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 semisynthetic cephalosporins and penicillins. Unfortunately, there are no cephalosporin acylases known that can efficiently hydrolyse the aminoadipic side chain of Cephalosporin C. In a previous directed evolution experiment, residue Asn266 of the glutaryl acylase from Pseudomonas *SY-77 was identified as being 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, 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 to the flexibility of the enzyme at this position. Mutants in which Asn266 was changed into Phe, Gln, Trp and Tyr displayed up to twofold better catalytic efficiency (k_{cat}/K_m)than the wild-type enzyme when adipyl-7-aminodesacetoxycephalosporanic acid (adipyl-7-ADCA) was used as substrate, due to a decreased K_m. Only mutants SY-77^{N266H} and SY-77^{N266M} showed an improvement of both catalytic parameters, resulting in 10- and 15-times higher catalytic efficiency with adipyl-7-ADCA, respectively. Remarkably, the catalytic activity (k_{cat}) of SY-77^{N266M} when using adipyl-7-ADCA as substrate was as high as when glutaryl-7-aminocephalosporanic acid (glutaryl-7-ACA) was used, and approaches commercially interesting activity. SY-77^{N266Q}, SY-77^{N266H} and SY-77^{N266M} mutants showed a modest improvement in hydrolysing Cephalosporin C. Since these mutants also have a good catalytic efficiency when adipyl-7-ADCA is used and are still active towards glutaryl-7-ACA, they can be regarded as broad substrate acylases. These results demonstrate that the combination of directed evolution for the identification of important positions, together with saturation mutagenesis for finding the optimal amino acid, is a very effective method for finding improved biocatalysts.*

Introduction

The most widely used antibiotics are semisynthetic 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 by *Penicillium chrysogenum* by a process that comprises several polluting chemical steps, followed by enzymatic deacylation by using penicillin acylase.[1] 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.[2] For the enzymatic deacylation of this novel β-lactam and CPC, novel acylases able to act on adipyl and amino-adipyl side chains, respectively, are needed. As the currently known acylases show little or no activity towards adipyl-7-ADCA and CPC,[3–5] it is of interest to investigate whether they can be created from other acylases, for example, 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.[6] Several variants were selected that showed considerable improvement in hydrolysing adipyl-7-ADCA. Sequencing of these variants demonstrated that mutation of Asn266 into His or Ser occurred frequently. This indicates that position 266 is important for the evolution of glutaryl acylase into an adipyl acylase, although this was not predicted from the crystal structure.[7–9]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 nonconservative mutations often show more potential to change the activity of an enzyme,[10] for example, from a glutaryl acylase to 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 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 using site-directed mutagenesis. Mutant enzymes were purified and catalytic parameters were determined by using glutaryl-7-ACA, adipyl-7-ADCA and CPC as substrates.

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Results

Preparation and purification of mutant enzymes

The megaprimer method was used to create the 19 different amino acid mutants at 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 remaining three mutants were made by site-directed mutagenesis with a specific primer. *Escherichia coli* DH10B cells containing the plasmid encoding the (mutant) enzyme were grown in 300 mL 2*YT medium at 25 °C. After 30 h the cells were harvested and sonicated. The soluble fraction was used to purify the enzyme in a three-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 under different conditions at 17–20 °C for 30–70 h in a volume of up to 2 litres failed. After 70 h at these low temperatures, the strains expressing these mutants reached only half the OD_{600} of strains bearing the wild-type (WT) gene in 30 h 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 insoluble fraction, whereas enzymes containing mutations N266I and N266V were hardly produced at all (data not shown). Consequently, these four mutants were discarded from further analysis.

