DOI: 10.1002/adsc.200700320

Evaluation of Different Glutaryl Acylase Mutants to Improve the Hydolysis of Cephalosporin C in the Absence of Hydrogen Peroxide

Fernando López-Gallego,[a] Lorena Betancor,[a] Charles F. Sio,[b] Carlos R. Reis,[b] Pol Nadal Jimenez,[b] Jose M. Guisan,[a] Wim. J. Quax,[b,*] and Roberto Fernandez-Lafuente[a,*]

[a] Departamento de Biocatalisis, ICP-CSIC, 28049 Madrid, Spain
Fax: (+34)-91-585-4760; E-mail: rfl@icp.csic.es
[b] Department of Pharmaceutical Biology, University of Groningen, Groningen, The Netherlands
E-mail: w.j.quax@rug.nl

Received: June 29, 2007; Published online: January 4, 2008

Abstract: 2-Oxoadipoyl-7-ACA is an intermediate in the conversion of cephalosporin C (CPC) to 7-aminocephalosporanic acid (7-ACA) when using a new route involving D-amino acid oxidase, catalase and glutaryl acylase. A key point in the reaction design is to avoid the accumulation of hydrogen peroxide in the reaction medium as the yields of 7-ACA decrease in the presence of this compound due to its low stability. Looking for an enzyme with improved activity towards 2-oxoadipoyl-7-ACA, different mutants of glutaryl acylase from *Pseudomonas* SY-77 with an improved activity towards adipoyl-7-ACA were evaluated. The best results on 2-oxoadipoyl-7-ACA hydrolysis were found with the double mutant Y178F+F375H, which showed a K_{cat} increase of 6.5-fold and a K_m decrease of 3-fold compared to the wild-type (wt) enzyme. When this enzyme was tested in the tri-enzymatic system to convert CPC into 7-ACA, this mutant permitted us to reach more than an 80% yield of 7-ACA using a 3-fold mass excess compared to DAAO; while the wt enzyme gave only a 40% yield. Therefore, the application of this new mutant to the one-pot conversion of CPC to 7-ACA gives very good result in terms of efficiency, yield and rate of the process.

Keywords: 7-ACA; D-amino acid oxidase; gutaryl acylase; hydrogen peroxide; semi-synthetic cephalosporins

Introduction

The semi-synthetic cephalosporins, which are produced from a cephalosporin nucleus, either 7-aminocephaloporanic acid (7-ACA) or 7-aminodesacetoxycephalosporanic acid (7-ADCA) are among the most used antibiotics. It was estimated that approximately 1.95 million kg of 7-ADCA and 2.14 million kg of 7-ACA were produced in 2000.[1]

Chemical deacylation of cephalosporin C (CPC),[2] a fermentation product, is the primary method used industrially to produce 7-ACA. This method is economically feasible using currently available chemical processes. However, due to the large amounts of hazardous chemicals used, there is a concern for environmental and safety reasons. To overcome these problems, efforts are being made to develop an efficient, entirely enzymatic process for the conversion of CPC to 7-ACA.

After many efforts,[3] the direct conversion of CPC to 7-ACA by cephalosporin C acylase has been successfully reported.[4] However, nowadays, the most widely used enzymatic route at the industrial level is the conversion of CPC to 7-ACA in a two-pot system with two different enzymes. Firstly, D-amino acid oxidase (DAAO) catalyzes the oxidative deamination of CPC yielding 7-β-(5-carboxy-5-oxopentanamido)-cephalosporanic acid (2-oxoadipoyl-7-ACA) and hydrogen peroxide. This latter product is able to catalyse the decarboxylation of 2-oxoadipoyl-7-ACA producing glutaryl-7-aminocephalosporanic acid (GL-7-ACA). Secondly, GL-7-ACA is hydrolysed to 7-ACA and glutaric acid by glutaryl acylase (GAC).[5] The main drawback of this system is the presence of hydrogen peroxide during the reaction, which leads to the inactivation of both enzymes, and prevents the use of just one reactor for the process, to avoid the exposition of GAC to this inactivating reagent.[6] Recently, a new route for the enzymatic conversion of CPC to 7-ACA was reported.[7] This is a tri-enzymatic, one-pot system where hydrogen peroxide is eliminat-

