plates AD-Leu (M9; 50 µg/ml chloramphenicol, 0.1% glycerol, and as a sole leucine source 0.1 mg/ml adipyl-leucine; LGSS Transferbureau Nijmegen, The Netherlands). Approximately 5000 viable *E. coli* DH10B cells containing the mutant plasmids were spread onto each plate. Of each library, a total of 10^5 transformants was plated. Cells were also plated onto control plates: GL-Leu (0.248 mg/ml glutaryl-leucine.2DCHA; Bachem), MLeu (0.04 mg/ml L-leucine; Sigma), and Min (no leucine at all). As a "positive" and a "negative" control, *E. coli* DH10B cells producing SY-77^{Y178H} and SY-77 wild type, respectively, were used. Plates were incubated at 30°C for at least 10 days. Every day new appearing colonies were marked. Colonies were selected on the basis of their date of appearance and size and streaked again on AD-Leu plates to ensure that unique colonies were obtained. Single colonies from these second plates were used to inoculate an overnight culture to determine acylase activity and to isolate plasmid DNA for sequence determination.

Determination of enzyme activity

10 ml of LB containing 50 µg/ml chloramphenicol and 0.1% glycerol was inoculated with 0.1 ml of an overnight culture and incubated at 30°C for 24 h. Cells were harvested (20 min, 3500 rpm) and incubated for 45 min in one-fifth volume of BugBuster (Novagen) at 20°C while vigorously shaking. Cell debris was removed by centrifugation (30 min, 13,000 rpm), and cell free extracts were stored at -20°C.

Enzyme activities were determined on glutaryl-7-ACA and adipyl-7-ADCA using the fluorescamine assay [99] in a 96-well format. All pipetting steps were performed by a Multiprobe II (Canberra Packard). 8 µl of cell free extract was mixed with 92 µl of 20 mM phosphate buffer, pH 7.5, and preheated at 37°C. The reaction was started by the addition of 100 µl of substrate in phosphate buffer, with a final concentration of 2 mM glutaryl-7-ACA or 5 mM adipyl-7-ADCA. After 60 and 120 min incubation at 37°C, an aliquot of 40 µl of reaction mixture was transferred to 140 µl of 0.5 M acetate buffer, pH 4.5, after which 20 µl of 1 mg/ml fluorescamine in acetone was added. After 60 min incubation at room temperature, the A_{380} was measured. Reaction mixtures without substrate or cell free extract were used to correct for absorption of the different reaction mixture components at 380 nm. Activities are given as the ratio between the hydrolysis of adipyl-7-ADCA and glutaryl-7-ACA (AD/GL) to account for variations in acylase concentration in the cell free extracts. The ratios were determined after 60 and 120 min of incubation. Values were accepted if they were consistent, and the average of these two ratios was used in further calculations. The mean and the S.D. of the ratio found in at least three independent

measurements were calculated.

Mutants with an AD/GL ratio of at least 150% of that of the wild type SY-77 acylase were purified as described below. The kinetic parameters of these mutants were determined by measuring the initial rate of hydrolysis on a range of substrate concentrations with a fixed amount of enzyme using the Multiprobe II for all pipetting steps. 140 µl of 20mM phosphate buffer, pH 7.5, with substrate (0.06–2 mM glutaryl-7-ACA or 0.2–10 mM adipyl-7-ADCA) was preheated at 37°C. 40 µl of phosphate buffer containing an appropriate amount of purified enzyme was added, starting the reaction. After 10 min of incubation at 37°C, 40 µl of 2.5 M acetate buffer, pH 4.5, was added to stop the reaction. 20 µl of 1 mg/ml fluorescamine in acetone was added, and the A_{380} was measured after 60 min incubation at room temperature.

Kinetic parameters were obtained by fitting the experimental data from Eadie-Hofstee plots, and the mean and S.D. values of at least four independent measurements were calculated. The k_{cat} was calculated using the theoretical molecular mass of the mature enzyme, 75.9 kDa.

Enzyme characterisation

Protein concentrations of the cell free extracts and purified samples were determined using the DC Protein Assay (Bio-Rad) in a 96-well plate with bovine serum albumin as the reference protein.

