Enzymatic synthesis of β-lactam antibiotics using penicillin-G acylase in frozen media

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Abstract Penicillin-G acylase (EC 3.5.1.11) from *Escherichia coli* catalyzed the synthesis of various β-lactam antibiotics in ice at −20°C with higher yields than obtained in solution at 20°C. The initial ratio between aminolysis and hydrolysis of the acyl-enzyme complex in the synthesis of cephalexin increased from 1.3 at 20°C to 25 at −20°C. The effect on the other antibiotics studied was less, leading us to conclude that freezing of the reaction medium influences the hydrolysis of each nucleophile-acyl-enzyme complex to a different extent. Only free penicillin-G acylase could perform transformations in frozen media: immobilized preparations showed a low, predominantly hydrolytic activity under these conditions.

Key words: Penicillin-G acylase; β-Lactam antibiotic; Acyl-enzyme complex; Frozen medium; Peptide synthesis

1. Introduction

An inherent problem in kinetically controlled peptide synthesis in aqueous solution by coupling of suitably activated derivatives of amino acids is competing hydrolysis [1–4][1]. Various kinetic models for these competing reactions have been proposed with or without binding of the nucleophile to the enzyme or acyl-enzyme complex [1,2,5–7]. The most common model is shown in Fig. 1A. The effect of the nucleophile concentration on the initial rate of product formation is consistent with the existence of a ternary nucleophile-acyl-enzyme complex [4,5]. The rate of hydrolysis of a nucleophile-acyl-enzyme complex and the corresponding acyl-enzyme complex without nucleophile bound to it is presumably different.

Peptide synthesis from activated donor substrates is kinetically controlled, i.e. the course of the reaction is determined predominantly by k_4 versus k_3 and k_5 (see Fig. 1A). Consequently, the yield is dependent on the properties of the enzyme, the temperature [8], the substrate concentration [1,2], the pH [1,3,8], the ionic strength [1,3,8] and the immobilization carrier [3,4,9]. The yield of a kinetic synthesis is defined as the conversion at the optimum of the product concentration (Fig. 1B).

The industrial application of kinetically controlled peptide synthesis is hampered by the necessity of separating the reaction products P and P_2 (Fig. 1A), which complicates the downstream processing, as well as by the recycling of the hydrolyzed donor which is generally required for economic reasons. Hence, the ratio of synthesis and hydrolysis (*S*/*H*) is a crucial reaction characteristic (Fig. 1B). A remarkable result concerning suppressing the hydrolytic side reaction in peptide synthesis was obtained by Jakubke and co-workers, who showed that proteases catalyze the synthesis of various peptides in frozen media, obtaining substantially higher yields than in the liquid phase [10]. This phenomenon could partially be explained by the existence of a liquid micro phase in the ice in which most solutes are present at high concentrations. Reduced water activity, changes in dielectric behavior, increased proton mobility and imposition of a favorable orientation of substrate and catalyst also were suggested to contribute to the increased yield in frozen media [11].

A prominent example of large-scale peptide synthesis is the manufacturing of semi-synthetic β-lactam antibiotics. The synthesis of the most widely used compounds of this class involves the formation of a peptide bond between an unnatural D-amino acid and the β-lactam nucleus, which is a cyclic derivative of L-cysteine-L-valine (Fig. 2). The traditional chemical processes for these antibiotics are currently being replaced by environmentally more attractive enzymatic alternatives. The catalyst in the coupling reaction is penicillin-G acylase (EC 3.5.1.11), in which a single N-terminal serine residue is responsible for the formation of the acyl-enzyme complex, as was concluded from the crystal structure of *Escherichia coli* penicillin-G acylase [12]. Minimization of unwanted competing hydrolysis is a major goal in the development of commercially viable biocatalytic routes to β-lactam antibiotics [13].

We reasoned that enzymatic coupling in frozen media could result in improved yields, by analogy with the protease-catalyzed peptide synthesis described above. In this paper, we present the results of our studies on penicillin-G acylase-catalyzed synthesis of antibiotics in frozen media.