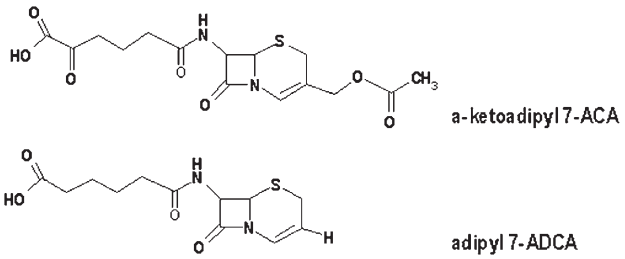
ed *in situ* due to the action of catalase (CAT) which is co-immobilised with DAAO.[8] The main advantage over the conventional system is the absence of hydrogen peroxide, avoiding enzyme inactivation by this compound and permitting the use of just one reactor. However, now the product of the DAAO oxidation is 2-oxoadipoyl-7-ACA, a product that is unstable and needs to be rapidly hydrolysed to 7-ACA, in order to obtain sufficiently high 7-ACA yields.

The main problem of this system is the relatively low specific activity of wild-type GAC towards 2-oxoadipoyl-7-ACA. As a consequence, a large excess of GAC over DAAO is needed to prevent accumulation of 2-oxoadipoyl-7-ACA.[7] Thus, any improvement on the GAC activity-affinity against this new compound may permit us to reduce the GAC/DAAO ratio and increase the global volumetric activity of the biocatalysts employed.

During the last years, several mutants of different glutaryl acylases from different microorganisms, mainly from *Pseudomonas* species, have been developed in order to improve the affinity and activity of the enzyme[9] towards adipoyl 7-ADCA. This compound is produced by feeding adipic acid to a *Penicilium chrysogenum,* which had been genetically modified to express an expandase enzyme,[10] and the hydrolysis of adipoyl-7-ADCA would yield 7-ADCA in an easy and environmental friendly way, opening a new route to produce the cephalosporin nucleus.

Thus, there are available several GAC mutants that present an important increase in the adipoyl-7-ADCA hydrolase activity.[9,11] In *Pseudomonas* SY-77 GAC, it was possible to identify some residues – Y178,[11b] N266, and P375[11a] – that are important for the spatial arrangement of adipoyl-7-ADCA in the substrate binding pocket of the enzyme. Adipoyl-7-ADCA has the same side-chain length as 2-oxoadipoyl-7-ACA and the only difference between both compounds is the carbonyl group in the α-carbon of 2-oxoadipoyl-7-ACA (Scheme 1).

Here, we have studied the catalytic properties of different SY-77 GAC mutants for their activity towards 2-oxoadipoyl-7-ACA and their impact on yield and conversion rate in the one-pot conversion of CPC to 7-ACA.



Scheme 1. Adipoyl-7-ADCA and 2-oxoadipoyl-7-ACA structures. The difference between the two substrates is mainly in their aliphatic side-chains. In the case of 2-oxoadipoyl-7-ACA, the side chain has a carbonyl group in the α-carbon, whereas the adipoyl-7-ADCA does not have this carbonyl group in its side-chain. The differences in the C-3 modifications between the two substrates do not translate in a different way to the interaction of both substrates with the binding pocket of the enzyme.

Result and Discussion

Kinetic Characterisation of GAC Activity towards 2-Oxoadipoyl-7-ACA

The kinetic parameters were determined for four mutants, two single mutants (Y178H and N266H) and two double mutants (Y178H+N266H and Y178F+F375H), and the wild-type (wt) enzyme towards glutaryl-7-ACA and 2-oxoadipoyl-7-ACA (Table 1).

The wt enzyme showed K_m and K_{cat} values on 2-oxoadipoyl-7-ACA that were 11-fold higher and 17-fold lower, respectively, as compared to glutaryl-7-ACA.

The N266H mutant showed a very high K_m for the 2-oxoadipoyl-7-ACA, even higher than the K_m of wt enzyme towards this substrate. On the other hand, the K_{cat} of the mutant N266H for 2-oxoadipoyl-7-ACA was slightly increased with respect to the same parameter for the wt enzyme. Due to these values of K_m and K_{cat} the catalytic efficiencies were very similar for both wt and N266H mutant.