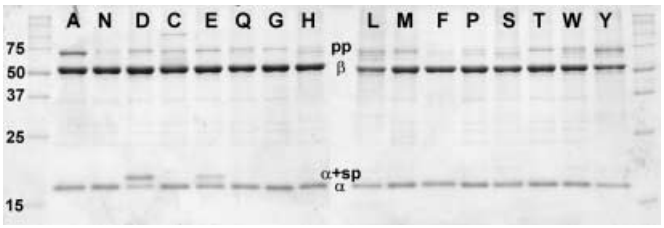

Figure 1. *Effects of mutation of Asn266 on the maturation of glutaryl acylase. Purified enzymes were boiled for 2 min in loading buffer then loaded onto a 12.5 % SDS-PAGE gel. The amino acid at position 266 is depicted above the lane. pp, propeptide; β, β-subunit; sp, spacer peptide; α, α-subunit.*

In order to determine possible interference of the remaining 16 amino acids at this position with enzyme maturation, 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 impaired processing of the propeptide, while mutations N266D and N266E result in less efficient cleavage of the spacer from the α-subunit. Other mutations do not seem to affect the processing of the enzyme.

Activity with adipyl-7-ADCA and glutaryl-7-ACA as substrate

The catalytic parameters of the purified mutant and WT enzymes were determined by using 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 exhibit considerable diversity of changed catalytic parameters. The most striking mutant is SY-77^{N266M}. Its catalytic efficiency when using adipyl-7-ADCA as substrate is 15 times higher than that of the WT enzyme. The K_m of this enzyme is 3.5 times lower and the k_{cat} is 4.5 times higher than WT parameters. The large hydrophobic amino acids Tyr, Trp and Phe at position 266 improve adipyl-7-ADCA hydrolysis by lowering K_m. Mutation N266H results in an almost tenfold improved efficiency in hydrolysing adipyl-7-ADCA, mostly due to a decreased K_m. The amino acid Gln at position 266, the side chain of which is one C atom longer than the side chain of the original Asn residue, induces a twofold decrease in the K_m for adipyl-7-ADCA hydrolysis. Substitution of Asn266 with the small amino acid Cys improves K_m, but decreases k_{cat} for adipyl-7-ADCA; this results in a catalytic efficiency that is similar to that of the WT enzyme. The other amino acids at position 266 have a decreased k_{cat} and/or a higher K_m when using adipyl-7-ADCA as substrate

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

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

[a] The mutant enzymes SY-77^{N266R}, SY-77^{N266I}, SY-77^{N266K} and SY-77^{N266V} did not produce enough enzyme to be purified, therefore their catalytic parameters could not be determined. The remaining 16 enzymes were purified > 90 %. The k_{cat} and K_m for 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.[6] n.d. = not detected.