2. Materials and methods

2.1. Materials

A penicillin-G acylase preparation of *E. coli* (420 U/ml) as well as immobilized *E. coli* penicillin-G acylase, assemblase (240 U/g), 6β-aminopenicillanic acid (6-APA) and 7β-amino-3′-desacetoxycephalosporanic acid (7-ADCA), ampicillin, amoxicillin, cephalexin and cefadroxil were provided by Gist-brocades (Delft, The Netherlands). D-(−)-phenylglycine methylester hydrochloric salt and D-(−)-4-hydroxyphenylglycine methylester were donated by DSM (Geleen, The Netherlands) as a gift. The immobilized penicillin-G acylase preparations PGA-450 and PGA-300 were kindly donated by Boehringer Mannheim (Penzberg, Germany).

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[1] The observed hydrolysis, i.e. acid formation, in kinetically controlled peptide synthesis is caused by two pathways: hydrolysis of the acyl donor (primary hydrolysis) and hydrolysis of the product (secondary hydrolysis).

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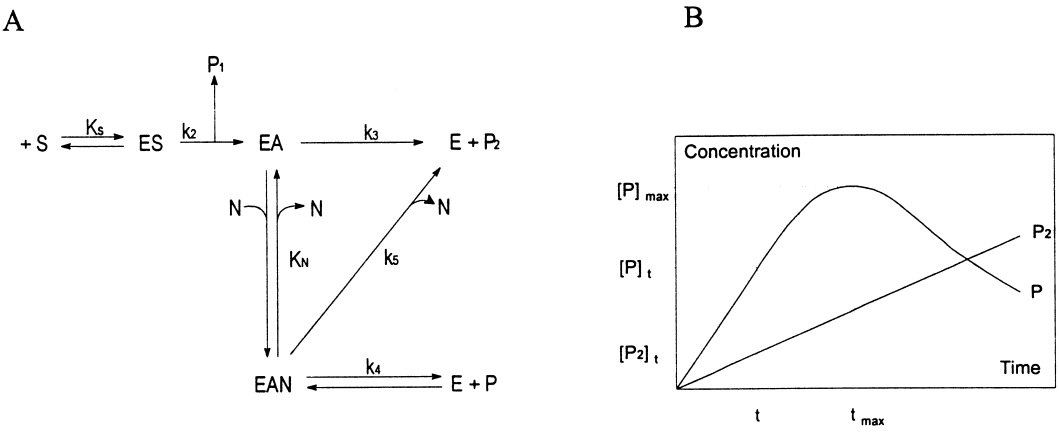

Fig. 1. Enzyme-catalyzed kinetically controlled peptide synthesis. (A) Scheme of the mechanism with binding of the nucleophile to the acyl-enzyme complex. E, enzyme; S, activated acyl donor; ES, Michaelis complex; EA, acyl-enzyme complex; P, peptide product; P_1, leaving group of activated acyl donor; P_2, hydrolysis product; N, nucleophile (acyl acceptor). (B) Scheme of the formation of synthesis product P and hydrolysis product P_1 in a kinetically controlled process. The ratio between synthesis and hydrolysis (*S*/*H*) is time dependent and is defined as $[P]_t/[P_2]_t$. The yield is defined at the optimum value of P as the ratio $[P]_{max}$/initial concentration of β-lactam nucleus.

2.2. Penicillin-G acylase assay

The activity of penicillin-G acylase was assayed in the hydrolysis of a 2% solution of penicillin-G potassium salt in 0.1 M phosphate buffer at a constant pH of 8.0 and temperature of 34°C with automated NaOH titration. One unit (U) of penicillin-G acylase liberates 1 μmol of phenylacetic acid per min.

2.3. Synthesis of β-lactam antibiotics

The pH of a solution containing side chain donor (20 mM) and β-lactam nucleus (20 mM) was adjusted to 7.5 with NaOH at room temperature. The solution was cooled to 0°C and 10 μl of *E. coli* penicillin-G acylase (4.2 U) was added to 0.99 ml of this solution in a polypropylene reaction vessel. The vessel was immediately transferred to an acetone/dry ice bath at −78°C for 2 min and then stored in the freezer at −20°C.

