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Kinetics of enzyme acylation and deacylation in the penicillin acylase-catalyzed synthesis of β-lactam antibiotics

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Penicillin acylase catalyses the hydrolysis and synthesis of semisynthetic β-lactam antibiotics via formation of a covalent acyl-enzyme intermediate. The kinetic and mechanistic aspects of these reactions were studied. Stopped-flow experiments with the penicillin and ampicillin analogues 2-nitro-5-phenylacetoxy-benzoic acid (NIPAOB) and D-2-nitro-5-[(phenylglycyl)amino]-benzoic acid (NIPGB) showed that the rate-limiting step in the conversion of penicillin G and ampicillin is the formation of the acyl-enzyme. The phenylacetyl- and phenylglycyl-enzymes are hydrolysed with rate constants of at least 1000 s^{-1} and 75 s^{-1}, respectively. A normal solvent deuterium kinetic isotope effect (KIE) of 2 on the hydrolysis of 2-nitro-5-[(phenylacetyl)amino]-benzoic acid (NIPAB), NIPGB and NIPAOB indicated that the formation of the acyl-enzyme proceeds via a general acid–base mechanism. In agreement with such a mechanism, the proton inventory of the k_{cat} for NIPAB showed that one proton, with a fractionation factor of 0.5, is transferred in the transition state of the rate-limiting step. The overall KIE of 2 for the k_{cat} of NIPAOB resulted from an inverse isotope effect at low concentrations of D_2O, which is overridden by a large normal isotope effect at large molar fractions of D_2O. Rate measurements in the presence of glycerol indicated that the inverse isotope effect originated from the higher viscosity of D_2O compared to H_2O. Deacylation of the acyl-enzyme was studied by nucleophile competition and inhibition experiments. The β-lactam compound 7-aminodesacetoxycephalosporanic acid (7-ADCA) was a better nucleophile than 6-aminopenicillanic acid, caused by a higher affinity of the enzyme for 7-ADCA and complete suppression of hydrolysis of the acyl-enzyme upon binding of 7-ADCA. By combining the results of the steady-state, presteady state and nucleophile binding experiments, values for the relevant kinetic constants for the synthesis and hydrolysis of β-lactam antibiotics were obtained.

Keywords: penicillin acylase; antibiotic synthesis; kinetic mechanism; kinetic isotope effect.

Penicillin acylase (PA) (EC 3.5.1.11) of *Escherichia coli* ATCC 11105 hydrolyses penicillin G to produce phenylacetic acid and 6-aminopenicillanic acid (6-APA). The latter compound can be used industrially as a precursor for the synthesis of semisynthetic β-lactam antibiotics. In synthetic reactions, 6-APA is coupled to an acyl group in a kinetically controlled conversion in which the acyl group is supplied as an activated precursor, e.g. an ester or an amide [1]. This condensation reaction can also be catalysed by PA and the yield of the desired product is strongly dependent on the mechanism and kinetic properties of the catalyst [2].

The catalytic mechanism of PA involves nucleophilic attack of the active-site serine, βS1, on the carbonyl carbon of the amide or ester bond of the substrate [3] (residues are labelled to indicate the subunit (α or β) and their position in the subunit). Via a tetrahedral intermediate that is stabilized by hydrogen bonds between the oxyanion hole residues βN241 and βA69 and the negatively charged oxygen of the substrate, an acyl-enzyme is formed concomitant with expulsion of the leaving group [3–5]. The acyl-enzyme may be deacylated by H_2O, yielding the hydrolysis product and the free enzyme. In this mechanism, the free N-terminal NH_2 group of the catalytic serine functions as the catalytic base, activating the nucleophile by proton abstraction in the acylation reaction. In the presence of a β-lactam nucleophile, such as 6-APA or 7-aminodesacetoxycephalosporanic acid (7-ADCA), the acyl group may be transferred to the β-lactam moiety rather than to H_2O, producing a semisynthetic antibiotic.

The kinetic scheme for PA-catalysed acyl transfer to H_2O or a β-lactam nucleophile can be described by the scheme developed for hydrolysis and synthesis of peptides by chymotrypsin and papain (Fig. 1) [2,6,7].