The cell free extracts were analysed by SDS-PAGE [110]. The 12.5% gel was stained with Coomassie Brilliant Blue. Identical samples were electroblotted onto a nitrocellulose membrane. The membrane was incubated with a polyclonal rabbit antibody against *Pseudomonas* SY-77 glutaryl acylase (Eurogentec S.A.) and subsequently with an alkaline phosphatase-conjugated goat anti-rabbit antibody. Bands corresponding to *Pseudomonas* SY-77 glutaryl acylase were coloured with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

Mutant and wild type enzyme were purified by three chromatography steps on a Duoflow system (Bio-Rad) using columns from Amersham Biosciences. *E. coli* DH10B containing the plasmids encoding the desired enzymes were grown for 24 h at 30°C in 300 ml of 2*YT medium supplemented with 50 µg/ml chloramphenicol and 0.1% glycerol. Cell free extract was made by sonication (10 min, output 4, 50% duty cycle on a Sonifier 250; Branson) and centrifugation (30 min at 14,000 rpm) and loaded onto a HiTrapQ column. The protein was eluted with a linear gradient of 0-1 M NaCl in 50 mM Tris-HCl, pH 8.8. After analysis on SDS-PAGE, the fractions containing the enzyme were loaded onto a HiTrap phenyl-Sepharose HP column. The protein was eluted with a gradient of 0.7-0 M $(NH_4)_2SO_4$. The pooled fractions containing the enzyme were desalted on a HiPrep 26/10 desalting column and reloaded onto a HiTrapQ column. After rinsing with 220 mM NaCl, the protein was eluted with 330 mM NaCl. The pooled fractions were stored at -20°C. Typical yields were 10 mg of more than 90% pure enzyme per litre of culture.

Results

Constructing the epPCR libraries

The conditions for the construction of the libraries were optimised. The highest PCR yields were obtained using 2.5 mM Mg^{2+} and an annealing temperature of 60°C for parts I, III, and V and 5 mM Mg^{2+} and 66°C for parts II and IV. Part I was subsequently mutagenised in the presence of 0.1-0.5 mM Mn^{2+}. A Mn^{2+} concentration of 0.5 mM

resulted in the desired mutation frequency of 0.7%, corresponding to approximately three base pair mutations per mutant. Since the relatively high GC content (67%) is equally distributed throughout the gene, we used 0.5 mM Mn^{2+} to mutagenise all parts. EpPCR gave a band of the correct size on an agarose gel for all five parts. Although both vector and insert were isolated from an agarose gel, cloning of the epPCR fragments into pMcSY-2 resulted in a significant part of contamination of parts II, III, and V with wild type sequences. Sequencing of at least 400 base pairs of 20 randomly picked clones from each bank revealed that all mutations were equally distributed throughout the gene (Figure 2). The mutational frequency of the different libraries varied from 0.2% for parts III and V to 0.7% for part I, resulting in 1-3 base pair mutations per mutant. The mutational bias was comparable with experiments under similar error-prone conditions as described before [111], although $X \rightarrow C$ ($X \neq C$) changes were lower than expected.

Since the transformation efficiency was not uniform for all five parts and plating of an equal amount of mutants onto each plate was desired, different volumes of the original transformation mixture were plated. All five libraries appeared to contain at least 10^5 transformants, which is enough to harbour every single mutant at least once [74].
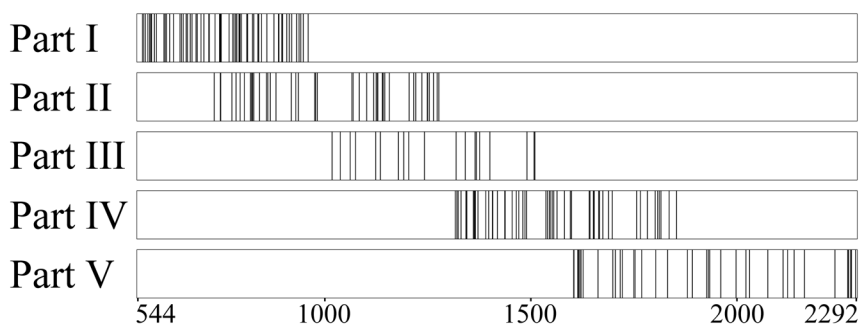

Figure 2. Distribution of mutations over the gene.
Of each epPCR part 20 clones were randomly picked and sequenced. Every identified mutated base is indicated by a line according to its position on the SY-77 gene.