Mutant Y178H showed a catalytic efficiency (K_{cat}/K_m) 6-fold higher towards 2-oxoadipoyl-7-ACA comparing to the catalytic efficiency of wt enzyme towards the same substrate. This fact is mainly due to the increment in the K_{cat} (K_m slightly decreased).

Table 1. Kinetics parameters of wt and different mutant GAC towards different substrates. The reactions were carried out as described in the Experimental Section at 37 °C and pH 7.5. The catalytic efficiency was defined as K_{cat}/K_m.

Enzyme	Glutaryl-7-ACA			2-Oxoadipoyl-7-ACA		
	K_m [mM]	K_{cat} [s^{-1}]	K_{cat}/K_m	K_m [mM]	K_{cat} [s^{-1}]	K_{cat}/K_m
wt	0.34±0.02	2.0±0.01	5.8	3.9±0.1	0.12±0.01	0.03
Y178H	0.8±0.05	1.4±0.1	1.7	2.2±0.1	0.4±0.02	0.18
N266H	0.4±0.03	2.33±0.1	6.1	5.0±0.3	0.24±0.01	0.047
Y178H+N266H	1.4±0.1	0.5±0.02	0.35	19.4±0.3	0.05±0.002	0.003
Y178F+F375H	2.81±0.1	0.33±0.1	0.12	1.27±0.1	0.77±0.04	0.63

When double mutant Y178H+N266H was analysed, the catalytic efficiency towards 2-oxoadipoyl-7-ACA was the lowest of all studied enzymes, even more, the catalytic efficiency decreased (10-fold) towards 2-oxoadipoyl-7-ACA comparing with the wt enzyme. This very low catalytic efficiency was due to both a K_m increase and a K_{cat} decrease (Table 1), although it has been reported to improve the enzyme performance against adipoyl-7-ACA.[12]

The double mutant Y178F+F375H showed the best results in all parameters studied towards 2-oxoadipoyl-7-ACA; an improvement of 3- and 6.5-fold in K_m, and K_{cat}, respectively, the values for K_m and K_{cat} being 1.27 and 0.77, respectively. The K_{cat} of this mutant towards 2-oxoadipoyl-7-ACA was around 40% of the K_{cat} of wt GAC towards glutaryl-7-ACA, while the K_m was only around double than of the K_m of the wt GAC towards glutaryl-7-ACA. This double mutant was obtained through rational randomisation of the most important amino acids implied in the interaction between the enzyme and the substrate, addressed to improve the efficiency against adipoyl-7-ADCA. Thus, this mutant also has a highly improved activity on 2-oxoadipoyl-7-ACA allowing us to go from a catalytic efficiency around 200-fold lower towards 2-oxoadipoyl-7-ACA than glutaryl-7-ACA (using wt enzyme), to only a factor of 9 (using double mutant Y178F+F375H). Considering that all these enzymes presented an improved performance against adipoyl-7-ACA, results suggest that the 2-oxo group in 2-oxoadipoyl-7-ACA may be playing an important role in the recognition of this substrate by the enzyme. Thus, a detailed modelling of the enzyme interactions was performed.

