DOI: 10.1002/cbic.200700521

Understanding Promiscuous Amidase Activity of an Esterase from *Bacillus subtilis*

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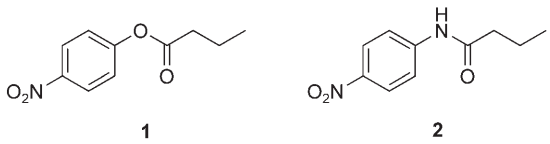
In the past few years, many enzymes have been identified that catalyze more than one chemical transformation; this feature is called catalytic promiscuity.[1] Usually, the reactions differ in the type of bond formation or cleavage and in the catalytic mechanism of bond making or breaking. Examples include the creation of an oxidase from a phosphatase by changing the metal ion to vanadate,[2] and C–C bond formation by an engineered lipase.[3] But the largest group of promiscuous enzymes involves functional-group analogues; for example, serine proteases are able to cleave ester bonds in addition to amide (peptide) bonds, or a lipase that is able to cleave amide bonds in addition to ester bonds.[1] Although the C–N and C–O bonds hydrolyzed differ chemically the catalytic mechanism is very similar and serine proteases have the same catalytic triad as lipases/esterases, which is composed of Ser-His-Asp (or Glu).[4] One example of promiscuous amidase activity is a lipase used in a large-scale process by the BASF, which efficiently performs kinetic resolution of racemic amines by enantioselective acylation.[5]

To investigate and find an explanation for the promiscuous amidase activity of an esterase we performed experiments combined with molecular modelling. A few years ago, we developed a high-throughput screening method in microtiter plate format to identify amidase activity.[6] The application of this assay to a range of lipases, esterases and peptidases revealed that several carboxylester hydrolases showed prominent activity in the hydrolytic cleavage of arylaliphatic acetamides. The most active enzymes were lipase B from *Candida antarctica* (CALB, E.C. 3.1.1.3) and an esterase from *Bacillus subtilis* (BS2, E.C. 3.1.1.3). Since BS2 can be easily over-expressed in *E. coli*,[7] and as a 3D structure of a close homologue, which differs in 11 out of 489 amino acids, is available,[8] we chose this esterase with promiscuous amidase activity as a model enzyme to alter and understand this feature. The esterase and amidase activities were studied by using the substrates *p*-nitrophenyl butyrate (**1**) and the corresponding anilide **2** as model compounds (Scheme 1). As both substrates differ only by the ester or amide bond, they represented ideal compounds for studying the promiscuous activity. The reaction outcome was determined spectrophotometrically since the substrates yield the chromophoric products *p*-nitrophenol and *p*-nitroaniline, respectively.



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Scheme 1. *p*-Nitrophenyl ester **1** and -amide **2** used as substrates for the comparison of amidase and esterase activity of BS2 and its mutants.

A molecular dynamics study of the wild-type enzyme hydrolyzing amide substrate **2** revealed that the amide transition state was stabilised by a hydrogen-bond network that consists of water molecules that reach from the substrate amide hydrogen and end at Glu188 (Figure 1, left). When Glu188 was substituted to the hydrophobic residue Phe (Figure 1, right) the amide transition-state stabilising water bridge was lost. From this modelling study, we hypothesised that the amide reaction would be clearly affected by the presence or absence of the transition-state stabilising hydrogen-bonded water network. The ester reaction, on the other hand, would not be as influenced since it lacks a corresponding hydrogen.

The hypothesis was evaluated experimentally by the generation of four mutants at position Glu188. Firstly, Glu188Asp was chosen since aspartate conserves the charge but has a shorter chain length than glutamate. This mutant should be able to retain a hydrogen-bond network. Secondly, a glutamine was introduced which has a neutral side chain that is the same length as that of glutamate and has the ability to participate in a water hydrogen-bond network. The two remaining mutants,

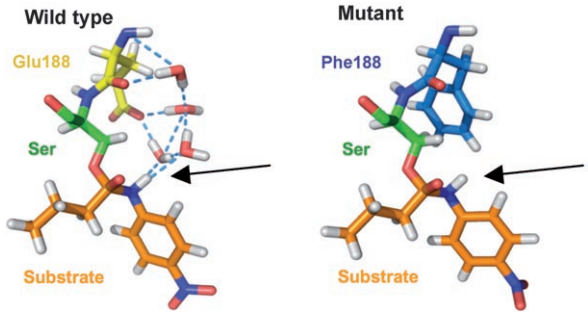

Figure 1. Snapshots from molecular-dynamics studies of the tetrahedral intermediate of amide **2** in the wild-type BS2 esterase (left) and the mutant Glu188Phe (right). The hydrogen-bond network that stabilises the substrate amide hydrogen in the wild-type enzyme is marked with an arrow. This stabilization was lost in the Glu188Phe mutant. The substrate's carbon atoms are shown in orange, the catalytic serine, which is covalently attached to the substrate, is in green. The wild-type amino acid Glu188 is shown in yellow (left) and the substitution Glu188Phe is in blue (right).