Selection on minimal medium containing adipyl-leucine

Transformation mixtures were plated onto minimal plates in the absence of leucine (Min) or in the presence of leucine (MLeu), adipyl-leucine (AD-Leu), or glutaryl-leucine (GL-Leu). On each plate 5000 mutant plasmid-bearing bacteria were plated. As a reference, *E. coli* DH10B cells producing SY-77 wild type and SY-77[Y178H] were used. Plates were examined every day, and colonies were marked and closely watched during the next days. After 3-4 days, small colonies appeared on all plates, the ones on Min and AD-Leu plates being somewhat smaller than those on MLeu and GL-Leu plates. On the Min plates, the spots did not grow upon further incubation, whereas colonies on the MLeu and GL-Leu plates increased in size until the 10th day. On the AD-Leu plates,

colonies of the positive mutant producing SY-77^{Y178H} increased in size until the 6th day, whereas the wild type strain colonies grew slowly but steadily until the 10th day. Colonies of the libraries grew at different rates.

On the 10th day, a range of small and large colonies was present on each plate containing leucine of any kind. The average size of the colonies on the AD-Leu plates from *E. coli* DH10B containing the library parts IV and V or the negative control pMcSY-2 was smaller than the average size of the colonies from the other mutant libraries and the bacteria bearing the positive mutant plasmid pMcSY-YH. The size and day of appearance were used as criteria to select colonies of every epPCR part. These colonies were again streaked onto AD-Leu plates to isolate single colonies. Colonies of cells producing SY-77 wild type and SY-77^{Y178H} were also plated onto this medium. Most colonies were selected from the plates of parts I and II. Of the 54 colonies picked, 50 grew on the second AD-Leu plate after five days. Plasmid DNA was isolated from overnight cultures of the putative mutants from the secondary plate and retransformed to *E. coli* DH10B. DNA sequence determination of these plasmids showed that eight plasmids had the wild type sequence. Furthermore, some mutants proved to have identical sequences, leaving 29 unique transformants with mutations throughout the whole gene. However, most mutations occurred in the first 200 amino acids of the β-subunit, which corresponds to the fact that most mutants were selected from the libraries of parts I and II, encoding the first 230 amino acids.

Activity measurements

A 24 hour culture of every mutant was harvested, and cell free extracts were made using BugBuster. Hydrolysis of both adipyl-7-ADCA and glutaryl-7-ACA were measured in triplicate in a medium throughput robotic assay. Several precautions were taken in order to maintain the reliability of the assay. To compensate for differences in acylase concentration in the cell free extracts, the ratio AD/GL was used to characterise the mutants. Furthermore, all measurements were done using the same amount of cell free extract, even those with lower protein concentrations, since BugBuster appeared to affect the hydrolysis of the adipyl substrate more than the glutaryl substrate. In some cases, this led to very low A_{380} values that did not differ significantly from the blank values. In all cases, the production of acylase was verified by blotting the cell free extract (data not shown). Some bacteria proved to produce no acylase at all, whereas other bacteria produced about three times less acylase, resulting in such a low enzyme activity that the ratio was not determinable. Amino acid mutations and activities of all 29 different mutants are given in Table 1.

The results from Table 1 strongly suggest that Asn266 is an important residue for substrate specificity. 22 of the 36 selected and sequenced transformants from parts I and II contain a mutation at this position. The two single mutants N266H and N266S have an AD/GL ratio of 0.19 and 0.14, respectively. Multiple mutants that were found comprising these mutations show the same ratio, implying that the additional mutations

Table 1. The AD/GL ratio of the selected mutants.