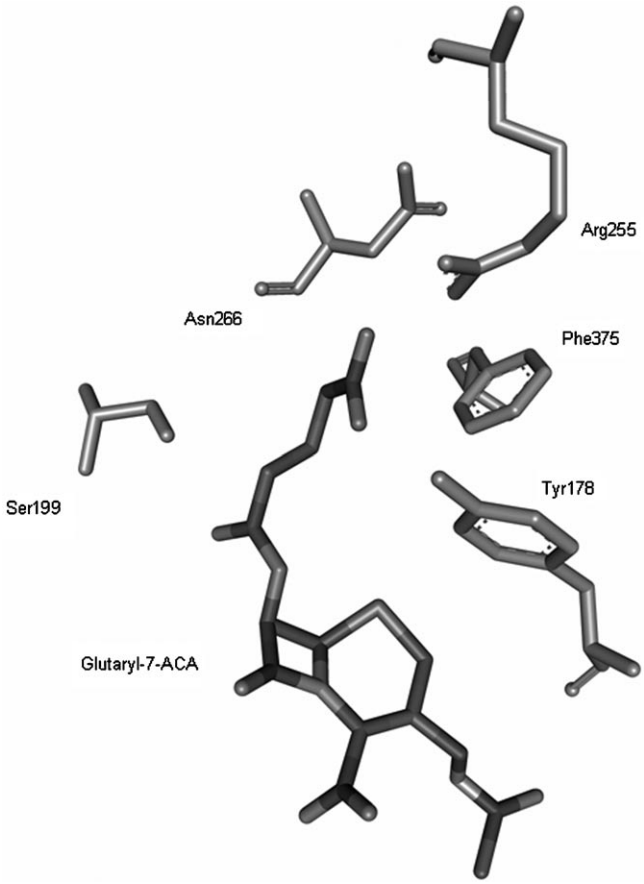


Figure 1. 3D structure of the binding of the side chain of glutaryl-7-ACA by glutaryl acylase. Residues Asn 266, Tyr 178 and Phe 375 were mutated yielding different single and double mutants. The residue Arg 255 interacts with the carboxy group of the GL-7-ACA side chain.

Models of the Active Site of Native and Mutated Glutaryl Acylase with Bound Substrates

Position 266 was demonstrated to be indirectly involved in binding the carboxyl group of the substrate side-chain *via* Arg255[11a] (Figure 1). Replacement of Asn266 by His was very favourable to the binding between the enzyme and the adipoyl-7-ADCA,[11a] but less favourable for binding to 2-oxoadipoyl-7-ACA (Table 1). The model does not give a straightforward explanation for this.

In contrast, certain mutations in position 178 improve the 2-oxoadipoyl-7-ACA hydrolase activity with regard to the wt enzyme. The change of Y178 by His was earlier described to increase the hydrolysis of adipoyl-7-ADCA,[11b] and was explained by assuming that histidine allows better accommodation of the longer side-chain of the new substrates (Figure 1).[11b] This also seems to apply to the hydrolysis activity towards 2-oxoadipoyl-7-ACA, which is improved 6 fold (Table 1).

The best results for both adipoyl-7-ADCA and 2-oxoadipoyl-7-ACA hydrolysis were obtained with double mutant Y178F+F375H. This mutant was obtained through a rational randomisation strategy.[12] Substitution of F375 by various residues was shown to improve adipoyl-7-ADCA hydrolysis activity.[13].

Both mutations would have a synergistic effect for the spatial arrangement of both adipoyl and 2-oxoadipoyl side-chains, explaining the improvements on the hydrolysis activity towards longer side-chain substrates (adipoyl-7-ADCA and 2-oxoadipoyl-7-ACA). Remarkably, the double mutant was able to hydrolyse 2-oxoadipoyl with a K_{cat} value which is 40% of the wt enzyme hydrolysis activity towards glutaryl-7-ACA. This is an interesting value to use this mutant in the one-pot tri-enzymatic synthesis to convert CPC to 7-ACA.

When looking in more detail at the 3D model of positions 178 and 375 of cephalosporin acylase it seems that in SY-77$^{Y178F+F375H}$ the scissile bond of 2-oxoadipoyl-7-ACA is placed at a more favourable po-