2.4. Analysis

The frozen reaction mixture was dissolved in 20 ml of a solution of 20 mM HCl in water/ethanol (3:1 v/v) and a measured amount of resorcinol dimethylether was added as internal standard. Samples were analyzed by high performance liquid chromotagrophy (HPLC) using a Waters M6000 pump, a 3×100 mm Nucleosil C-18 column and a Shimadzu SPD-6A UV detector at 215 nm. The eluent was prepared by adjusting the pH of a 0.68 g/l solution of KH_2PO_4 in acetonitrile/water (30:70 v/v) containing 0.68 g/l sodium dodecylsulphate to 3.0 with phosphoric acid. The flow rate was 1.0 ml/min. Retention times (in min) were as follows: D-(−)-4-hydroxyphenylglycine (1.5), D-(−)-phenylglycine (1.6), 6β-aminopenicillanic acid (2.3), 7β-amino-3′-desacetoxycephalosporanic acid (2.5), amoxicillin (3.9), cefadroxil (4.2), D-(−)-4-hydroxyphenylglycine methylester (5.8), resorcinol dimethylether (6.6, internal standard), ampicillin (10.5), cephalexin (11.5) and D-(−)-phenylglycine methylester (12.7).

3. Results and discussion

The synthesis of ampicillin, amoxicillin, cephalexin and cefadroxil from the corresponding side chain methyl esters and β-lactam nuclei (Fig. 2) was performed at 20°C and in ice at −20°C using penicillin-G acylase from *E. coli* as the catalyst. No inactivation of the enzyme was observed during the time of reaction under these conditions. The reactions were monitored over time and the maximum yields, initial synthesis rates and *S*/*H* are listed in Table 1.

The yields of ampicillin, amoxicillin and cephalexin were significantly higher at −20°C compared to 20°C. In contrast, the rate of cefadroxil formation was so slow at −20°C that the yield could not be determined. The initial ratio of cephalexin synthesis to hydrolysis of its side chain donor (*S*/*H*) increased markedly in ice compared with reaction in liquid medium at 20°C. In contrast, the initial *S*/*H* for ampicillin and amoxicillin only showed a small increase and for cefadroxil, a decrease was even observed.

Reaction in frozen media depressed the synthesis rates. Consequently, the reaction times were much longer in spite of a 5-fold increase in catalyst concentration. Furthermore, the absolute synthesis rates of the four products, which were of the same order of magnitude at 20°C, depended strongly on the nature of the side chain donor when the reaction was carried out at −20°C. At 20°C, the hydroxyl group in 4-hydroxyphenylglycine methyl ester exerted a slightly activating effect, as has been noted previously [14]. This was ascribed to an interaction of the 4-hydroxyl group with a serine residue in

Table 1
Synthesis of semi-synthetic β-lactam antibiotics mediated by penicillin-G acylase from *E. coli* in ice at −20°C compared with synthesis in liquid medium at 20°C

	−20°C			20°C		
	Yield (%)	$V_{synthesis}$ (μmol/U/h)	*S*/*H*	Yield (%)	$V_{synthesis}$ (μmol/U/h)	*S*/*H*
Ampicillin	61 (6.5 h)	0.48	1.3	16 (0.9 h)	5.5	0.7
Cephalexin	80 (46 h)	0.24	25	22 (0.8 h)	11	1.3
Amoxicillin	38 (6 days)	0.016	1	10 (0.7 h)	8.3	0.5
Cefadroxil	N.D.	0.001	1	22 (0.7 h)	17	2.5

Reaction conditions: 20 mM β-lactam nucleus, 20 mM side chain methyl ester, pH 7.5, penicillin-G acylase concentration: 0.84 U/ml (20°C), 4.2 U/ml (−20°C). The yield is defined as in Fig. 2. $V_{synthesis}$ and *S*/*H* were determined from the initial linear part of the conversion.

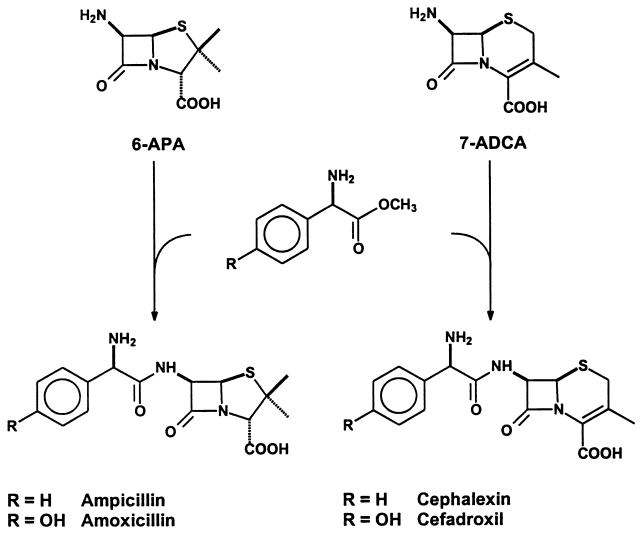

Fig. 2. Synthesis of semi-synthetic antibiotics by enzymatic acylation of β-lactam nuclei with side chain methyl ester.