Mutants of the five epPCR libraries were selected on minimal medium with adipyl-leucine as sole leucine source. DNA sequences of these selected mutants were determined. Furthermore, cell free extracts were made using BugBuster (Novagen). The activity of the cell free extracts from all mutants on adipyl-7-ADCA and glutaryl-7-ACA was determined in a robotic assay using fluorescamine. The ratio of these values (AD/GL) is used as a measure for the improvement of the enzyme towards adipyl-7-ADCA. The mean +/- S.D. ($n \geq 3$) is given. All selected transformants from Part V appeared to have wild type sequence. Multiple mutants were genetically separated and both DNA sequence and AD/GL ratio were determined.

Part	Mutation	Activity (AD/GL)
I	N266H (2x)	0.19 ± 0.01
	N266H (2x)	N.D.[1]
	N266H, D308N, S320Y	0.19 ± 0.01
	N266H, K314R	0.19 ± 0.00
	N266H, E317V	0.19 ± 0.01
	N266H, S320T	0.19 ± 0.00
	F229L, V237A	0.15 ± 0.01
	F229L[2]	0.15 ± 0.00
	V237A[2]	0.08 ± 0.00
	N266S (2x)	0.14 ± 0.01
	S223P, M271L	0.11 ± 0.00
	M271L[2]	0.12 ± 0.00
	A246V	0.11 ± 0.01
	D193V	0.09 ± 0.00
	Q291L	0.09 ± 0.01
	Q291R	0.09 ± 0.01
	S223P	0.08 ± 0.00
	G207*, Q218L	N.D.[1]
	N266H, S201A	N.D.[1]
	T225S	N.D.[1]
II	N266H (4x)	0.19 ± 0.01
	N266H (1x)	N.D.[1]
	N266H, S301T	0.19 ± 0.01
	N266H, A307E	0.19 ± 0.01
	N266H, E399V, T421K	0.19 ± 0.01
	M271V, Q291K, T374S	0.16 ± 0.01
	M271V, Q291K[2]	0.09 ± 0.00
	T374S[2]	0.12 ± 0.00
	N266S	0.14 ± 0.01
	N266S, F258L, T310S, E399D	0.12 ± 0.03
	M347L	0.12 ± 0.00
	E420V	0.09 ± 0.01
	N226S, F256I, Y426N	N.D.[1]
III	F375L (3x)[3]	0.45 ± 0.02
	W450R	0.09 ± 0.01
IV	M480L, R527L	0.09 ± 0.01
	S639A	0.09 ± 0.01
	V646A	0.08 ± 0.01
pMcSY-77 (WT)		0.09 ± 0.00
pMcSY-YH		0.24 ± 0.00

Notes:

[1] N.D. No activity could be detected; analysis by Western blotting indicated that no acylase was produced.

[2] These mutants were made by site directed mutagenesis in order to determine the mutation responsible for the higher ratio in the double mutant.

[3] F375L was found three times resulting from different base pair substitutions: 2x **C**TC, 1x TT**A**

do not affect the substrate specificity.

Although much less frequently found, mutation F375L results in the best AD/GL ratio found in these epPCR libraries. It has a five times better ratio than the wild type enzyme and even a two times better ratio than our positive control SY-77[Y178H] [28]. Other clones having an improved AD/GL ratio are the single mutants and SY-77[A246V] and SY-77[M347L] and the multiple mutants SY-77[F229L+V237A], SY-77[S223P+M271L], and SY-77[M271V+Q291K+T374S]. We genetically separated the multiple mutants and measured the AD/GL activity ratio of the resulting mutants in order to pinpoint the mutation that contributes most to the altered activity. The results as shown in Table 1 show that the single mutants SY-77[F229L] and SY-77[M271L] alone give the same activity ratio as their respective double mutants. This suggests that the second mutations have no effect on the activity of the mutants. Separating M271V and Q291K from T374S resulted in a lower total ratio than the triple mutant, indicating a synergistic effect of the mutations. Therefore, the triple mutant was used to determine k_{cat} and K_m.