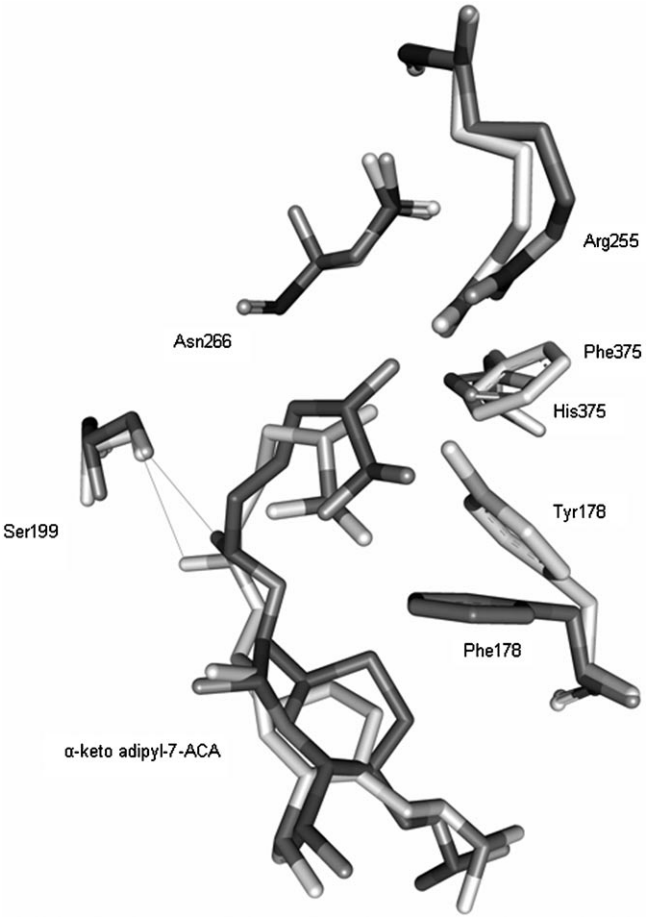


Figure 2. Structural impression of the mutational effects. Overlay of 2-oxoadipoyl-7-ACA and active site residues in the structural models of wild-type glutaryl acylase proteins SY-77$^{Y178F\ F375H}$ (dark grey) and refined wild-type (light grey).

sition with respect to the Ser199 when compared with wt (Figure 2).

Also the presence of polar residues allowing additional hydrogen bonds may stabilise the position of the side-chain and thereby also of the scissile bond towards the catalytic serine.

The best result was therefore obtained when simultaneously sufficient space for the longer side-chain was created and formation of new hydrogen bonds is allowed.

Use of the Mutant Enzyme in the Tri-Enzymatic Conversion of CPC to 7-ACA

The conversion of CPC to 7-ACA was carried out using a tri-enzymatic system in only one reactor comparing wt GAC and the mutant enzyme Y178F+F375H (Figure 3). The specific activities towards 2-oxoadipoyl-7-ACA of both immobilised enzymes were 0.058 and 0.58 IU/mg, respectively, hydrolysing

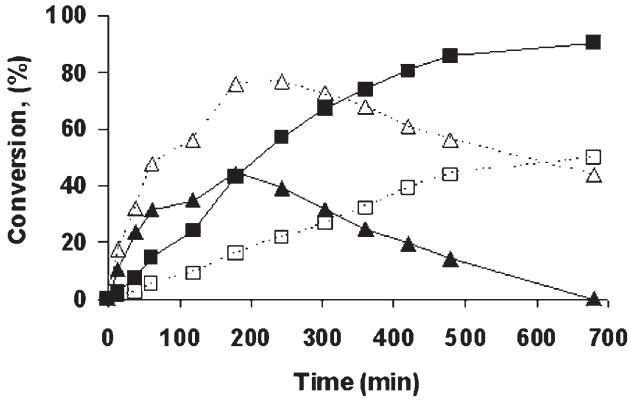


Figure 3. One pot 7-ACA production with native (open symbols and dotted lines) and mutant Y178F/F375H (full symbols and solid lines).The reactions were carried with 3-fold mass excess of GAC (for mutant and native enzyme) with respect to DAAO. Samples were withdrawn at different times and analysed by HPLC: 7-ACA (*squares*). 2-oxoadipoyl-7-ACA (*triangles*).

10 mM of substrate. The specific activity of DAAO to carry out the oxidative deamination of CPC was much higher than the specific activity of wt GAC to carry out the hydrolysis of 2-oxoadipoyl-7-ACA (around 20-fold higher). Moreover, DAAO will be acting against a high concentration of substrate while GAC will be acting against very low concentrations of 2-oxoadipoyl-7-ACA (because we intend to prevent accumulation of this product).

When the one-pot system was carried out with the immobilised mutant GAC Y178F+F375H as biocatalyst, the reaction time with mutant GAC was 3.5-fold shorter than the one with wt GAC to get the 50% yield of 7-ACA (Figure 3). Moreover, the maximum accumulation of 2-oxoadipoyl-7-ACA in the reaction (using a ratio of GAC/DAO of 3) was of 80% with the wt GAC and 40% with the mutant one (Figure 3). Even more interestingly, the mutant enzyme yielded a 95% conversion of 7-ACA while the native enzyme yielded only around 60% (Figure 3).