Assuming rapid binding, and applying a steady state assumption for EAc and EacN, gives the following

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Abbreviations: PA, penicillin acylase; PAPNA, phenylacetyl-*p*-nitroanilide; NIPAB, 2-nitro-5-[(phenylacetyl)amino]-benzoic acid; NIPGB, D-2-nitro-5-[(phenylglycyl)amino]-benzoic acid; 6-APA, 6-aminopenicillanic acid; 7-ADCA, 7-aminodesacetoxycephalosporanic acid; PGA, D-phenylglycinamide; PAA, phenylacetic acid amide; NIPAOB, 2-nitro-5-phenylacetoxy-benzoic acid; PNA, *p*-nitrophenylacetate; PGPNA, D-phenylglycine-*p*-nitroanilide; pNPA, *p*-nitrophenylacetate; KIE, kinetic isotope effect.

Enzymes: penicillin acylase (PA; EC 3.5.1.11).

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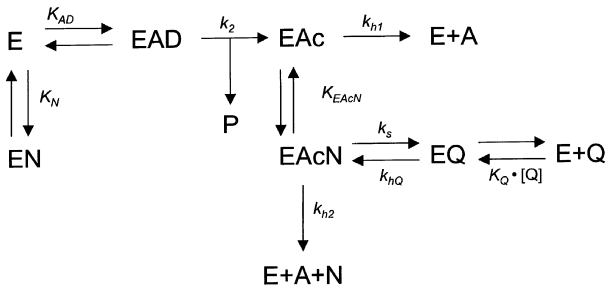

Fig. 1. Kinetic scheme describing the synthesis and hydrolysis reactions catalysed by penicillin acylase, adapted from a similar scheme for chymotrypsin catalysed reactions [7]. In this Scheme A(D) is the acyl donor, N the β-lactam nucleophile, Q the product of aminolysis (i.e. the antibiotic) and A the product of hydrolysis (i.e. the acid of the acyl donor). EAD, EN, and EQ are the enzymes with substrates noncovalently bound, whereas EAc and EAcN represent the acyl-enzyme intermediate with and without bound nucleophile. The rate constants for the acylation of the enzyme are given by k_2, k_{2N} and k_{hQ}, whereas k_s, k_{h1} and k_{h2} are the rate constants for deacylation. K_Q, K_{AD} and K_N, K_{EAcN} are the binding constants of the substrates to the free enzyme and the acyl-enzyme, respectively.

expression for yield of the desired product, i.e. the maximum product accumulation $[Q]_{max}$ [8]:

$$[Q]_{max} = \frac{K_Q}{k_{hQ}} \cdot \frac{k_2}{K_{AD}} \cdot \frac{[N] \cdot k_s}{k_{h1} \cdot K_{EAcN} + k_{h2} \cdot [N]} \cdot [AD] \qquad (1)$$

In this equation [AD] and [N] are the concentrations of acyl donor and nucleophile, respectively, at the point where $[Q]_{max}$ is reached.

Equation (1) shows that the kinetic constants for the acylation by the substrate AD or the product Q and the competition for the acyl-enzyme between H_2O and a second nucleophile N are important factors with respect to the formation of transacylation products. Although several studies have appeared that have addressed the kinetic properties of the enzyme and mutants [4,9–13], investigations into the individual rate and binding constants underlying the kinetic properties and biocatalytic performance in the synthesis and hydrolysis of antibiotics are scarce [11,14,15].

We attempted to determine the kinetic constants of PA, which are relevant for the hydrolytic and the synthetic reactions, to obtain more insight into the interactions in the active site that influence synthetic performance. Using stopped-flow experiments and nucleophile binding studies we were able to obtain values for the kinetic constants for hydrolysis and synthesis of penicillin G, ampicillin and cephalexin. Furthermore, insight into the nature of the transition state in the rate-determining steps of the reaction was obtained by analysing solvent kinetic isotope effects.

