Chapter 3

Mutational analysis of a key residue in the substrate specificity of a cephalosporin acylase

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β-lactam acylases are crucial for the synthesis of semi-synthetic cephalosporins and penicillins. Unfortunately, however, there are no cephalosporin acylases known that can efficiently hydrolyse the amino-adipic side chain of this compound. In a previous directed evolution experiment residue Asn266 of the glutaryl acylase from *Pseudomonas* SY-77 was identified as a residue important for substrate specificity. In order to explore the function of this residue in substrate specificity, we performed a complete mutational analysis of position 266. Codons for all amino acids were introduced in the gene and 16 proteins that could be functionally expressed in *Escherichia coli* were purified to homogeneity and their catalytic parameters were determined. The mutant enzymes displayed a broad spectrum of affinities and activities, pointing towards the flexibility of the enzyme at this position. Mutants in which Asn266 was changed into Phe, Gln, Trp and Tyr displayed up to a 2-fold better catalytic efficiency (k_{cat}/K_m) on adipyl-7-aminodesacetoxycephalosporanic acid (adipyl-7-ADCA), due to a decreased K_m. Only mutants SY-77^{N266H} and SY-77^{N266M} showed an improvement of both catalytic parameters, resulting in a 10- and 15-times higher catalytic efficiency on adipyl-7-ADCA, respectively. Remarkably, the catalytic activity (k_{cat}) of SY-77^{N266M} on adipyl-7-ADCA is as high on glutaryl-7-aminocephalosporanic acid (glutaryl-7-ACA), and approaches commercially interesting activities. On Cephalosporin C, mutants SY-77^{N266Q}, SY-77^{N266H} and SY-77^{N266M} show a modestly improved hydrolysis. Since these mutants also have a good catalytic efficiency on adipyl-7-ADCA and still activity towards glutaryl-7-ACA, they can be regarded as broader substrate acylases. These results demonstrate that a combination of directed evolution for the identification of important positions and saturation mutagenesis for finding the optimal amino acid is very effective for finding improved biocatalysts.

Introduction

The most widely used antibiotics are semi-synthetic cephalosporins and penicillins. Key intermediates in the synthesis of cephalosporins are 7-aminocephalosporanic acid (7-ACA) and 7-aminodesacetoxycephalosporanic acid (7-ADCA). 7-ACA is obtained by removing the side chain from Cephalosporin C (CPC) produced by the fungus *Acremonium chrysogenum* in a two step enzymatic process. 7-ADCA is produced from penicillin G made by *Penicillium chrysogenum* involving several polluting chemical steps followed by enzymatic deacylation using 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 strain that produces adipyl-7-ADCA [18]. For the enzymatic deacylation of this novel β-lactam and CPC, novel acylases with activity on adipyl and amino-adipyl side chains, respectively, are needed. As the presently identified acylases show little or no activity towards

adipyl-7-ADCA and CPC [13,25,102], it is of interest to investigate whether they can be created from another acylase, e.g. glutaryl acylase. In a former study we used directed evolution as a tool to identify important residues for the transformation of a glutaryl acylase into an adipyl acylase [116]. Several variants were selected and showed a considerable improvement of the hydrolysis of adipyl-7-ADCA. Sequencing of these variants demonstrated that the mutation of Asn266 into His or Ser occurred frequently. This indicates that position 266 is important for the evolution of the glutaryl acylase into an adipyl acylase, although this was not predicted from the crystal structure [30,32,115].
Since we used a random mutagenesis strategy introducing on average 1-2 point mutations per gene in our directed evolution experiment, it was not possible to obtain all 20 amino acids on this position. Furthermore, the amino acid substitutions obtained by point mutations are usually conservative, while non-conservative mutations often comprise more potential to change the activity of an enzyme [117], e.g. from a glutaryl acylase towards an adipyl acylase or even a CPC acylase. The side chain of residue 266 may influence the enzyme specificity either by a polar interaction with the charged carboxyl group of the substrate side chain or by a better accommodation of the hydrophobic part of this side chain. In order to fully explore the importance of this position for the substrate specificity of the enzyme, we changed the amino acid at position 266 into all other amino acids by a site-directed mutagenesis approach. The mutant enzymes were purified and catalytic parameters were determined on glutaryl-7-ACA, adipyl-7-ADCA and CPC.