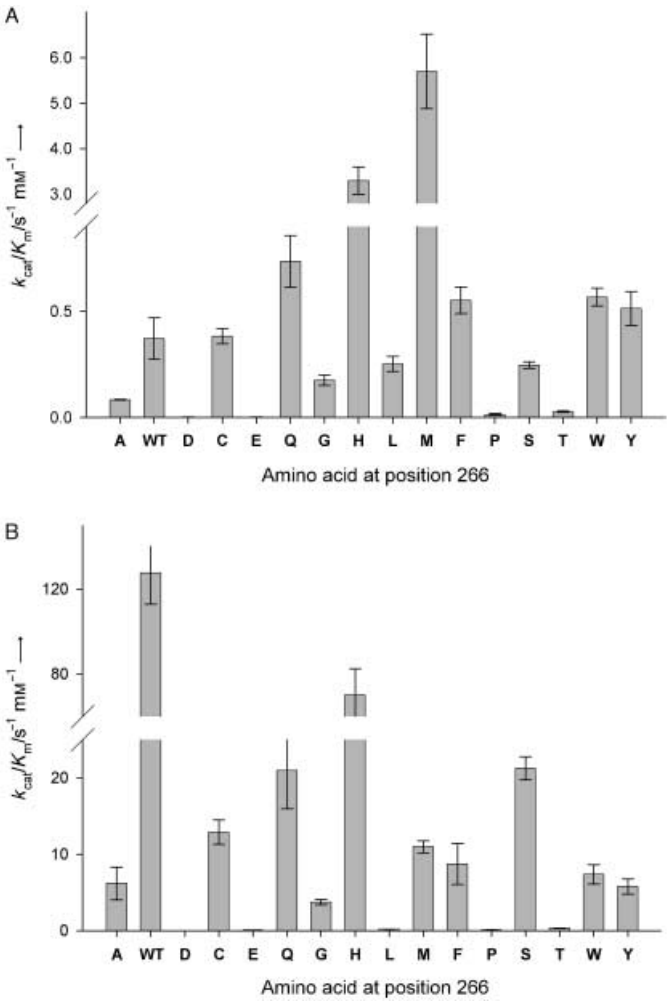

Figure 2. *Catalytic efficiencies of purified enzymes on adipyl-7-ADCA (A) and glutaryl-7-ACA (B). Catalytic parameters were determined by an automated fluorescamine assay performed at different substrate concentrations. The kinetic parameters were calculated from Eadie–Hofstee plots and listed in Table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

resulting in a lower catalytic efficiency. Changing Asn266 into any other amino acid leads in all cases to a lower activity towards glutaryl-7-ACA. The extent of this decrease in k_{cat} and/or K_m is distinct for each amino acid.

Conversion of Cephalosporin C

To determine its activity on CPC, 20 μg of each mutant and the WT enzyme were incubated at 37 °C with 10 mM CPC. The WT enzyme and mutants SY-77^{N266Q}, SY-77^{N266H}, SY-77^{N266M} and SY-77^{N266W} hydrolysed the substrate after 20 h. These activities were too low for K_m and k_{cat} determination. In order to avoid long incubation times that might 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 CPC conversion in the first 5 h was determined and normalised with respect to WT enzyme (Figure 3). Mutants SY-77^{N266Q} and SY-77^{N266H} displayed almost a 100% improvement in converting CPC compared to WT enzyme, whereas SY-77^{N266M}

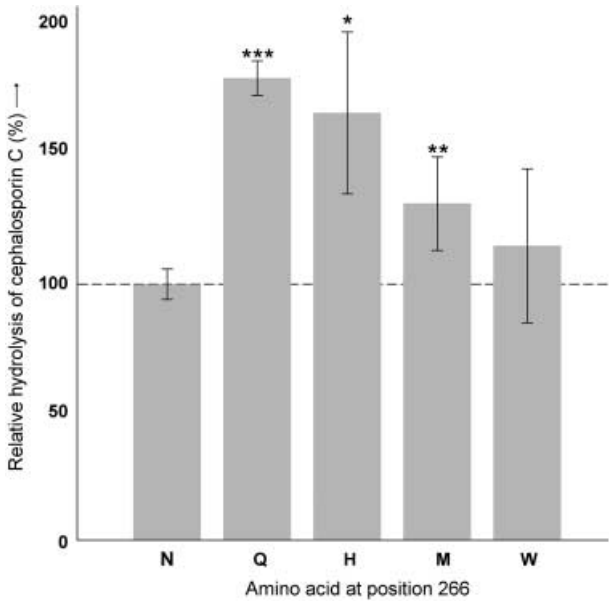

Figure 3. *Hydrolysis of 10 mM CPC by selected Asn266 mutants in the first 5 h. The slopes of hydrolysis curves were calculated for at least six different experiments. Conversion of CPC by WT enzyme was set at 100%. Values were tested for statistically significant differences by Student's t-test; * = $p < 0.01$, ** = $p < 0.005$, *** = $p < 0.0005$, mutant N266W was not significantly different.*

improved by 30%. The conversion rate of SY-77^{N266W} is not significantly different from that of WT enzyme.