Materials and methods

Kinetic measurements

All kinetic experiments were done with purified penicillin acylase of *E. coli* ATCC 11105, which was obtained as described previously [4]. The enzyme concentration was determined by measuring A_{280} and using $\varepsilon = 210\,000\ M^{-1}\cdot cm^{-1}$. The conversion of the chromogenic substrates 2-nitro-5-[(phenylacetyl)amino]-benzoic acid (NIPAB) ($\Delta\varepsilon_{405nm} = 9.09\ mM^{-1}.cm^{-1}$), phenylacetyl-*p*-nitroanilide (PAPNA) ($\Delta\varepsilon_{405nm} = 13\ mM^{-1}\cdot cm^{-1}$), 2-nitro-5-phenylacetoxy-benzoic acid (NIPAOB) ($\Delta\varepsilon_{405nm} = 11.4\ mM^{-1}\cdot cm^{-1}$), D-2-nitro-5-[(phenylglycyl)amino]-benzoic acid (NIPGB) ($\Delta\varepsilon_{405nm} = 9.09\ mM^{-1}\cdot cm^{-1}$), D-phenylglycine-*p*-nitroanilide (PGPNA) and *p*-nitrophenylacetate (*p*-NPA) ($\Delta\varepsilon_{405nm} = 13\ mM^{-1}\cdot cm^{-1}$) was followed by measuring the increase in absorbance change at 405 nm, on a Perkin Elmer Bio40 UV/VIS spectrophotometer at 30 °C in 50 mM phosphate buffer, pH 7.0. The stopped-flow experiments were carried out on an Applied Photophysics SX.17MV spectrophotometer, with a 10-mm optical path length. The stopped-flow cell was thermostatically controlled at 30 °C. Stock solutions of all ester substrates were made in acetonitrile and diluted to the appropriate concentration in 0.5% acetonitrile in 50 mM phosphate buffer, pH 7.0, prior to the start of kinetic experiments. Conversion of non chromogenic substrates was followed using HPLC as described [4]. Nucleophile competition experiments were carried out by mixing enzyme with solutions of acyl donor and nucleophile. The enzymatic conversions were followed by HPLC and the V_s/V_h ratios were calculated from the initial rates of the formation of synthesis and hydrolysis products.

Buffers for experiments in D_2O were prepared as follows: solutions of 50 mM KH_2PO_4 and $K_2HPO_4{\cdot}3H_2O$ were made by dissolving the appropriate amount of salt in D_2O. Both solutions were combined to yield a buffer solution with a pH meter reading of 6.6. H_2O/D_2O mixtures were made by mixing 50 mM potassium phosphate buffers in D_2O, pH 6.6 with 50 mM potassium phosphate buffers in H_2O, pH 7.0. The values for Δε were corrected for the influence of D_2O on the extinction coefficients, which was measured with all H_2O/D_2O mixtures that were used. Proton inventories were carried out at pL 7.0, which is in the pH-independent region for PA catalysed hydrolysis reactions [9].

Fitting of kinetic parameters to the data was done using the program SCIENTIST (Micromath Inc., version 2.0). The goodness of fit and the information content of the data were checked by inspecting the value for the model selection criterion, standard deviations for the 95% confidence interval of the parameter values and the correlation matrix for the fitted parameters [16]. These parameters were calculated using the Statistics procedure implemented in the SCIENTIST program.

Chemicals

NIPAB, *p*-NPA, phenylacetic methyl ester and phenylacetic acid ethyl ester were purchased from Sigma Chemical Company. NIPAOB, NIPGB and 5-(2-amino-2-phenylacetoxy)-2-nitro-benzoic acid (NIPGBester) were purchased from Syncom (Groningen, the Netherlands). PGPNA, ampicillin, penicillin G, 6-APA and 7-ADCA were obtained from DSM-Gist (Delft, the Netherlands). PAPNA was prepared as follows: phenylacetylchloride was added dropwise to an equimolar amount of 4-nitroaniline dissolved in chloroform containing one equivalent of triethyl amine. After refluxing for 3 h, the mixture was extracted with 1 M

HCl and 1 M NaOH. After drying and evaporation a white-yellow solid was obtained that was recrystallised from methanol/ether. Mp. 114–115 °C (uncorr.). ^{1}H NMR (300 MHz, dimethylsulfoxide *d*6) δ (p.p.m.): 3.71 (s, 2H, CH_2); 7.26–7.35 (m, 5H, CH); 7.82 (d, J = 9.0 Hz, 2H, CH); 8.20 (d, J = 9.0, 2H, CH); 10.77 (brs, 1H, NH). Phenylacetamide was prepared as follows: phenylacetylchloride was added dropwise to concentrated ammonia solution. This gave the product as a white precipitate that was filtered off and dried to constant weight. Mp. 152–153 °C (uncorr.). ^{1}H NMR (300 MHz, D_2O) δ (p.p.m.): 3.65 (s, 2H, CH_2); 7.35–7.50 (m, 5H, CH).