Characterisation of purified mutants

Mutants with a significantly different enzymatic activity from wild type (i.e. with an AD/GL ratio higher than 0.12) were purified. The K_m and k_{cat} values of these mutants on both adipyl-7-ADCA and glutaryl-7-ACA were determined (Table 2). The ratio of the k_{cat} values of the purified enzymes on adipyl-7-ADCA and glutaryl-7-ACA is in agreement with the AD/GL ratio determined with the cell free extracts. Previously, the protein concentration was determined with the Bradford assay. However, due to precipitation phenomena, it was decided to use the DC protein assay with these purified acylase samples. This resulted in approximately 35% higher protein concentration values and thus in a decreased k_{cat} value for SY-77 wild type compared with an earlier study [28]. SY-77 wild type preparations purified on different days were found to vary up to 20% in enzymatic parameters. Even when taking this variation into account, most mutants show enzymatic properties that are significantly different from SY-77 wild type.

Table 2. K_m and k_{cat} values of purified enzymes with an AD/GL ratio of $\geq$ 0.12.

Mutant enzymes with an AD/GL ratio of $\geq$ 0.12 were purified >90%. The V_{max} and K_m on adipyl-7-ADCA and glutaryl-7-ACA were determined by measuring the initial rate of hydrolysis on a range of substrate concentrations with a fixed amount of enzyme as described earlier [28]. WT, wild type enzyme.

	Adipyl-7-ADCA		Glutaryl-7-ACA	
	k_{cat} (s^{-1})	K_m(mM)	k_{cat} (s^{-1})	K_m(mM)
WT	0.46 ± 0.02	0.96 ± 0.03	4.3 ± 0.2	0.033 ± 0.004
N266H	0.55 ± 0.02	0.14 ± 0.02	3.3 ± 0.1	0.016 ± 0.004
N266S	0.34 ± 0.02	0.42 ± 0.04	2.2 ± 0.1	0.094 ± 0.008
F375L	0.67 ± 0.05	0.82 ± 0.04	1.1 ± 0.1	0.27 ± 0.06
F229L	0.47 ± 0.02	0.52 ± 0.04	3.1 ± 0.1	0.048 ± 0.002
M271V + Q291K + T374S	0.61 ± 0.04	0.7 ± 0.1	3.5 ± 0.1	0.043 ± 0.004

The k_{cat} values on adipyl-7-ADCA of SY-77[F375L] and the triple mutant SY-77[M271V+Q291K+T374S] are significantly higher than that of wild type, whereas the corresponding K_m values of SY-77[N266H], SY-77[N266S], and SY-77[F229L] are significantly lower than that of wild type. The catalytic efficiency of all mutants is increased for adipyl-7-ADCA with an exceptionally high value for SY-77[N266H]. With respect to the activity on glutaryl-7-ACA, none of the mutants shows an improved k_{cat}, whereas only mutant SY-77[N266H] exhibits a lower K_m value than SY-77 wild type. Most interestingly, this results in a higher catalytic efficiency of mutant SY-77[N266H] on both substrates adipyl-7-ADCA and glutaryl-7-ACA (Figure 3).

Discussion

In this paper, we describe the evolution of a glutaryl acylase towards an adipyl acylase, in order to arrive at an efficient hydrolysis of adipyl-7-ADCA into 7-ADCA for the synthesis of semi-synthetic cephalosporins [112]. We performed random mutagenesis on five overlapping parts of the gene, encoding the β-subunit of the acylase. This resulted in five mutant libraries of approximately 10^5 individual clones, which appeared to be large enough to contain every single mutant at least once [74]. Sequencing of 20 randomly picked clones of each part showed that mutation frequencies lie between one and three base pair substitutions per mutant for all parts. Furthermore, the mutations were scattered throughout the gene (Figure 2).

The mutant libraries were transformed into the leucine deficient *E. coli* strain DH10B, after which the transformants were selected for growth capability on minimal medium containing adipyl-leucine. Unfortunately, cells that produced no mutated acylase with activity towards adipyl-leucine appeared to be able to form small colonies, most likely by using the leucine that was liberated into the medium by lysis of dead, mostly non-transformed cells. Consequently, the selection for growth on AD-Leu plates was less clear cut than desired. Nevertheless, we could identify 54 colonies that excelled in early growth and colony size. 50 colonies out of these were able to grow on a second selective plate with adipyl-leucine as a sole leucine source. These transformants were screened for their hydrolysis activity on the substrates glutaryl-7-ACA and adipyl-7-ADCA. All acylase-producing transformants were active on the β-lactam substrates, in contrast to earlier reports describing similar selection procedures [68,113]. This strengthens the hypothesis that the side chain is the most important moiety for substrate binding, as was already suggested from the crystallographic models of penicillin G acylase [114] and cephalosporin acylase [30].