the otherwise hydrophobic pocket of penicillin-G acylase [12]. In contrast, at −20°C, the reaction appeared to be strongly inhibited by the 4-hydroxyl group. Taking into consideration that the structure of the enzyme will be more rigid in frozen medium, we tentatively conclude that the substituent forces the acyl donor into an orientation that is unfavorable for reaction. The selectivity for the acyl acceptor also changed in frozen medium. Whereas at 20°C, 7-ADCA was twice as reactive as 6-APA, the order of reactivity was reversed at −20°C.

The observed phenomena caused by freezing of the medium cannot be explained by a change in activity of the reactants by the freeze concentration effect and decrease of temperature, because this would imply that a comparable trend should have been observed for all antibiotics studied. We conclude from our results that the effect of freezing on substrate selectivity and the initial *S*/*H* finds its major origin in a structural change of the enzyme and therefore, an altered kinetic behavior of the respective nucleophile-acyl-enzyme complexes. For protease-catalyzed peptide synthesis in frozen media, it was previously concluded that the freeze concentration effect was insufficient to explain the increased yields [11]. By comparison of different proteases and nucleophiles, Littlemore et al. concluded that freezing affects the structure of the enzyme in a beneficial way only for certain nucleophiles [15].

Freezing of the reaction medium was found to affect the secondary hydrolysis of the product. Ampicillin slowly hydrolyzed in the presence of relatively high concentrations of phenylglycine methyl ester in the course of a synthesis reaction at 20°C, whereas in ice at −20°C, almost no product hydrolysis was observed at all (Fig. 3A) even at a very low donor concentration. The absence of secondary hydrolysis was also observed in α-chymotrypsin-catalyzed peptide synthesis in frozen media [10,16,17]. This was ascribed to a reduction of amidase activity of the enzyme relative to esterase activity at low temperatures, the mechanism of which is still unexplained. Comparison between the rate of enzymatic hydrolysis of D-phenylglycine methyl ester and ampicillin at 20°C and −20°C showed indeed a 200-fold decrease of the amidase/esterase ratio in ice compared to 20°C (data not shown). The yield of these antibiotics in ice is therefore predominantly determined by the contribution of primary hydrolysis.

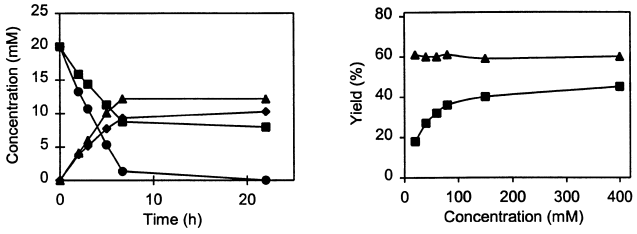

Fig. 3. Synthesis of ampicillin in frozen medium at −20°C at pH 7.5 using 4.2 U/ml *E. coli* penicillin-G acylase. (A) Substrate concentration: 20 mM 6-APA, 20 mM D-phenylglycine methyl ester, (■) 6-APA, (●) D-phenylglycine methyl ester, (♦) D-phenylglycine, (▲) ampicillin. (B) Effect of increasing substrate concentration on the yield in ice at −20°C (▲) compared with liquid medium at 20°C (■). The reaction was started with equimolar concentrations of 6-APA and D-phenylglycine methyl ester.