Results and Discussion

Steady-state kinetic parameters for phenylacetylated and phenylglycylated substrates

The hydrolysis of acyl donors and antibiotics by PA proceeds via a two step mechanism, involving acylation of the active-site serine by the substrate and subsequent hydrolysis of the acyl-enzyme. In the absence of a nucleophile other than water, the steady-state rate of production of [A] by hydrolysis of the substrate [AD], according to Scheme 1, is given by [17]:

$$\frac{d[A]}{dt} = \frac{k_{cat} \cdot E_0 \cdot [AD]}{K_m + [AD]} \quad (2)$$

in which

$$k_{cat} = \frac{k_2 \cdot k_{h1}}{k_2 + k_{h1}} \quad (3)$$

and

$$K_m = \frac{K_{AD} \cdot k_{h1}}{k_2 + k_{h1}} \quad (4)$$

If deacylation of the enzyme is much slower than acylation, i.e. $k_{h1} \ll k_2$, the k_{cat} is given by k_{h1} and burst kinetics for the release of the first product P will be observed [17]. If, however, only acylation of the enzyme is rate-limiting, i.e. $k_2 \ll k_{h1}$, the value for k_{cat} is given by k_2 and an effect of the leaving group of the substrate on the k_{cat} can be observed.

To investigate whether acylation or deacylation is the rate-limiting step in the conversion of phenylacetylated and phenylglycylated substrates, we determined the steady-state kinetic parameters for a series of amides and esters, with phenylglycine or phenylacetic acid as the acyl group and different leaving groups (Table 1) (Fig. 2).

For the hydrolysis of amides of phenylacetic acid, different k_{cat} values were observed for substrates with different leaving groups. The k_{cat} values for the methyl and ethyl ester of phenylacetic acid were approximately fivefold higher than for the best amide substrate, phenylacetamide, which is in agreement with the fact that amide bonds are in general more stable than ester bonds. The k_{cat}/K_m for NIPAOB was more than threefold higher than for penicillin G, which makes NIPAOB the best substrate known so far for penicillin acylase. The k_{cat} values for phenylacetic acid methyl ester and phenylacetic acid ethyl ester were only slightly lower than the k_{cat} of NIPAOB, indicating that increasing the reactivity of the carbonyl function of the substrate by using *p*-hydroxynitrobenzoic acid as the leaving group, did not lead to a higher rate of conversion. For esters of phenylglycine the k_{cat} was similar to the k_{cat} for phenylglycine amide, indicating that the higher reactivity of the ester bond also did not lead to an increased rate of conversion.

These results indicate that for hydrolysis of the phenylacetylated amides, the rate-limiting step is the formation of the acyl-enzyme and k_{cat} is given by k_2. For esters of phenylacetic acid and esters or amides of phenylglycine, in which a leaving group effect was almost absent, the k_{cat} may be set by k_2, k_{h1} or a combination of k_2 and k_{h1}.

If k_2 and k_{h1} are in the same order of magnitude a presteady-state burst of product formation should be observed from which the values for k_2 and k_{h1} can be calculated [18,19]. To obtain the values for k_2 and k_{h1}, we performed stopped-flow experiments with the chromogenic

Table 1. Steady-state parameters of penicillin acylase for the hydrolysis of esters and amides of phenylacetic acid and phenylglycine. The structures of the chromogenic substrates are shown in Fig. 2. The reaction conditions are given in the Materials and methods section. Values represent means of three experiments. The standard deviation was in all cases within 10% of the mean values.

Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM \cdot s^{-1}$)
Amides			
NIPAB	15	0.015	1000
PAPNA	14	0.13	107
Phenylacetamide	50	0.180	277
Penicillin G	42	0.007	6000
NIPGB	15	1.7	8.82
PGPNA	3.1	1.7	1.82
Phenylglycine amide	30	40	0.75
Ampicillin	30	2.5	12
p-NPA	1	0.029	34
Esters			
NIPAOB (NIPABester)	200	0.011	18181
Phenylacetic acid methyl ester	190	0.16	1187
Phenylacetic acid ethyl ester	170	0.11	1545
NIPGBester	– [a]		–
Phenylglycine methyl ester	50	12.5	4
Phenylglycine ethyl ester	24	12	2

[a] Chemically unstable.

