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Hybrid penicillin acylases with improved properties for synthesis of β-lactam antibiotics

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Abstract

Penicillin acylase (PA) from *Escherichia coli* can catalyze the acylation of 6-aminopenicillanic acid (6-APA), a conversion that is applicable in the biocatalytic preparation of semi-synthetic β-lactam antibiotics such as ampicillin. The efficiency of this kinetically controlled conversion, in which an amide or ester acts as the acyl donor, is dependent on the kinetic properties of the enzyme. To further improve the synthetic properties of PAs, family gene shuffling was performed with the PA-encoding genes of the PAs from *E. coli*, *Kluyvera cryocrescens* and *Providencia rettgeri*. Of these three PAs, the *E. coli* enzyme possessed the best properties for the synthesis of ampicillin. Shuffled recombinant libraries were pre-screened for activity by growth selection, followed by testing the catalytic performance in ampicillin synthesis using HPLC. Three clones with improved synthetic properties were selected and sequence analysis showed that the shuffled genes were hybrids of the PA-encoding genes from *E. coli* and *K. cryocrescens*, with additional point mutations. The hybrid enzymes displayed a 40–90% increase in the relative rate of acyl transfer to the β-lactam nucleus during ampicillin synthesis. This increase was not accompanied by a reduction of synthetic activity that has previously been reported for mutants of *E. coli* PA constructed by site-directed mutagenesis. Similar improvements in acyl transfer were obtained for the synthesis of amoxicillin, cephalexin and cefadroxil, making the new hybrid enzymes interesting candidates for the biocatalytic synthesis of several β-lactam antibiotics.

Keywords: Penicillin acylase; Ampicillin; Cephalexin; Synthesis; Biocatalysis; Gene shuffling; Directed evolution

1. Introduction

Penicillin acylase (penicillin amidohydrolase, EC 3.5.1.11) is a serine hydrolase that is used for the biocatalytic production of 6-aminopenicillanic acid (6-APA) by hydrolysis of penicillin G, which is obtained by fermentation. Penicillin acylases (PA) are present in various bacteria, archea and fungi [1–3], and they are likely to be involved in the metabolism of aromatic carbon compounds [4,5]. The *pac* gene encodes a preprotein consisting of a signal peptide, an α-chain, a spacer peptide and a β-chain and maturation of this PA preprotein yields a heterodimer consisting of the α- and β-chain of 23 and 63 kDa, respectively. In 2000, the crystal structure of the precursor protein was solved and it provided evidence for an autocatalytic processing mechanism of the preprotein [6]. Earlier X-ray analysis had already shown that the hydroxyl group of the N-terminal serine of the β-subunit becomes acylated during the first half reaction, and deacylated when the acyl enzyme is cleaved in the second half reaction [7]. The free NH_2 of this serine is proposed to act as a base that facilitates proton abstraction.

The penicillin nucleus 6-APA is a key intermediate for the preparation of several semi-synthetic β-lactam antibiotics. Chemical coupling of an appropriate acyl group to 6-APA is accompanied by the use of hazardous and polluting chemicals, needs to be done at low temperature to prevent formation of side-products, and requires a large amount of energy [8–10]. The use of biocatalysis would make this conversion more environmentally benign. Penicillin acylase can also be applied for the coupling of an acyl group to 6-APA. Unfortunately, the synthetic properties of the well-studied PA from *Escherichia coli* ATCC 11105 are only moderate, which causes incomplete conversion of the substrates to products. Since the enzymatic coupling is a kinetically controlled process, the yield of the reaction is dependent on the kinetic properties of the enzyme, and alternative enzymes may be more suitable. Penicillin acylases have also

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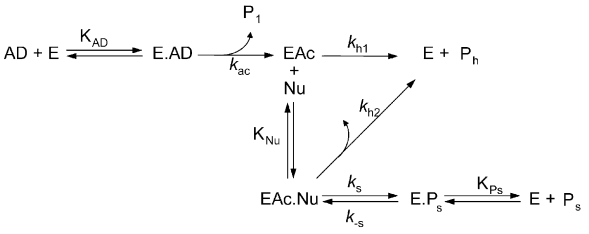

Fig. 1. Kinetic scheme describing the synthesis and hydrolysis reactions catalysed by PA. Abbreviations: E is the free enzyme, E.AD is the non-covalent enzyme-acyl donor complex, P_1 is the product released during acylation of the enzyme, EAc is the acyl-enzyme intermediate, A the hydrolysis product, EAc.Nu is the non-covalent acyl-enzyme–nucleophile complex, E.P_s is the non-covalent enzyme–product complex and P_s is the synthesis product (e.g., antibiotic). K_{Nu}, K_{AD} and K_{Ps} are the binding constants of substrates and products indicated, and k_{ac}, k_{h1}, k_s, k_{-s} and k_{h2} are the rate constants of the reaction steps.

been found in *Kluyvera cryocrescens* and *Providencia rettgeri*, but the synthetic properties of these enzymes are not well documented.