Sixteen unique mutants showed a significantly higher AD/GL activity ratio than SY-77 wild type (Table 1). Five mutants with a 50% increased AD/GL ratio were purified. These mutants have an enhanced catalytic efficiency on adipyl-7-ADCA with respect to wild type (Figure 3), resulting from a significant improvement of either K_m or k_{cat} (Table 2).

A

Adipyl-7-ADCA

k_{cat}/K_m $(s^{-1}$ $mM^{-1})$

WT N266H N266S F375L F229L M271V+ Q291K+ T374S

B

Glutaryl-7-ACA

k_{cat}/K_m $(s^{-1}$ $mM^{-1})$
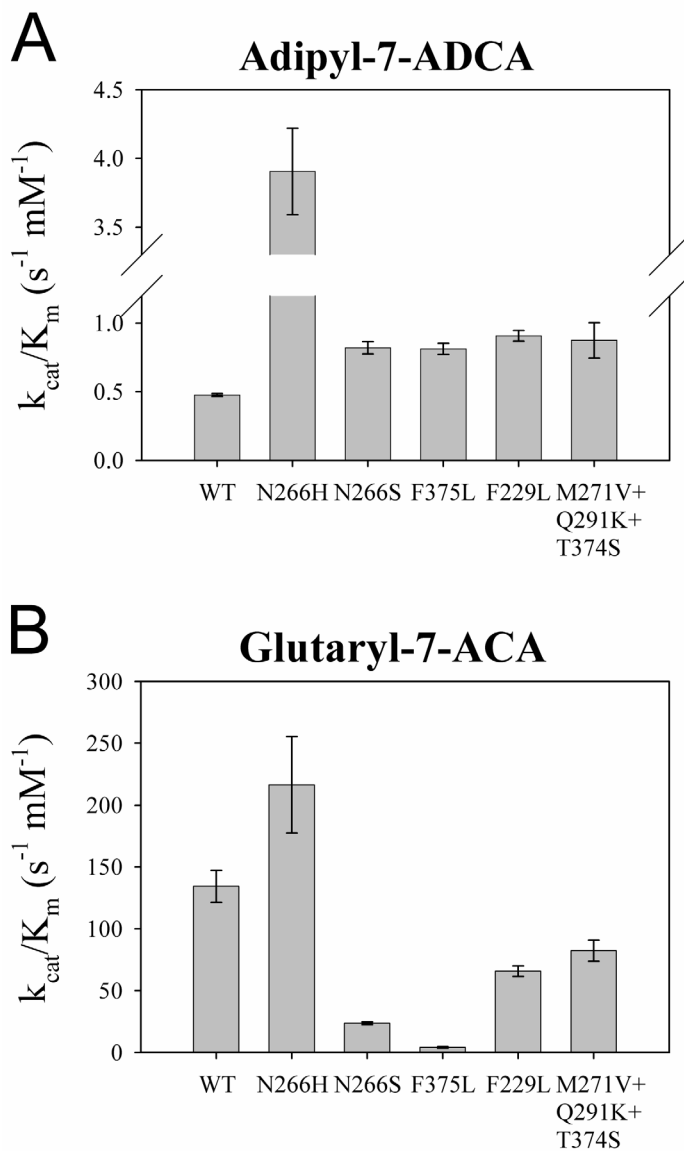
WT N266H N266S F375L F229L M271V+ Q291K+ T374S

Figure 3. Catalytic efficiency of mutated enzymes on adipyl-7-ADCA and glutaryl-7-ACA.
The catalytic efficiencies of the purified, mutated acylases on adipyl-7-ADCA (A) and glutaryl-7-ACA (B) were calculated from the data given in Table 2. The vertical axe in A is broken to illustrate more clearly the differences in catalytic efficiencies for all mutants.