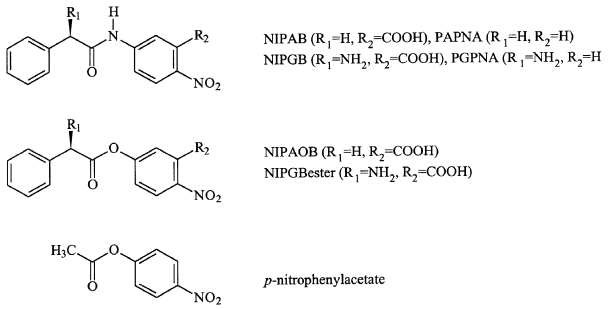

Fig. 2. Structures of the chromogenic substrates used in this study. NIPGBester was hydrolysed by penicillin acylase, but due to high rates of solvolysis, no kinetic parameters could be determined.

substrates NIPAOB and NIPGB. No initial burst of product formation was observed during the hydrolysis of both compounds, indicating that the acylation rate is much slower than the rate of deacylation. The theoretical maximum of the amplitude of the burst phase is equal to the total enzyme concentration. When $k_{h1} = 5\ k_2$, only 2% of the maximum of the amplitude of the burst phase can be observed which is considered to be the lower limit of detection. Since the k_{cat} for the hydrolysis of NIPAOB is 200 s^{-1}, hydrolysis of the acyl-enzyme must take place at a rate of at least 1000 s^{-1}. Likewise, the lower limit for the hydrolysis of the phenylglycylated enzyme is at least 75 s^{-1}, which is 5 k_{cat} for the hydrolysis of NIPGB. Attempts to obtain a more accurate estimate for the lower limit of k_{h1}, by using the more reactive ester of NIPGB in kinetic experiments, failed because of the high rates of spontaneous hydrolysis of this compound.

The only substrate of PA for which the steady-state rate of hydrolysis was preceded by an exponential burst phase was *p*-NPA, indicating that for this substrate the rate of formation of the covalent intermediate is faster than the hydrolysis rate, in agreement with results obtained by Morillas *et al.* (1999) (Fig. 3).

These results indicate that in the hydrolysis of acyl donors and antibiotics the breakdown of the acyl-enzyme is much faster than the rate of formation of the acyl-enzyme. It follows from Eqns (3 and 4) that under these conditions the K_m is equal to the dissociation constant of the substrate, K_{AD} or K_Q, and that the k_{cat} for hydrolysis equals the rate constant for acylation.

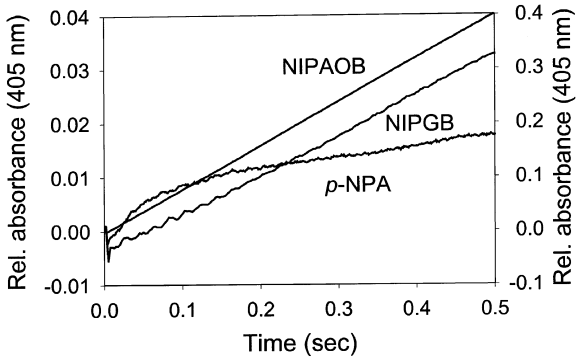

Fig. 3. Stopped-flow traces using NIPGB, NIPAOB and p-NPA. For NIPGB and NIPAOB, the final enzyme concentration was 4 μM and substrate concentration was 10·K_m. For p-NPA the final enzyme concentration was 3 μM and substrate concentration was 400 μM.

Proton transfer in the acylation reaction

To obtain further information about the rate-limiting reactions, we determined the solvent deuterium kinetic isotope effect (KIE) on these reactions. The k_{cat} for the hydrolysis of NIPAB, NIPGB and NIPAOB all displayed a normal solvent deuterium KIE of 2, whereas no effect of D_2O on the K_m was observed. For the substrates NIPAB and NIPAOB, a proton inventory was recorded by measuring the k_{cat} in mixtures of H_2O and D_2O (Fig. 4).

The relation between the value of a rate constant and the mole fraction D_2O can be described by the simplified form of the Gross–Butler equation [20],

$$\frac{k_x}{k_0} = \prod_i^v (1 - x + x\phi_i^T) \qquad (5)$$

In this equation k_x is the rate constant at D_2O fraction x, k_0 the rate in H_2O, Φ_i^T the transition state fractionation factor at the i^{th} exchangeable hydrogenic site, and v the number of protons involved in the reaction [20]. If one proton is in flight in the transition state, a plot of k_x/k_0 vs. x gives a straight line, whereas a second or multiple order polynomial is indicative of more than one proton being transferred in the transition state.