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Glu188Ala and Glu188Phe, have uncharged side chains of different sizes and both are unable to participate in a stabilising hydrogen-bond network. All mutants were expressed in a similar manner as wild-type BS2 esterase and purified to homogeneity by using a His-tag.

As can be seen in Table 1, both esterase and amidase specificities, k_{cat}/K_M, decreased for all four BS2 esterase mutants compared to the wild-type esterase. Figure 2 shows that mutants

Table 1. Specificities of BS2 wild-type esterase and mutants towards ester **1** and amide **2.** The increase in transition-state energy, $\Delta\Delta G^{\neq}$, caused by the mutations was calculated from the k_{cat}/K_M values.

	k_{cat}/K_M [mM min^{-1}]		$\Delta\Delta G^{\neq}(k_{cat}/K_M)$ [kJ mol^{-1}]	
mutant	ester	amide	ester	amide
wild type	40700	3.3	0.0	0.0
Glu188Gln	13500	0.62	2.7	4.0
Glu188Asp	850	0.13	9.6	8.0
Glu188Ala	2340	0.03	7.1	12.0
Glu188Phe	6160	0.03	4.7	11.7

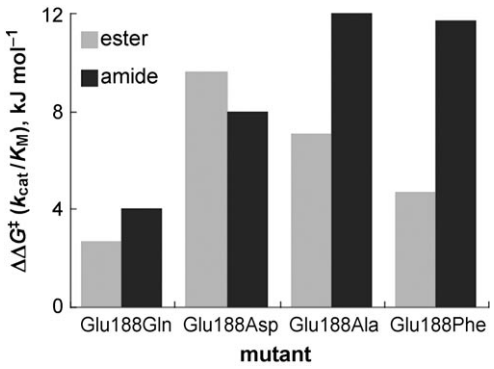

Figure 2. The increase in transition-state energy for the mutants compared to the wild-type BS2 esterase. Esterase activity is shown in light grey and amidase activity in dark grey. For Glu188Gln and Glu188Asp, the energy increase was roughly the same for both esterase and amidase reactions. For the mutants with hydrophobic side chains, Glu188Ala and Glu188Phe, the amidase reaction was suppressed to a greater extent than the esterase reaction.

Glu188Gln and Glu188Asp both lost esterase and amidase activity with a similar change in transition-state energy, $\Delta\Delta G^{\neq}$ (k_{cat}/K_M). This result is in accordance with our hypothesis, which suggested that both these mutants should retain the ability to form a hydrogen-bond network that is beneficial for the amidase reaction and invariant to the esterase reaction. However, for the two mutants Glu188Ala and Glu188Phe, the influence of the point mutations was much more severe for the amidase activity. This is also in line with the hypothesis with which we predicted a more prominent decrease in amidase activity for the mutants since they both lose the hydrogen-bond network.

Hydrogen bonds that involve the amide hydrogen in the transition state have been proposed to be important for an increased reaction rate since substrate-assisted catalysis has been observed when methoxyacetate esters were used in lipase-catalysed amidation.[9] In that case a 280-fold increase in rate was observed, which corresponds to an energy difference of 14 kJ mol^{-1} in transition state. The effect observed with the latter example was higher than with the present case with BS2 esterase. The energy difference in stabilisation might be explained by the higher degree of organisation needed to create the hydrogen-bond network involving several molecules—such an organised system suffers from entropic penalty. In the methoxyacetate case no extra molecule needs to be coordinated and the formed hydrogen bond can be more effectively used to stabilise the transition state.

In this work we have shown that a hydrogen-bond network can stabilize amide hydrolysis catalysed by an esterase; this affords a molecular explanation for the promiscuous behaviour of this enzyme.