Materials and Methods

Bacterial strains and plasmids and DNA manipulations

The plasmid pMcSY-2 [116] and the leucine deficient *Escherichia coli* DH10B (Invitrogen) were used for the cloning of the libraries and the expression of the SY-77 acylase.
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).
Mutants were made using the megaprimer method described by Landt *et al.* [42]. All PCR reactions were performed in a Mastercycler® gradient thermocycler (Eppendorf) using recombinant *Pfu* polymerase (Stratagene). A 5'-universal primer, CGCCGGACTTCGAGATCTA and a 3'-mutagenic primer, CATGCCGTTGACGGT**SNN**GGTGATGCCCATCCG, were used in the first PCR using the following program: 3 min at 95°C, 30 cycles of 45 s at 95°C, 30 s at 48°C, 30 s at 72°C and a final step of 10 min at 72°C. The resulting fragment was purified and used as the 5'-primer in a second PCR together with a 3'-universal primer GATTGGTGACCCGCGGCA. In this reaction the following program was used: 3 min at 95°C, 30 cycles of 45 s at 95°C, 1 min at 53°C, 1 min at 72°C and a final step of 10 min at 72°C. The second PCR product was purified and cloned into pMcSY-2 using *Bgl*II and *Sst*II. Resulting plasmids were sequenced in order to determine the amino acid at position 266. Amino acids, which were not obtained in the random

primer PCR, were made by using the 3'-mutagenic primer with the specific codon at the NNS position. The cloned DNA fragment was sequenced on both strands afterwards.

Purification of enzymes

Mutant and wild type enzyme were purified in three chromatography steps on a Duoflow system (Bio-Rad) using columns from Amersham Biosciences as described before [116]. Typically, *E. coli* DH10B containing the plasmids encoding the desired enzymes were grown in 2*YT medium (300 ml, 30 hours, 25°C) supplemented with chloramphenicol (50 µg/ml) and glycerol (0.1%). Cell free extract was made by sonication (10 min, output 4, 40% duty cycle on a Sonifier 250, Branson) and centrifugation (30 min, 17000 g). The cell free extract was blotted onto a membrane and stained with polyclonal rabbit antibody against purified SY-77 glutaryl acylase (Eurogentec S.A.) as described before [116]. Mutants that showed a low concentration of acylase were grown again for up to 70 hours at 17°C–20°C in 2 litre of the same medium. Protein concentrations of the purified samples were determined using the DC Protein Assay (Bio-Rad) with bovine serum albumin as the reference protein. The samples were analyzed on a 12.5% SDS-PAGE gel stained with Coomassie Brilliant Blue [110].

Enzyme characterization

The kinetic parameters of the purified wild type and mutant enzymes on glutaryl-7-ACA and adipyl-
7-ADCA were tested in an automated fluorescamine assay as described before [116]. Hydrolysis of CPC was determined by the fluorescamine assay, using the highest possible concentration of substrate with respect to background values. 20 µg of enzyme was added to a reaction mixture of 300 µl containing CPC (10 mM) in phosphate buffer (20 mM, pH 7.5). After 20 and 40 hours incubation at 37ºC, an aliquot of 40 µl reaction mixture was transferred to 140 µl acetate buffer (0.5 M, pH 4.5), after which 20 µl of fluorescamine in acetone (1 mg/ml) was added. After incubation at room temperature (60 min) the A_{380} was measured. The enzymes, which showed some hydrolysis in this assay, were used in a more accurate assay. In this assay, 75 µg of enzyme was used in a 300 µl reaction mixture containing CPC (10 mM) in phosphate buffer (20 mM, pH 7.5). Samples of 40 µl were taken from this reaction mixture every hour for five hours. In these samples hydrolysis of CPC was also measured using the automated fluorescamine assay. Data were analysed by linear regression. The slopes were calculated from two experiments with at least three different sets of data points. The significance of deviations in CPC hydrolysis activity was tested by the Student's t-test.