It thus appears that the selection procedure leads to the isolation of mutants with changes in both kinetic parameters. This seems logical, bearing in mind that the used concentration of adipyl-leucine (0.4 mM) was of the same order of magnitude as the K_m. All mutations that cause a significant increase in AD/GL ratio are localised in close vicinity of the substrate binding site (Figure 4A). Two hot spot positions were found to be mutated with high frequency: Asn266 and Phe375. The latter has been described before as one of the potentially important residues that determine substrate specificity and is thought to interact directly with the substrate [30]. Asn266, however, has not yet been proposed as a target residue for changing substrate specificity and is likely to interact in an indirect manner with the substrate. Its frequent appearance in our collection of selected transformants underscores therefore the strength of random in comparison with structure-based site-directed mutagenesis.

It is striking that mutations in residue Asn266 are found with such high frequency; it was mutated in two-thirds of the selected and sequenced transformants originating from the two parts in which they could be found, parts I and II. This large number was not a consequence of some sequence bias, as proven by sequence determination of randomly picked colonies. Based on mutational efficiency, this residue could have been mutated into several other amino acids (for instance, mutation of one base in the triplet encoding Asn266 can result in Asp, His, Ile, Lys, Ser, Thr, and Tyr). However, apparently only enzymes comprising either of the two different mutations N266H and N266S could pass the selection procedure. The importance of residue Asn266 can be explained with the aid of the reported structures of substrate-bound glutaryl acylase. From these structures, it is clear that Arg255 is crucial for the binding of substrate in the wild type enzyme [32,115]. Arg255 is positioned correctly towards the substrate by a hydrogen bond with residue Asn266 via Tyr351. Replacing Asn266 with His or Ser will alter this network of hydrogen bonds, resulting in an altered positioning of Arg255 toward the substrate. In the case of the N266S mutant, this results in an increased catalytic efficiency for adipyl-7-ADCA and a decreased catalytic efficiency for glutaryl-7-ACA. Mutant SY-77^{N266H} has a unique characteristic: its 8-fold improved catalytic efficiency on adipyl-7-ADCA is accompanied by an improved catalytic efficiency on glutaryl-7-ACA. All other characterised mutants have a decreased catalytic efficiency on glutaryl-7-ACA. Mutant SY-77^{N266H} may accept more substrates and as such be a preferable template for other directed evolution studies.

The enzyme bearing mutation F375L resulted in the best AD/GL ratio found using these epPCR libraries. It was only found in part III and not in part II, which may be due to the prominent presence of the Asn266 mutants in part II. SY-77^{F375L} has a 5-fold higher AD/GL activity ratio compared with the wild type enzyme. This ratio is mainly due to the decreased k_{cat} on glutaryl-7-ACA and not so much the increased k_{cat} on adipyl-7-ADCA. This result emphasises the requirement of enzymatic characterisation in order to be able to identify mutants with the desired properties. The F375L mutation corresponds to the notion that the bulky Phe375 should be changed into a smaller residue in order to provide space for the extra carbon [32]. The enlarged side chain
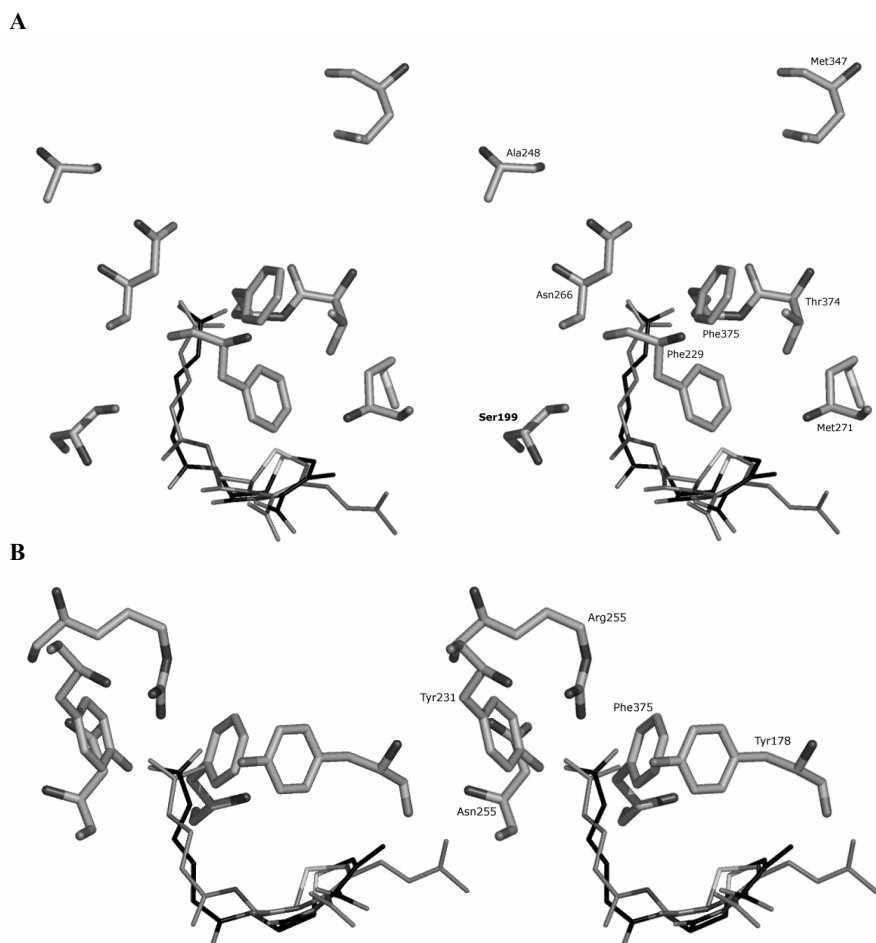
A