However, the difference between the 2-oxoadipoyl-7-ACA hydrolase activity of wt and mutant (Y178F+F375H) GAC in the one-pot system is lower than one could expect regarding the catalytic efficiency improvement. This fact could be due to different causes. For example, the wt and mutant GAC acts over a very low concentration of 2-oxoadipoyl-7-ACA, thus, the most important kinetic parameter would be the K_m (3-fold higher for the mutant than for the wt enzyme). Moreover, the system with wt enzyme accumulated more 2-oxoadipoyl-7-ACA than the system with mutant enzyme. This fact means that the 2-oxoadipoyl-7-ACA concentration using wt GAC is much higher than the concentration over which the mutant

enzyme is working. In this way, although the mutant enzymes was 18-fold more efficient than the wt one when the kinetics parameters were calculated, in the one-pot system, the mutant enzyme was 3.5-fold more efficient than the wt one.

Conclusions

Some important GAC residues implied in the recognition and hydrolysis of 2-oxoadipoyl-7-ACA have been detected , permitting us to produce a mutant glutaryl acylase having much better properties for the hydrolysis of 2-oxoadipoyl-7-ACA than the wild-type enzyme. However, the mutant enzymes were selected for the hydrolysis of adipoyl-7-ADCA, a substrate somehow similar but not identical to the new one. Thus, saturation mutations in these positions could permit us to obtain new mutants with even better properties than the Y178F+F375.

Experimental Section

Chemicals

The β-lactam substrates like glutaryl-7-ACA, adipoyl-7-ADCA and CPC (as sodium salt) were kindly donated by DSM (Netherlands). Fluorescamine was from Sigma–Aldrich (Lousina, USA). Agarose 4 BCL was supplied by Pharmacia

D-Amino acid oxidase from *Trigonopsis variabilis* (DAAO) was obtained from Recordati (Milan, Italy). Catalase from bovine liver (CATb) was purchased from Fluka (Buch, Switzerland). Glyoxyl agarose 4-BCL (40 μmoles of glyoxyl groups/g support) and polyethyleneimine 600 KDa-agarose 4BCL supports were prepared according to previous reports.[14] The Kromasil C-8, (5 μm, 250×4.6 mm) column was purchased from Analisis vínicos (Tomelloso, Spain). Amonium acetate (HPLC grade) was purchased from Merck & Co. Acetonitrile was purchased from Biosolve (Netherlands). All other reagents were of analytical grade.

Preparation of 2-Oxoadipoyl-7-ACA

5 mL of 50 mM of CPC pH 7 were incubated with 0.1 g of co-immobilized derivative DAAO/CATb prepared as described in ref.[7] The conversion of CPC to 2-oxoadipoyl-7-ACA was checked by HPLC. When 100% of CPC had been consumed, the suspension was vacuum filtered and the supernatant was immediately used in the kinetics characterisation experiments.

Production of Different Glutaryl Acylase Mutants from *Pseudomonas* SY-77

Different mutants were constructed and produced as previously detailed. The mutant Y178H was described in ref.[11b], the mutant Y178H+N266H (Thesis Linda Otten) and the mutant Y178F+F375H were described in ref.[12] and the mutant N266H was described in ref.[11a]

Purification of Enzymes

Mutant and wt enzymes were purified by three chromatography steps on a Duoflow system (Bio-Rad) using columns from http://www.jbc.org/cgi/inline?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 $\mu g\,mL^{-1}$ 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).After this treatment, the cell-free extract was loaded onto a HiTrapQ column. The proteins were eluted with a linear gradient of 0–1 M NaCl in 50 mM Tris-HCl, pH 8.8. Afterwards, 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. The pooled fractions in 50 mM Tris-HCl, pH 8.8 were stored at −20 °C. Typical yields were 10 mg of more than 90% pure enzyme per liter of culture.