Modelling of the (mutant) enzymes

In order to get a structural impression of the mutational effects we made an overlay of selected active site residues in the structural models of the glutaryl acylase proteins containing mutations N266Q, N266M and N266W. The wild type structure was also refined under the same circumstances to account for differences in crystal structures and modelled structures. The models were constructed on the basis of wild type glutaryl acylase(PDB entry 1FM2) using the molecular graphics and modelling program DS Modeling (Accelrys, San Diego, CA, USA). The structures were refined using the DS CHARMm® module by energy minimisation consisting of 150 steps of steepest descent followed by 5000 iterations of the Adopted Basis-set Newton-Raphson algorithm.

Results

Preparation and purification of the mutant enzymes

The megaprimer method was used in to create the 19 different amino acid mutants on position 266. First, a primer with a randomised codon 266 was used and 96 transformants were sequenced. This resulted in 17 different amino acids at this position in a single mutagenesis round. The missing three mutants were made by site-directed mutagenesis using a specific primer. *E. coli* DH10B cells containing the plasmid encoding the (mutant) enzyme were grown in 300 ml 2*YT-medium at 25°C. After 30 hours the cells were harvested and sonicated. The soluble fraction was used to purify the enzyme in a 3-step protocol. Typically, a yield of more than 10 mg enzyme per litre was reached with a purity of at least 90%.

The enzymes containing mutations N266R, N266I, N266K and N266V were hardly produced under these conditions. Attempts to obtain these mutant enzymes by growing them in different experiments at 17°C-20°C for 30-70 hours in a volume up to two litres failed. At these low temperatures after 70 hours the strains expressing these mutants reached only half the OD_{600} of the strains bearing the wild type gene in 30 hours at 25°C. Furthermore, they still hardly produced any enzyme in the soluble fraction. Western blots of SDS-PAGE gels with cell lysates of these mutants showed that SY-77^{N266R} and SY-77^{N266K} were mainly present in the non-soluble fraction, whereas the enzymes containing mutations N266I and N266V were hardly produced at all (data not shown). Consequently, these four mutants were discarded from further analysis.

In order to determine possible interference of the remaining 16 amino acids at this position with the maturation of the enzymes, 3 µg of purified enzyme was loaded onto an SDS-PAGE gel and stained with Coomassie Brilliant Blue. From these gels (Figure 1) it is clear that mutation N266A results in an impaired processing of the propeptide, while mutations N266D and N266E result in a less efficient cleavage of the spacer from the α-subunit. The other mutations do not seem to affect the processing of the enzyme.

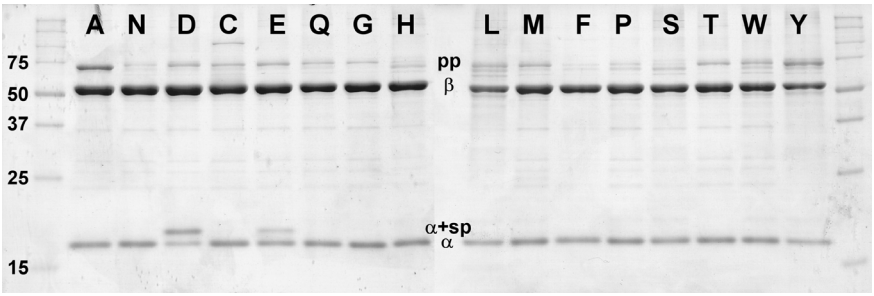

Figure 1. Effects of mutation of Asn266 on the maturation of glutaryl acylase.
The purified enzymes were boiled for 2 minutes in loading buffer and loaded onto a 12.5% SDS-PAGE gel. The amino acid at position 266 is depicted above the lane. pp, propeptide; β, β-subunit; sp, spacer peptide; α, α-subunit.