Youshko et al. [11] proposed that a kinetically controlled synthesis catalyzed by PA can be represented by the scheme shown in Fig. 1. Essentially the same scheme was used to describe the synthesis and hydrolysis of peptides by chymotrypsin [12], papain [13], and carboxypeptidase Y [14]. An activated acyl donor (AD), usually an amide or a methylester of a phenylacetic acid derivate, is attacked by Oδ of βSer1 of the enzyme to form an acyl-enzyme intermediate (EAc) (Fig. 2). This covalent intermediate can either be hydrolyzed by water, yielding the free acid (P_h) of the acyl donor, or aminolyzed by a β-lactam nucleus (Nu), yielding the desired product (P_s). The formed antibiotic can also be hydrolyzed by the enzyme, liberating the β-lactam nucleus (Nu) and the free acid (P_h). Due to this competitive product hydrolysis, the concentration of antibiotic will reach a maximum $[P_s]_{max}$, after which it will decrease again (Fig. 3). Since no antibiotic is present at the beginning of the reaction, product hydrolysis can be neglected in the initial phase of the conversion. Hence, the rate of formation of P_s (v_{P_s}) divided by the rate of formation of P_h (v_{P_h}) represents the preference of the acyl-enzyme for the β-lactam nucleus over water in deacylation and is given by Youshko et al. [15]:

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

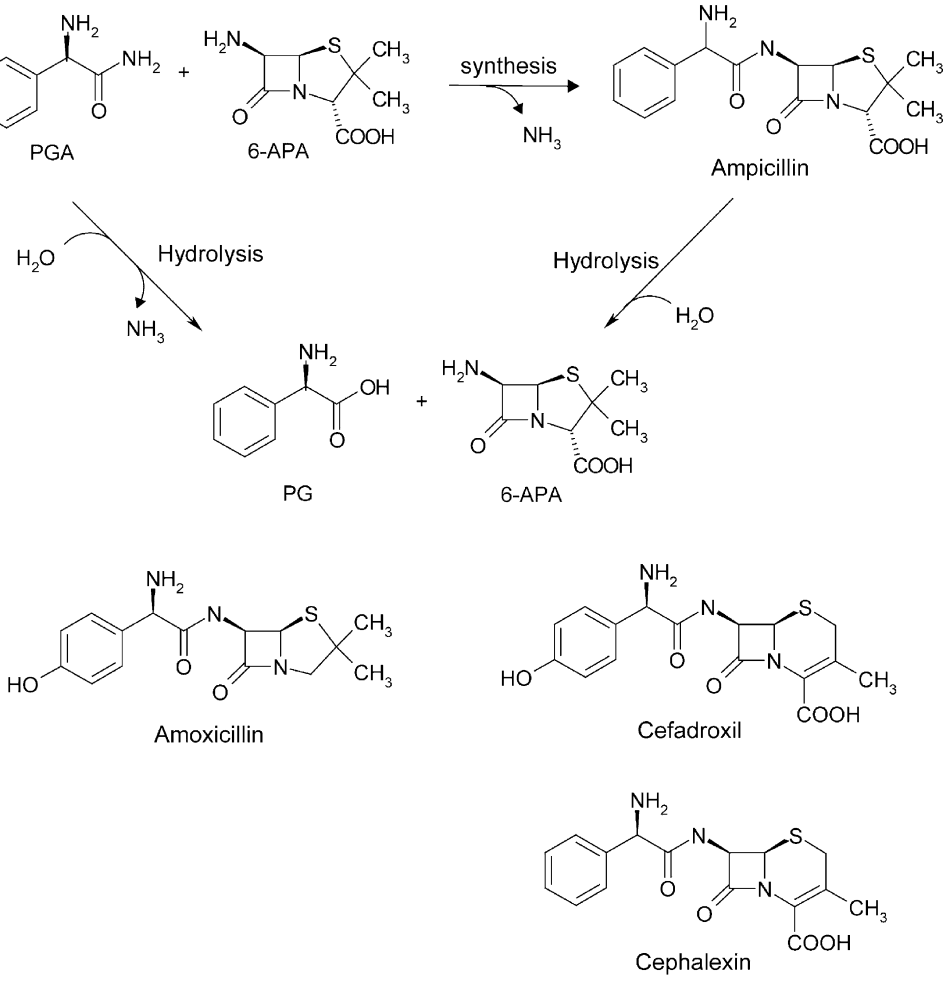

Fig. 2. Kinetically controlled synthesis of ampicillin. Penicillin acylase can couple the activated side chain (PGA) to the β-lactam nucleus (6-APA). Both the activated side chain and the formed product can be hydrolyzed to phenylglycine (PG). Structures of the other semi-synthetic β-lactam antibiotics that were synthesized by the hybrids are given as well.