Characterisation of Wild-Type and Mutant Enzymes

Enzyme activities were determined towards glutaryl-7-ACA and 2-oxoadipoyl-7-ACA using the fluorescamine assay,[15] in a 96-well format. All pipetting steps were performed by a Multiprobe II (Camberra Packard). The kinetic parameters of these mutants with the two different substrates were determined by the measuring of the initial hydrolysis rate on a range of substrate concentrations with a fixed amount of enzyme. 140 μL of 20 mM phosphate buffer, pH 7.5, with different concentrations of different substrates (0.005–0.5 mM glutaryl-7-ACA and 0.02–6.25 mM 2-oxoadipoyl-7-ACA) were preheated at 37 °C. 40 μL of phosphate buffer containing an appropriate amount of purified enzyme were added, starting the reaction. After 30 and 60 min of incubation at 37 °C, 40 μL of the reaction mixture were withdrawn and mixed with 140 μL of 2.5 M sodium acetate pH 4.5 to stop the reaction and 20 μL of 1 $mg\,mL^{-1}$ fluorescamine in acetone. The absorbance was measured at 380 nm after 60 min of incubation at room temperature with the fluorescamine. Kinetics parameters were obtained by fitting the experimental data from Lineweaver–Burk plots, and the mean values of at least four independent measured were calculated. The K_{cat} was calculated using the theoretical molecular mass of the mature enzyme, 75.9 kDa.

Models of the Active Site of Native and Mutated Glutaryl Acylase with Bound Substrates

Modelling was performed using Discovery Studio 1.7 (Accelrys, San Diego, CA, USA). To get a structural view of the mutational effects we made an overlay of selected active site residues using the structural models of the glutaryl acylase proteins containing the mutations Y178F and F375H. The wild-type structure was also refined using the same parameters to account for differences in the modelled structures.

The model for the enzyme was constructed on the basis of the free *P.diminuta* KAC-1 (PDB ID 1M2). Hydrogens were added automatically and the environment of the acylase was modelled as vacuum. Models of the substrates were con-

structed and energy minimised using the CHARMm force field.[16] Minimisation was done using a dielectric constant of 1 and a non-bonded cut-off distance of 12 Ångstroms. The substrate was docked using the grid based approach CDOCKER[17] using as coordinates the binding site reported for glutaryl-7-ACA (PDB ID 1JVZ). Initially the enzyme was fixed and the atoms of the substrate were allowed to move. In subsequent rounds of minimisation the constraints on the amino acids forming the active site were removed and replaced by distance constraints based on the reported distances observed in the complex with the glutaryl-7-ACA.[18] The structures were refined by energy minimisation consisting of 150 steps of steepest descent followed by 2500 iterations of the adopted basis-set Newton–Raphson algorithm.

Immobilisation of the Enzymes

The co-immobilisation of DAAO and CATb, 1 mg of DAAO and 10 mg of CATb per gram of support, and the immobilisation of glutaryl acylase (1 mg of GAC/g of support) (wt and Y178F+F375H mutant) were carried out as previously described.[7,14] More than 90% of the offered protein was immobilised in both cases, and we could recover more than 90% of the offered activity and the kinetics constants did not change after the immobilisation process in both wt and mutant glutaryl acylase.

Control of the Reaction

The products of the reactions were determined through HPLC with Kromasil C8 [5 μm, 250×4.6 mm) column, mobile phase 20 mM ammonium acetate pH 5.2; acetonitrile (95:5 v/v)]. The retention times of the different products were: 4.1 min for CPC, 4.9 min for 7-ACA, 6.7 min for 2-oxoadipoyl-7-ACA and 12.3 min for GL-7-ACA.

Conversion of CPC to 7-ACA by the Tri-Enzymatic System

Different ratios (2, 3, 5, 7,5 and 15) between the mass of GAC and DAAO were added to 2 mL of 5 mM CPC in 0.1 M potassium phosphate buffer at pH 8 and 25 °C. We used one DAO biocatalyst co-immobilised with CATb on glyoxyl agarose (1 mg DAAO:10 mg CATb/g biocatalyst) and other glutaryl acylase biocatalyst (wt or mutant) (1 mg GAC/g biocatalyst) immobilised on PEI(25 kDa) and treated with glutaraldehyde.[14] The suspension was gently stirred and the pH was maintained at 8.0 by adding 4 M NaOH during all the process. Periodically, samples were withdrawn and analysed *via* HPLC as described above. Glutaryl-7-ACA did not appear through the reaction indicating that the co-immobilisation of DAAO and CAT was working perfectly.

Acknowledgements

This project has been funded by the Spanish CICYT (PPQ 2002–01231A) and by STW, with the grant GBI4704 and part was also funded by EU grant EST-020278–2. We gratefully recognise a predoctoral fellowship and a short-term fellowship for Fernando Lopez-Gallego from Spanish MCYT.