B

Figure 4. Three-dimensional views on the positions of mutated residues.
A. Three-dimensional view of the mutated residues identified after selection for adipyl activity. The glutaryl acylase SY-77 was modelled with both adipyl-7-ADCA (black) and glutaryl-7-ACA (grey) using the crystal model of the complex with glutaryl-7-ACA. The catalytic residue Ser199 is depicted to illustrate its position relative to the scissile bond of the substrates.
B. Three-dimensional view of five selected residues surrounding the carboxylate group of the side chain. These residues appear to be important to position the carboxylic head of the substrate by direct or indirect interactions.
Both figure 4A and 4B were created using the molecular graphics program PyMOL (W.L. DeLano; available on the World Wide Web at www.pymol.org).
A colour print of this picture can be found on http://www.jbc.org/cgi/content/full/277/44/42121.

binding pocket increases the degrees of freedom of the bound substrate. This may result in a better fit of the alternative adipyl side chain but a decreased binding of the original glutaryl moiety of the substrate.

In the SY-77[F229L] mutant, the altered residue lies close to the active site residue Tyr231, which was shown to be crucial for the binding of the side chain of glutaryl-7-ACA [32]. Phe229 lies also in close proximity to the β-lactam nucleus. Replacing this amino acid with the smaller leucine will alter the configuration of the substrate binding pocket. A similar effect can be expected for the triple mutation M271V+Q291K+T374S. Met271 lies close to Val268, which has interactions with both the side chain and the nucleus of glutaryl-7-ACA. In addition, Thr374 is the neighbour of Phe375, which interacts with the side chain of glutaryl-7-ACA.

In this study, residues Asn266 and Phe375 of the β-subunit were found to be important targets for changing side chain specificity of glutaryl acylase. In a similar study concerning the α-subunit, Tyr178 was pointed out as an important residue [28]. Mutation of each of these three residues resulted in an improved activity toward adipyl-7-ADCA. Combined with residues Tyr231 and Arg255, which are known to be crucial for the binding of the side chain of glutaryl-7-ACA [32,115], these residues form a pocket in which the carboxylate group of the side chain is embedded (Figure 4B). Residues Tyr178, Tyr231, Asn266, and Phe375 seem to form the corners of a square plane, with the carboxylate group in the centre, which is held in place by the positive charge of Arg255 above this plane. As described before [32], the rest of the aliphatic side chain is positioned tightly in the active site by hydrophobic interactions with several residues. Our results in this and our previous study [28] indicate that the accommodation of larger side chains requires modification in this part of the side chain binding pocket, while keeping the scissile bond properly positioned toward the catalytic Ser199. Therefore, a logical step in evolving this enzyme toward a further improved adipyl acylase or even a cephalosporin C acylase seems to be a combination of mutations of these residues.

Acknowledgments