Experimental Section

All chemicals were purchased from Fluka (Buchs, Switzerland), Sigma (Steinheim, Germany) and Merck (Darmstadt, Germany), unless stated otherwise. Restriction enzymes and polymerases were obtained from New England BioLabs (Beverly, MA, USA) and Roboklon GmbH (Berlin, Germany). MWG-Biotech (Ebersberg, Germany) provided the primers and performed the sequence analysis. NMR spectroscopy measurements were performed by using a Bruker ARX300 device.

Synthesis of *p*-nitrobutyranilide: *p*-Nitroaniline (1 g, 7.2 mmol) in dioxane (10 mL) was added, dropwise, to a solution of butyryl chloride (300 mg, 2.8 mmol) in dioxane (6 mL) and the mixture was stirred for 15 min. After stopping the reaction by adding ice-cold water (25 mL), the product was washed with diluted hydrochloric acid and water. Chromatography on silica gel (hexane/dichloromethane, 25:1) yielded **2** as a yellowish powder (714 mg, 48%).

Expression and analysis of BS2 esterase: The recombinant esterase BS2 and its Glu188 mutants were produced in *E. coli* as described.[7] The protein content was determined by using Bradford reagent with bovine serum albumin as the standard. Protein purification was performed by using immobilized metal ion affinity chromatography by taking advantage of the His-tag cloned into the mature esterase; pH shift was used for protein elution. Proteins were also analyzed by SDS-PAGE and activity staining as described.[10]

Site-directed mutagenesis: For site-directed mutagenesis complementary primers bearing the nucleotides to be changed and the vector pG-BS2, which encoded the wild-type BS2, were used for PCR with *Pfu* DNA polymerase, under the following reaction conditions: 1) 30 s at 95 °C, 2) 18 cycles: 50 s at 95 °C, 60 s at 55 °C, 10 min at 68 °C. The PCR mixture was then treated with *Dpn*I to digest the nonmethylated template DNA. The plasmids that encoded the mutant BS2 were transformed into competent *E. coli* cells, and the mutant clones were produced as described.[7] The following primers were used:

Glu188Ala: 5′-GTCGTTACATTGAACTATCGGCTG-3′

Glu188Asp: 5′-GTAACAGTATTTGGAGATTCCGCCGGCGGGATGAG-3′

Glu188Gln: 5′-CGTAACAGTATTTGGACAGTCCGCCGGCGGGATGAG-3′

Glu188Phe: 5′-CGTAACAGTATTTGGATTCTCCGCCGGCGGGATGAG-3′

Determination of enzyme activity in the hydrolysis of *p*-nitrophenyl derivatives: Enzyme activity was determined in microtiter plates by hydrolysis of *p*-nitrophenyl butyrate (**1**) for esterase activity and the anilide **2** for amidase activity. Substrate solution (30 μL, 1 mM) was added to enzyme solution (270 μL) with a final protein concentration of 66 ng mL^{-1} in phosphate buffer (100 mM, pH 7.5) and DMSO (10%, *v/v*). Release of *p*-nitrophenol (ε 14.6 × 10^3 M^{-1} cm^{-1}) and *p*-nitroaniline (ε 9.1 × 10^3 M^{-1} cm^{-1}) was quantified by using a fluorimeter (Thermofisher scientific, Langensebold, Germany) at 410 nm. One unit of activity was defined as the amount of enzyme that released 1 μmol product per min under assay conditions. Kinetic data were determined by using purified wild-type and mutant BS2 at four substrate concentrations (0.1, 0.4, 0.7, 1.0 mM).

Molecular modelling: A model of the highly homologous esterase BsubpNBE from *B. subtilis* was generated by adding two missing loops (residues 64–71 and 413–417), which were resolved in the organophilic mutant 5-6c8 (PDB code: 1C7J), to the crystal structure (PDB code: 1QE3) of the wild-type enzyme and then minimizing the overall system. A molecular dynamics study with a water box was performed by using the program YASARA (http://www.yasara.org).

Acknowledgements

This work was supported by the Deutscher Akademischer Austauschdienst (DAAD, Germany), The Swedish Research Council, The Swedish Foundation for International Cooperation in Research and Higher Education (STINT, Sweden) and the Deutsche Bundesstiftung Umwelt (Germany; Grants AZ13159 and AZ13162).

Keywords: amidases · *Bacillus subtilis* · catalysis · esterases · molecular modeling

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Received: September 3, 2007
Published online on November 19, 2007