Discussion

In a previous study with error-prone PCR, amino acid Asn266 of the glutaryl acylase of *Pseudomonas* SY-77 was pointed out as one of the residues that defines substrate specificity.[6] This result was surprising since Asn266 was not thought to be directly involved in substrate binding, but could interact via Tyr351 with Arg255, which accommodates the carboxylate head of the side chain.[8,9] In order to elucidate the role of Asn266 in substrate specificity, we 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 maturation and hydrolysis of several substrates. The catalytic properties varied over a broad spectrum (Table 1, Figures 2 and 3); this points to the active role of position 266 in enzymatic activities.

Since the maturation of an enzyme is crucial for its activation,[11] 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 improper folding of the enzyme. The presence of Ile or Val at position 266 causes low enzyme production in contrast to other aliphatic residues. It is possible that branching at 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; this indicates that the first intramolec-

ular 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 α-subunit) is supposed to involve a similar catalytic mechanism as hydrolysis of the substrate.[11,12] Therefore, it would be expected that changes in residues involved in catalysis also interfere with enzyme processing. However, most of the purified mutant proteins do not show the uncleaved 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 Glu188 for Arg255.[13]

Remarkably, the K_m of the enzyme in hydrolysing adipyl-7-ADCA has improved in half of the purified mutants, thus 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, only 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 4.5-fold higher k_{cat} and 3.5-fold lower K_m, resulting in 15-fold better catalytic efficiency (Figure 2). The k_{cat} for hydrolysing adipyl-7-ADCA is the same as that of glutaryl-7-ACA for this mutant and approaches enzymatic activities that can be 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 to WT enzyme and show a 1.5 to twofold 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). In a similar way to SY-77^{N266M}, SY-77^{N266Q} appears to displace Arg255, allowing better accommodation of the adipyl side chain. The catalytic properties of this mutant, when using adipyl-7-ADCA as substrate, are however not as good as those of 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} show similar k_{cat} values and bind adipyl-7-ADCA twice as well as WT enzyme. 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 enlargement of the substrate binding pocket and probably also by increasing hydrophobic interactions.

None of the mutants shows an increased activity or affinity for the preferred substrate, glutaryl-7-ACA. Evolution optimised this amino acid, logically resulting in decreased activity upon substitution, as is seen in most mutagenesis studies.[15] Only mutants SY-77^{N266H} and SY-77^{N266S}, which were already found in the directed evolution experiment, have similar catalytic parameters as WT enzyme when using glutaryl-7-ACA as substrate; this indicates 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.

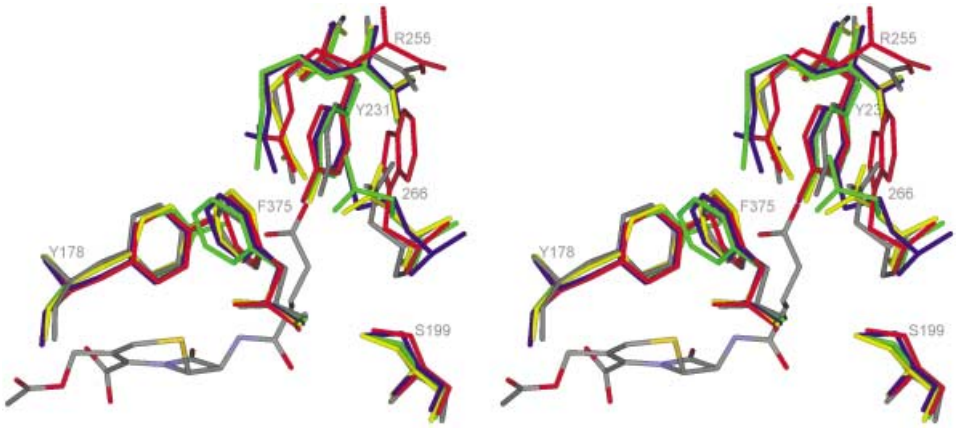

Figure 4. *Structural impression of the mutational effects. Overlay of selected active site residues in the structural models of glutaryl acylase proteins SY-77^{N266Q} (green), SY-77^{N266M} (blue), SY-77^{N266W} (red) and refined wild-type (yellow). The models were constructed on the basis of* wild-type glutaryl acylase *(grey; PDB entry 1FM2) by 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).*