Activity towards adipyl-7-ADCA and glutaryl-7-ACA

The catalytic parameters of the purified mutant and wild type enzymes were determined on both adipyl-7-ADCA and glutaryl-7-ACA. The activity and affinity constants (k_{cat} and K_m) are listed in Table 1, the catalytic efficiencies (k_{cat}/K_m) are depicted in Figure 2. Interestingly, the library of mutants at position 266 exhibits a considerable diversity of changed catalytic parameters. The most striking mutant is SY-77^{N266M}. Its catalytic efficiency on adipyl-7-ADCA is 15-fold higher than that of wild type. The K_m of this enzyme is 3.5-times lower and the k_{cat} is 4.5-times higher than wild type parameters. The large hydrophobic amino acids Tyr, Trp and Phe at position 266 improve adipyl-7-ADCA hydrolysis by lowering the K_m. Mutation N266H results in an almost 10-fold improved catalytic efficiency on adipyl-7-ADCA, mostly due to a decreased K_m. The amino acid Gln at position 266, which side chain is one C atom longer than the original side chain of Asn, induces a 2-fold decrease of the K_m for adipyl-7-ADCA hydrolysis. Substitution of Asn266 with the small amino acid Cys improves K_m, but decreases k_{cat} on adipyl-7-ADCA resulting in a catalytic efficiency that is similar to wild type. The other amino acids at position 266 have a decreased k_{cat} and/or a higher K_m on adipyl-7-ADCA resulting in a lower catalytic efficiency. Changing Asn266 into any other amino acid in all cases leads to a lower activity towards glutaryl-7-ACA. The extent of this decrease on k_{cat} and/or K_m is distinct for each amino acid.

Table 1. K_m and k_{cat} values on adipyl-7-ADCA and glutaryl-7-ACA of purified enzymes.

The mutant enzymes SY-77^{N266R}, SY-77^{N266I}, SY-77^{N266K} and SY-77^{N266V} did not produce enough enzyme to be purified and catalytic parameters could not be determined. The remaining 16 enzymes were purified >90%. The k_{cat} and K_m on both substrates were determined by measuring the initial rate of hydrolysis on a range of substrate concentrations with a fixed amount of enzyme as described earlier [116]. N.D., not detectable.

AA at position 266	adipyl-7-ADCA		glutaryl-7-ACA	
	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)
Asn (WT)	0.41 ± 0.01	1.2 ± 0.3	4.0 ± 0.3	0.031 ± 0.002
Ala	0.18 ± 0.02	2.2 ± 0.2	1.4 ± 0.1	0.26 ± 0.08
Asp	0.002 ± 0.000	2.2 ± 0.9	N.D.	N.D.
Cys	0.31 ± 0.03	0.80 ± 0.03	2.0 ± 0.1	0.16 ± 0.03
Glu	0.023 ± 0.002	13.8 ± 5.1	0.025 ± 0.002	0.29 ± 0.00
Gln	0.46 ± 0.04	0.62 ± 0.13	2.4 ± 0.2	0.12 ± 0.03
Gly	0.23 ± 0.02	1.4 ± 0.3	2.0 ± 0.4	0.54 ± 0.13
His	0.47 ± 0.01	0.14 ± 0.01	3.1 ± 0.3	0.044 ± 0.008
Leu	0.25 ± 0.02	1.0 ± 0.2	0.082 ± 0.011	0.43 ± 0.04
Met	1.9 ± 0.1	0.33 ± 0.05	1.8 ± 0.1	0.17 ± 0.02
Phe	0.37 ± 0.04	0.6 ± 0.1	0.9 ± 0.2	0.10 ± 0.00
Pro	0.061 ± 0.005	4.4 ± 1.2	0.13 ± 0.01	1.3 ± 0.3
Ser	0.24 ± 0.02	0.99 ± 0.05	1.6 ± 0.1	0.075 ± 0.005
Thr	0.15 ± 0.01	5.7 ± 1.2	0.49 ± 0.14	1.5 ± 0.5
Trp	0.33 ± 0.01	0.59 ± 0.06	1.3 ± 0.1	0.18 ± 0.04
Tyr	0.33 ± 0.01	0.65 ± 0.10	0.61 ± 0.06	0.11 ± 0.01