From an industrial point of view, the use of immobilized penicillin-G acylase in frozen systems could have advantages above free enzyme, because an immobilized catalyst is more efficiently separated from the products and is also more stable as well as recycable. Hence, we performed the synthesis of ampicillin with several commercially available immobilized *E. coli* penicillin-G acylase formulations in ice at −20°C. The synthesis rates were very low and *S*/*H* was 4–10 times lower compared with the free enzyme (Table 2). The 1000-fold decrease in rate can be explained by limiting diffusion of the substrates to the heterogeneous catalyst, because the immobilized enzyme is not included in the liquid micro phase to the same extent as the free enzyme. We note that secondary hydrolysis of the product, which is known to be promoted by diffusional limitation in carrier materials, is unlikely to contribute to the low *S*/*H*, because product hydrolysis was found to be negligible in ice in combination with free enzyme. The low *S*/*H* values of immobilized formulations can be explained

Table 2
Ampicillin synthesis in frozen medium at −20°C using immobilized *E. coli* penicillin-G acylase preparations

	$V_{synthesis}$ (μmol/U/h)	*S*/*H*
PGA-300	0.8×10^{-4}	0.14
PGA-450	5.1×10^{-4}	0.2
Assemblase	6.2×10^{-4}	0.3
Free *E. coli* Penicillin-G acylase	0.48	1.3

Reaction conditions: 20 mM 6-APA, 20 mM D-phenylglycine methyl ester hydrochloric salt, pH 7.5, penicillin-G acylase concentration: 16 U/ml. $V_{synthesis}$ and *S*/*H* were determined from the initial linear part of the conversion.

Table 3
Ampicillin synthesis in frozen medium at −20°C using various concentrations of free *E. coli* penicillin-G acylase

Penicillin-G acylase concentration	$V_{synthesis}$ (μmol/U/h)	*S*/*H*
4.2 U/ml	0.43	1.3
2.1 U/ml	0.22	1.3
1.05 U/ml	0.19	1.3
0.42 U/ml	0.16	1.3

Reaction conditions: 20 mM 6-APA, 20 mM D-phenylglycine methyl ester hydrochloric salt, pH 7.5, penicillin-G acylase concentration: 16 U/ml. $V_{synthesis}$ and *S*/*H* were determined from the initial linear part of the conversion.

by the relatively low nucleophile concentration in the frozen phase, because the nucleophile is primarily present in the liquid micro phase, leading to an increase of the primary hydrolysis.

The influence of the enzyme concentration on the rate and *S*/*H* of ampicillin synthesis in ice (Table 3) shows that the synthesis rate per unit enzyme increases with an increasing enzyme concentration, but that the initial *S*/*H* is not affected in the range of penicillin-G acylase concentrations studied. This can be rationalized by assuming that the fraction of enzyme present in the liquid microphase is increasing with increasing the total concentration. Enzyme in the frozen phase is assumed to react very slowly, as was observed for the immobilized preparation.

The reactant concentrations that we have used so far are much lower than commonly used in practical synthesis [13]. Hence, we compared the effect of reactant concentration on ampicillin synthesis in ice and in liquid medium (Fig. 3B). Using equimolar concentrations of reactants, we observed that the maximum yield of ampicillin in ice is not influenced by the increased concentration, whereas in liquid medium at 20°C, the yield increased with the substrate concentration. This is in agreement with results observed for α-chymotrypsin-catalyzed peptide synthesis in ice, i.e. peptide yields in frozen media are determined by the acyl donor/acceptor ratio and not by the absolute concentration [17]. We further observed that the rate of synthesis in ice reached a plateau value at 60 mM substrate concentration, whereas the rate in liquid medium increased proportionally with a concentration up to at least 400 mM (data not shown).

Synthesis of β-lactam antibiotics in a frozen medium at −20°C resulted in improved yields and a more efficient use of the side chain donor compared with reaction in solution at room temperature. We propose that the observed effect of freezing on substrate selectivity and the initial *S*/*H* is primarily due to a structural change of the enzyme and therefore, an altered kinetic behavior of the respective nucleophile-acyl-enzyme complexes. It is still unclear, however, to which extent this is a result of freezing or merely a lower temperature.

Antibiotic synthesis in ice requires a soluble catalyst: immobilized preparations were hardly active. In contrast to reaction in liquid medium, the yield of ampicillin synthesis in ice did not improve with an increasing reactant concentration. Nevertheless, we have shown that the turnover frequency (g of product/g of catalyst/h) of antibiotic synthesis in ice can surprisingly be increased by using higher concentrations of enzyme.

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