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Saturation mutagenesis reveals the importance of residues αR145 and αF146 of penicillin acylase in the synthesis of β-lactam antibiotics

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Abstract

Penicillin acylase (PA) from *Escherichia coli* can catalyze the coupling of an acyl group to penicillin- and cephalosporin-derived β-lactam nuclei, a conversion that can be used for the industrial synthesis of β-lactam antibiotics. The modest synthetic properties of the wild-type enzyme make it desirable to engineer improved mutants. Analysis of the crystal structure of PA has shown that residues αR145 and αF146 undergo extensive repositioning upon binding of large ligands to the active site, suggesting that these residues may be good targets for mutagenesis aimed at improving the catalytic performance of PA. Therefore, site-saturation mutagenesis was performed on both positions and a complete set of all 38 variants was subjected to rapid HPLC screening for improved ampicillin synthesis. Not less than 33 mutants showed improved synthesis, indicating the importance of the mutated residues in PA-catalyzed acyl transfer kinetics. In several mutants at low substrate concentrations, the maximum level of ampicillin production was increased up to 1.5-fold, and the ratio of the synthetic rate over the hydrolytic rate was increased 5–15-fold. Moreover, due to increased tendency of the acyl–enzyme intermediate to react with β-lactam nucleophile instead of water, mutants αR145G, αR145S and αR145L demonstrated an enhanced synthetic yield over wild-type PA at high substrate concentrations. This was accompanied by an increased conversion of 6-APA to ampicillin as well as a decreased undesirable hydrolysis of the acyl donor. Therefore, these mutants are interesting candidates for the enzymatic production of semi-synthetic β-lactam antibiotics.

Keywords: Penicillin acylase; Antibiotic synthesis; Ampicillin; Directed evolution; Protein engineering; Mutagenesis

1. Introduction

The β-lactam family of antibiotics, including penicillins, is the most important class of antibacterial compounds in clinical application. The narrow bactericidal spectrum of naturally occurring penicillin G, its low acid stability, and emerging resistance problems have triggered the development of semi-synthetic penicillins since the late 1940s, leading to the introduction of ampicillin in 1961 (Bergan, 1984; Levy, 1998; Nayler, 1991). Nowadays, ampicillin is one of the most widely used semi-synthetic β-lactam antibiotics with an estimated market of 20,000 tonnes year^{-1} (Bruggink, 2001). Its chemical synthesis is done under harsh conditions using reactive intermediates and organic solvents at a low temperature, causing high downstream processing costs and processes that are environmentally undesirable (Bruggink et al., 1998). Therefore, biocatalytic production processes for semi-synthetic antibiotics are highly desirable. The first step of such a process involves the PA-catalyzed cleavage of penicillin G into phenylacetic acid (PAA) and the penicillin nucleus 6-aminopenicillanic acid (6-APA). In the second step, 6-APA is coupled to a phenylglycine side group, yielding ampicillin. This second step can also be catalyzed by PA if an ester or amide of phenylglycine is used (Bruggink et al., 1998; Kasche, 1986; Alkema et al., 2002a; Youshko et al., 2000). However, the synthetic capacities of known PAs are only modest and need to be improved for economically

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competitive usage in large-scale production of penicillins and cephalosporins.

The enzyme-catalyzed synthesis of β-lactam antibiotics can be carried out in either an equilibrium-controlled or a kinetically controlled conversion (Svedas et al., 1980a,b; Kasche, 1986). In an equilibrium-controlled conversion the product concentration cannot be influenced by the properties of the enzyme as the enzyme only affects the rate at which conversion occurs. The level of product accumulation that can be reached is governed by the thermodynamic equilibrium, which is unfavorable in case of ampicillin synthesis (Svedas et al., 1980a; Schroen et al., 1999). In a kinetically controlled conversion, however, the enzyme catalyzes the transfer of the acyl group from the activated acyl donor to a nucleophilic acceptor (6-APA or a cephalosporin-derived nucleus). For the preparation of semi-synthetic penicillins, the acyl donor is usually the amide or methyl ester of an aromatic carboxylic acid. In this case, the level of product accumulation is governed by the catalytic properties of the enzyme and high non-equilibrium concentrations of the acyl-transfer product can transiently be obtained (Svedas et al., 1980b; Youshko et al., 2002a,b). The ability of PA to catalyze effective acyl transfer to β-lactam antibiotic nuclei is very much dependent on the reaction conditions (Ferreira et al., 2004; Ospina et al., 1996; Park et al., 2000), and the type of PA and therefore can be influenced by mutating the enzyme's active site (Alkema et al., 2002b; Gabor and Janssen, 2004).