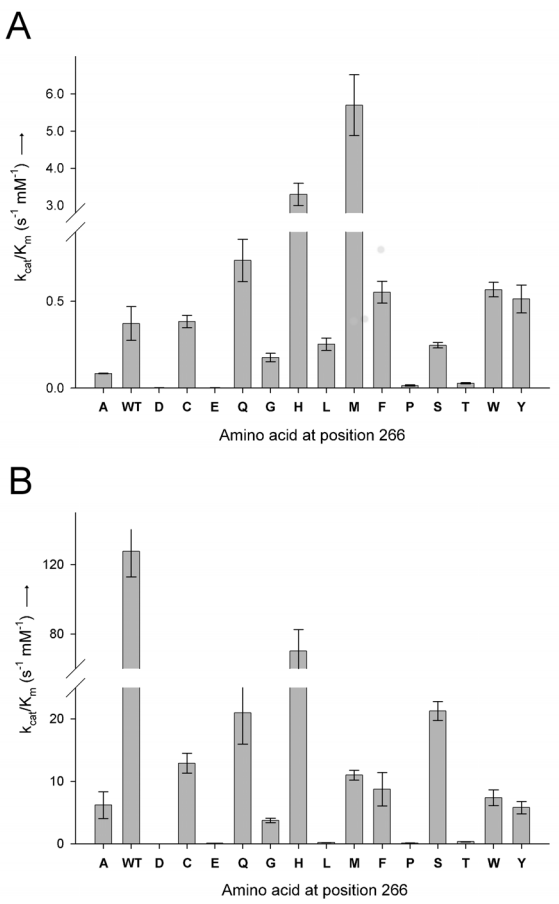

Figure 2. Catalytic efficiencies of purified enzymes on adipyl-7-ADCA (A) and glutaryl-7-ACA (B).
The catalytic parameters were determined by an automated fluorescamine assay performed at different substrate concentrations. The kinetic parameters were calculated from Eadie-Hofstee plots and listed in Table 1. The given values are a mean ± S.D. of at least three independent measurements.

Conversion of Cephalosporin C

For the determination of activity on CPC, 20 µg of all mutant and wild type enzymes were incubated at 37°C with 10 mM CPC. The wild type enzyme and the mutants SY-77^{N266Q}, SY-77^{N266H}, SY-77^{N266M} and SY-77^{N266W} showed hydrolysis after 20 hours. These activities were too low to be able to determine K_m and k_{cat}. In order to avoid long incubation times that may lead to enzyme destabilisation, the conversion rate of the different mutants was calculated from an assay with more enzyme (75 µg). The mean slope of the conversion of CPC in the first five hours was determined and normalised

towards wild type (Figure 3). Mutants SY-77^{N266Q} and SY-77^{N266H} displayed almost a two times improved conversion of CPC over wild type, whereas SY-77^{N266M} has improved 30%. The conversion rate of SY-77^{N266W} is not significantly different from wild type.

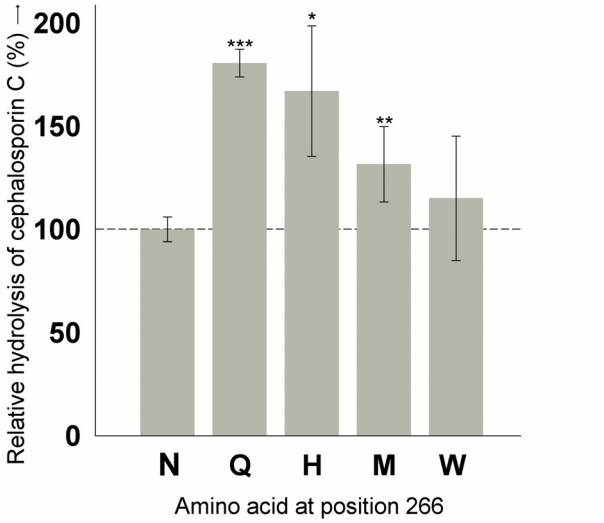

Figure 3. Hydrolysis of 10 mM CPC by a few selected Asn266 mutants in the first five hours.
The slopes of the hydrolysis curves were calculated for at least six different experiments. Conversion of CPC by wild type was set at 100%. Values were tested for statistical significant difference by the Student's t-test; * = $p < 0.01$, ** = $p < 0.005$, *** = $p < 0.0005$, mutant N266W was not significantly different.

Discussion

In a previous study using error prone PCR, amino acid Asn266 of the glutaryl acylase of *Pseudomonas* SY-77 was pointed out as one of the residues defining substrate specificity [116]. This result was surprising since Asn266 was indicated not to be directly involved in substrate-binding, but could interact via Tyr351 with Arg255, which accommodates the carboxylate head of the side chain [32,115]. In order to elucidate the role of Asn266 in substrate specificity, we have mutated Asn266 into the 19 other amino acids and expressed them in *E. coli*. Sixteen of the 20 different enzymes could be expressed in a soluble form and were purified. These 16 acylases were characterised with respect to their activity in the maturation process and the hydrolysis of several substrates. The catalytic properties varied over a broad spectrum (Table 1, Figures 2 and 3) pointing at the active role of position 266 in the enzymatic activities.