References

[1] R. P. Elander, *Appl. Microbiol. Biotechnol.* **2003**, *61*, 385–392.
[2] H. P. B. Fechtig, H. Bickel, E. Vischer, *Helv. Chim. Acta* **1968**, *51*, 1109–1120.
[3] a) A. Matsuda, K. Toma, K. Komatsu, *J. Bacteriol.* **1987**, *169*, 5821–5826; b) B. B. Oh, *Biochem. Biophys. Res.Commun.* **2003**, *310*, 19–27.
[4] L. Pollegioni, S. Lorenzi, E. Rosini, G. L. Marcone, G. Molla, R. Verga, W. Cabri, M. S. Pilone, *Protein Sci.* **2005**, *14*, 3064–3076.
[5] a) H. D. Conlon, J. Baqai, K. Baker, Y. Q. Shen, L. B. Wong, R. Noiles, W. Raush, *Biotechnol. Bioeng.* **1995**, *46*, 510–513; b) A. A. Nikolov, *Enzyme Microb. Technol.* **1994**, *16*, 1037–1041; c) A. Parmar, H. Kumar, S. S. Marwaha, J. F. Kennedy, *Crit. Rev. Biotechnol.* **1998**, *18*, 1–12.
[6] a) I. de La Mata, F. Ramon, V. Obregon, M. P. Castillon, C. Acebal, *Enzyme Microb. Technol.* **2000**, *27*, 234–239; b) E. S. Dey, J. R. Miller, S. Kovacevi, K. Mosbach, *Appl. Biochem. Biotech.* **1991**, *27*, 239–250.
[7] F. Lopez-Gallego, L. Betancor, A. Hidalgo, C. Mateo, R. Fernandez-Lafuente, J. M. Guisan, *Adv. Synth. Catal.* **2005**, *347*, 1804–1810.
[8] R. Fernandez-Lafuente, V. Rodriguez, J. M. Guisan, *Enzyme Microb. Technol.* **1998**, *23*, 28–33.
[9] C. F. Sio, W. Quax, *Curr. Opin.Biotechnol.* **2004**, *15*, 349–355.
[10] L. Crawford, A. M. Stepan, P. C. McAda, J. A. Rambosek, M. J. Conder, V. A. Vinci, C. D. Reeves, *Biotechnol. (NY)* **1995**, *13*, 58–62.
[11] a) L. G. Otten, C. F. Sio, J. Vrielink, R. H. Cool, W. J. Quax, *J. Biol. Chem.* **2002**, *277*, 42121–42127; b) C. F. Sio, A. M. Riemens, J. M. van der Laan, R. M. Verhaert, W. J. Quax, *Eur. J. Biochem.* **2002**, *269*, 4495–4504.
[12] C. F. Sio, *Mutans and homologs of cephalosporin acylase*, *PhD Thesis*, University of Groningen, **2004**.
[13] C. F. Sio, L. G. Otten, R. H. Cool, W. J. Quax, *Biochem. Biophys. Res. Comm.* **2003**, *312*,755–760.
[14] a) J. M. Guisan, *Enzyme Microb Technol.* **1988**, *10*, 375–382; b) C. Mateo, O. Abian, R. Fernandez-Lafuente, J. M. Guisan, *Biotechnol. Bioeng.* **2000**, *68*, 98–105; c) N. Alonso, F. Lopez-Gallego, L. Betancor, A. Hidalgo, C. Mateo, J. M. Guisan, R. Fernandez-Lafuente, *J. Mol. Catal. B: Enzym.* **2005**, *35*, 57–61.
[15] F. Reyes, M. J. Martinez, J. Soliveri, *J. Pharm. Pharmacol.* **1989**, *41*, 136–137.
[16] A. D. J. MacKerell, B. R. Brooks, C. L. I. Brooks, L. Nilsson, B. Roux, Y. Won, M. Karplus, *CHARMM: The Energy Function and Its Parameterization with an Overview of the Program*, in: *The Encyclopedia of Computational Chemistry*, (Ed.: P. v. R. Schleyer), John Wiley, Chichester, **1998**, pp 271–277.
[17] G. Wu, D. H. Robertson, C. L. Brooks, M. J. Vieth, *Comput. Chem.* **2003**, *24*, 1549–1562.
[18] Y. Kim, W. G. J. S. Hol, *Chem. Biol.* **2001**, *8*, 1253–1264.