Both structural and kinetic data have shown that PA catalyzes the conversion of amides and esters via an acyl–enzyme intermediate, in which residue Ser1 of the β-subunit is esterified to the acyl group (Duggleby et al., 1995; Konecny et al., 1983). Either the amino group of an added external nucleophile (6-APA) or water can attack the acyl enzyme, yielding the desired acyl-transfer product (antibiotic) or the hydrolyzed acyl donor, respectively. The ratio between the rate of synthesis, v_{Ps} and rate of hydrolysis, v_{Ph}, is an important parameter for evaluating the synthetic performance of PA. Since the initial value of this ratio, $(v_{Ps}/v_{Ph})_{ini}$, or the so-called synthesis/hydrolysis ratio (Youshko et al., 2001, 2002a), is dependent both on the kinetic properties of the enzyme and the concentration of the nucleophilic acceptor (i.e. 6-APA), it can be used to compare different enzyme variants. The maximum level of product accumulation that is transiently achieved is a second important parameter that is used for comparison of PA variants.

Youshko et al. (2002a) have shown that the course of acyl transfer is predicted by three enzyme-dependent parameters, α, β_0 and γ, as well as initial concentrations of acyl donor and nucleophile (β-lactam nucleus). The $(v_{Ps}/v_{Ph})_{ini}$ is hyperbolically dependent on the nucleophile concentration of the nucleophile (nucleus) according to Eq. (1):

$$\left(\frac{v_{Ps}}{v_{Ph}}\right)_{ini} = \frac{\beta_0[Nu]}{1+\beta_0\gamma[Nu]} \quad (1)$$

The hyperbolic dependence reflects a situation in which the acyl–enzyme complex that is formed during PA-catalyzed acyl transfer can still be hydrolyzed by water even if nucleophile is bound to it, e.g. at saturating nucleophile concentrations. Under these saturating conditions, the $(v_{Ps}/v_{Ph})_{ini}$ reaches a maximum value $(1/\gamma)$. Thus, γ should be low for good synthesis. The parameter β_0, which represents the preference of the acyl enzyme to react with nucleophile instead of water, should be high. The relative preference of the free enzyme for synthetic product versus the acyl donor is expressed with the specificity parameter α (Eq. (2)), which describes competition between two substrates (acylated β-lactam antibiotic and acyl donor) for the enzyme. It should obviously be low.

$$\alpha = \frac{(k_{cat}/K_m)_{Ps}}{(k_{cat}/K_m)_{AD}} \quad (2)$$

These three parameters describe the concentration of the β-lactam antibiotic during the course of the conversion according to Eq. (3).

$$\frac{d[Ps]}{d[Ph]} = \frac{\beta_0[Nu][AD] - \alpha[Ps](1+\beta_0\gamma[Nu])}{(1+\beta_0\gamma[Nu])([AD]+\alpha[Ps])}, \quad (3)$$

with $[AD]_0 = [AD] + [Ps] + [Ph]$ and $[Nu]_0 = [Nu] + [Ps]$, where [AD], [Nu], [Ps] and [Ph] are, respectively, the concentrations of acyl donor, nucleophile, product of synthesis (antibiotic) and product of hydrolysis. $[AD]_0$ and $[Nu]_0$ are the initial concentrations of acyl donor and nucleophile.

The selectivity of the enzyme for the nucleophile is governed by the active-site geometry. Done et al. (1998) reported that the crystal structure of PA can adopt two distinct and energetically favored conformations, the open and closed form. A 16 amino acid long α-helix becomes interrupted between residues αM142 and αA143, allowing a movement of the last part of the helix upon substrate binding. The closed conformation (helical state) is adopted in the ligand-free enzyme or if a small ligand occupies the substrate-binding site, whereas after binding of larger ligands, such as 3,4-dihydrophenylacetic acid and *m*-nitrophenylacetic acid, the enzyme is in the open conformation (coil form). In the latter conformation, residues αM142–αF146 are repositioned towards the solvent and the α-helix is partially unfolded. Upon binding of penicillin G to the inactive mutant βN241A, in which the oxyanion hole is corrupted because it is partly formed by the side chain oxygen of N241, the enzyme also adopts the open conformation (Alkema et al., 2000; McVey et al., 2001). In the helical form, αR145:NH_2 is hydrogen bonded to the main chain carbonyl oxygen of residue βF24. Upon binding of penicillin G, this hydrogen bond is replaced by one between an oxygen atom of the carboxylate group of the ligand and αR145:NH_2 via two bridging water molecules (Alkema et al., 2000), as residue αR145 orients itself into the solvent and residue αF146 moves 3.5 Å towards the solvent (Fig. 1). Such intricate changes may influence the pK_a values of groups close to the active site, which is important for catalysis (Morillas et al., 1999).

The extensive repositioning of αR145 and αF146 upon substrate binding as well as the interactions between these residues and the bound β-lactam ring prompted us to investigate the effect of mutations at these positions on the synthetic properties of penicillin acylase. For example, the intricate structural changes could well influence nucleophile binding and reactivity as well as sensitivity of the acyl–enzyme intermediate towards hydroly-