Since the maturation of the enzyme is crucial for its activation [118], we first examined the effect of the different mutations on the maturation process. Mutants SY-77^{N266R} and SY-77^{N266K} were only produced as insoluble precursor proteins. Probably, the longer side chains and positive charge result in a non-proper folding of the enzyme. The presence of Ile or Val at position 266 causes a low production of the enzyme, in contrast to other aliphatic residues. It is possible that the branching at the Cβ interferes with the folding process. Mutant SY-77^{N266A} is soluble, but not fully maturated. A significant fraction of this purified protein is in the propeptide form indicating that the first intramolecular cleavage step (between the spacer and the β-subunit) is affected (Figure 1). The second step in the maturation process (cleavage of the spacer peptide from the β-subunit) is supposed to involve a similar catalytic mechanism as the hydrolysis of the substrate [29,118]. Therefore, it would be expected that changes in residues involved in catalysis also interfere with the processing of the enzyme. However, most of the purified mutant proteins do not show the non-cleaved form of α-subunit plus spacer peptide. Obviously, the long period of cell growth and protein purification could give slower processing mutants enough time to mature. Thus, small changes in maturation efficiency would not be detected. Only the change of Asn266 into the charged residues Asp and Glu clearly results in poor cleavage of the spacer peptide from the α-subunit (Figure 1). It seems that these amino acids do not interfere with the folding of the enzyme, but are in competition with the carboxylate group of the Glu188 for the Arg255 [119].

Remarkably, the K_m of the enzyme towards adipyl-7-ADCA has improved in half of the purified mutants, pointing at the flexibility and importance of position 266. Only mutants SY-77^{N266E}, SY-77^{N266P} and SY-77^{N266T} have a strongly increased K_m. Concerning the catalytic activity, merely two mutants, SY-77^{N266H} and SY-77^{N266M}, show a significantly increased k_{cat}. The most striking mutant is SY-77^{N266M}, which shows a 4.5 times higher k_{cat} and a 3.5 times lower K_m, resulting in a 15-times better catalytic efficiency (Figure 2). The k_{cat} on adipyl-7-ADCA is the same as the k_{cat} on glutaryl-7-ACA of this mutant and approaches enzymatic activities that are commercially exploited [14]. Although the side chain of Met is one methyl group longer than Asn, it is flexible and unbranched. Modelling of this amino acid at position 266 in the enzyme suggests that mutation N266M only affects the position of the side chains of Arg255 and Met266 (Figure 4). It is likely that Met266 can have direct hydrophobic interactions with adipyl-7-ADCA. In addition, it clearly displaces the side chain of Arg255, thereby enlarging the substrate binding pocket and creating more space for the longer adipic side chain.

Five other mutants maintain a similar k_{cat} on adipyl-7-ADCA as wild type and show a 1.5 to 2-times decreased K_m. SY-77^{N266H} and SY-77^{N266Q} both have a larger amino acid at position 266 that is able to form hydrogen bonds. In the original crystal structure, the longer side chain of mutant amino acid Gln266 would not fit in the same orientation as Asn. Application of energy minimisation to this mutant enzyme structure bends the Gln side chain towards Arg255, resulting in a more favourable position for hydrogen

bonding with either the substrate or Arg255 (Figure 4). Similar to SY-77^{N266M}, SY-77^{N266Q} appears to displace Arg255, allowing a better accommodation of the adipyl side chain. The catalytic properties on adipyl-7-ADCA of this mutant are however not as good as those from SY-77^{N266M}, probably due to the polar character of the Gln side chain. The large aromatic amino acids in the mutants SY-77^{N266F}, SY-77^{N266W} and SY-77^{N266Y} express similar k_{cat} values and bind adipyl-7-ADCA 2-fold better than wild type. These amino acids are more likely to change the position of the substrate by hydrophobic interactions. Modelling of the mutant with the largest of these three amino acids, SY-77^{N266W}, revealed that the position of Trp266 nearly overlaps with the original position of Asn (Figure 4). Incorporation of this large aromatic side chain does however cause a displacement of the backbone of Arg255 and its closest neighbours away from the substrate. Mutation N266W thus seems to improve the accommodation of the longer adipic side chain by an enlargement of the substrate binding pocket and likely also by increasing the hydrophobic interactions.