Although the importance of residue 266 became evident from a selection experiment with the adipyl side chain, we hypothesised that this residue might also 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 WT enzyme (Figure 3). This activity is, however, still two

orders of magnitude lower than the hydrolysis of glutaryl-7-ACA. Due to 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 WT enzyme. 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 have 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 more radical changes in enzyme activity to be found. 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 commercially interesting activity towards adipyl-7-ADCA, and appears to make the realisation of an industrially applicable CPC acylase feasible.

Experimental Section

Bacterial strains and plasmids and DNA manipulations: Plasmid pMcSY-2[6] and the leucine deficient *E. coli* strain DH10B (Invitrogen) were used for the cloning of libraries and the expression of SY-77 acylase.

Molecular DNA techniques were executed by following standard protocols.[16] Enzymes used for DNA manipulations were purchased from New England Biolabs and Invitrogen and applied according to the manufacturer's instructions. Plasmid DNA was isolated by using the Qiaprep Spin Miniprep Kit (QIAGEN). DNA was extracted from agarose gels by using the QIAquick Gel Extraction Kit (QIAGEN).

Mutants were made by using the megaprimer method described by Landt et al.[17] All PCR reactions were performed in a Mastercycler® gradient thermocycler (Eppendorf) with recombinant *Pfu* polymerase (Stratagene). A 5′-universal primer, 5′-CGCCGGACTTCGAGATCTA-3′, and a 3′-mutagenic primer, 5′-CATGCCGTTGACGGT-**SNN**GGTGATGCCCATCCG-3′, were used in the first PCR with 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, 5′-GATTGGTGACCCGCGGCA-3′. 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 by using *Bgl*II and *Sst*II. Resulting plasmids were sequenced in order to determine the amino acid at position 266. Amino acids that were not obtained with 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 WT enzymes were purified in three chromatography steps on a Duoflow system (Bio-Rad) by using columns from Amersham Biosciences as described before.[6] Typically, *E. coli* DH10B containing the plasmids encoding the desired enzymes were grown in 2*YT medium (300 mL, 30 h, 25 °C) supplemented with chloramphenicol (50 μg mL^{-1}) 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, 17 000 *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.[6] Mutants that showed a low concentration of acylase were grown again for up to 70 h at 17–20 °C in 2 L of the same medium. Protein concentrations of purified samples were determined by using the DC Protein Assay (Bio-Rad) with bovine serum albumin as the reference protein. Samples were analyzed on a 12.5 % SDS-PAGE gel stained with Coomassie Brilliant Blue.[18]

Enzyme characterization: Kinetic parameters of purified WT and mutant enzymes on glutaryl-7-ACA and adipyl-7-ADCA were tested in an automated fluorescamine assay as described before.[6] Hydrolysis of CPC was determined by the fluorescamine assay, with the highest possible concentration of substrate with respect to background values. Enzyme (20 μg) 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 h incubation at 37 °C, a 40 μL aliquot of reaction mixture was transferred to acetate buffer (140 μL, 0.5 M, pH 4.5), after which fluorescamine in acetone (20 μL, 1 mg mL^{-1}) was added. After incubation at room temperature for 60 min, the A380 was measured. The enzymes that 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 5 h. In these samples, hydrolysis of CPC was also measured by 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 Student's t-test.

Modelling of (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 and modelled structures. The models were constructed on the basis of wild-type glutaryl acylase (PDB entry 1FM2) by 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.

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Keywords: antibiotics · enzyme catalysis · mutagenesis · protein engineering · substrate specificity

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