None of the mutants show an increased activity or affinity for the preferred substrate glutaryl-7-ACA. Evolution optimised this amino acid, logically resulting in a decreased activity upon changing, as is seen in a lot of mutagenesis studies [108]. Only mutants SY-77^{N266H} and SY-77^{N266S}, which were already found in the directed evolution experiment, do have similar catalytic parameters on the glutaryl substrate as wild type enzyme indicating that these mutations hardly affect the overall structure of the substrate-binding pocket of the enzyme. This is not surprising considering the fact that these amino acids could be created by a single base pair substitution, which usually leads to conservative mutations and preservation of enzyme activity.

Although the importance of residue 266 became evident from a selection experiment using the adipyl side chain, we hypothesised that this residue also might influence the binding of the amino-adipyl side chain of CPC. We found mutants SY-77^{N266H}, SY-77^{N266Q} and SY-77^{N266M} to have a small but significant increase in hydrolysis activity of CPC over wild type (Figure 3). This activity is, however, still two orders of magnitude lower than the hydrolysis of glutaryl-7-ACA. As a consequence of these low activities we could not determine whether these improvements stem from effects on either K_m or k_{cat}.

Interestingly, the same mutants SY-77^{N266Q}, SY-77^{N266H} and SY-77^{N266M} also have an improved activity on adipyl-7-ADCA and SY-77^{N266H} even displays almost the same activity on glutaryl-7-ACA as wild type. Apparently, these three mutated enzymes have expanded their substrate range. These results seem to confirm the hypothesis that a CPC acylase can be made from a glutaryl acylase, but that one amino acid substitution is not sufficient.

In conclusion, we used saturation mutagenesis to complement a previous directed evolution approach in which residue Asn266 was identified as a key residue for substrate specificity. This allowed the analysis of a larger sequence space at this particular position and finding of more radical changes in enzyme activity. The

biochemical and structural effects of the different amino acids at position 266 were analysed and underlined its importance for substrate recognition. This strategy allowed the isolation of a mutant enzyme with a commercial interesting activity towards adipyl-7-ADCA, and appears to make the realisation of an industrial applicable CPC acylase feasible.

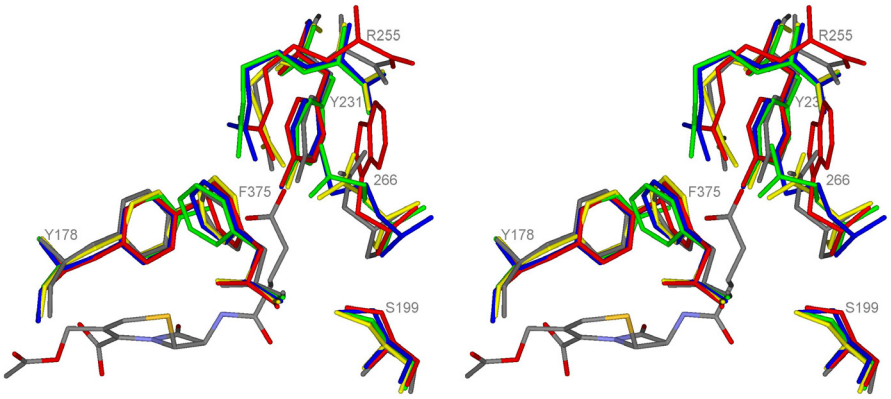

Figure 4. Structural impression of the mutational effects.
Overlay of selected active site residues in the structural models of glutaryl acylase proteins SY-77^{N266Q} (green), SY-77^{N266M} (blue), SY-77^{N266W} (red) and refined wild type (yellow). The models were constructed on the basis of wild type glutaryl acylase (grey; PDB entry 1FM2) using the molecular graphics and modelling program DS Modeling (Accelrys, San Diego, CA, USA). For clarity, glutaryl-7-ACA was added (coordinates from PDB entry 1JVZ).

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