A linear dependence of k_x/k_0 on x was observed for the k_{cat} for NIPAB, suggesting that one proton is transferred in the transition state of the reaction. Fitting to the Gross–Butler equation yielded a fractionation factor of 0.5 for the proton that is exchanged. This fractionation factor is indicative of general acid/base catalysis mechanism where the proton is less tightly bound in the transition state than in the reactant state [21], in agreement with the mechanism of PA-catalysed hydrolysis as proposed by Duggleby *et al.* (1995). The proton that gives rise to the normal isotope effect may be the proton that is transferred from the seryl oxygen to the seryl amino group during activation of the nucelophilic serine, or the proton that is donated to the leaving group during collapse of the tetrahedral intermediate.

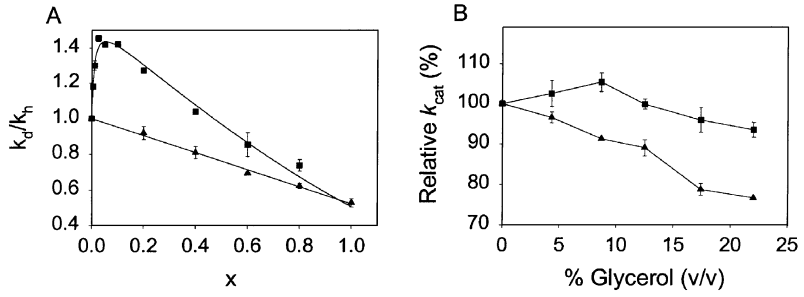

Fig. 4. Effect of D_2O and glycerol on the hydrolysis of NIPAB and NIPAOB. (A) Proton inventories for k_{cat} of NIPAB and NIPAOB. k_{cat} values were determined at a substrate concentration of 1 mM, which is 100-fold higher than the K_m for these substrates at varying molar fractions of D_2O (×). ▲, NIPAB; ■, NIPAOB. The symbols and standard deviations represent the mean of three independent experiments. The lines are the best fit to the data using Eqn (5) (NIPAB) and Eqn (6) (NIPAOB). (B) The figure shows k_{cat} values for NIPAB (▲) and NIPAOB (■) in phosphate buffer with increasing concentrations of glycerol.

A concerted mechanism in which the proton would be directly transferred from the seryl oxygen to the leaving group, mediated by the hydrogen bonding with the seryl NH_2 group but without protonation of this group, may also cause a linear proton inventory.

For the hydrolysis of NIPAOB a different effect of D_2O was found. We observed an increase of k_{cat} at small mole fractions of D_2O, whereas there was an almost linear decrease of the k_{cat} at larger mole fractions of D_2O. Unlike with NIPAB, these data could not be fitted to Eqn (5), or to a modified Gross–Butler equation taking medium effects into account [22]. The higher viscosity of D_2O compared to H_2O and the isotope effects on H-bonds have been described to influence the enzyme structure, flexibility and stability [23–27]. D_2O effects on rate constants that result from changes in solvent viscosity instead of proton transfer in the rate-limiting step have been described for chymotrypsin, NAD-malic enzyme and alkaline phosphatase [28–30]. It is conceivable that the increase in activity of PA may be caused by small structural changes in the active site at low concentrations of D_2O, making enzyme–substrate interactions more favourable for catalysis than in H_2O.

To study the influence of solvent viscosity on the hydrolysis of NIPAB and NIPAOB, the k_{cat} values for these substrates at various glycerol concentrations were determined (Fig. 4). It appeared that the k_{cat} for NIPAOB increased to a maximum of 105% at 9% glycerol whereas the k_{cat} for NIPAB decreased with increasing concentrations of glycerol. To account for viscosity effects on enzyme activity, Eqn (6) was fitted to the data. In this equation a term is included that describes the rate enhancement originating from solvent properties. It is assumed that the rate enhancement with NIPAOB as the substrate due to the presence of D_2O is maximal at a certain mole fraction of D_2O, given by k', where K_x is the mole fraction of D_2O at which half of the maximal rate enhancement is obtained.

$$\frac{k_x}{k_0} = \left(\frac{K_x + k' \cdot x}{K_x + x}\right) \cdot \prod_i^v (1 - x - x\phi_i^T) \qquad (6)$$

The second term is the simplified form of the Gross-Butler equation and describes the decrease in rate due to proton transfer in the transition state. Fitting Eqn (6) to these data yielded values of 1.58 ± 0.03 for k' and 0.0078 ± 0.0016 for K_x. The best fit was obtained assuming a two-proton transfer mechanism, suggesting that two protons are transferred in the transition state of this reaction. However, a distinction between a one-proton and a multiple-proton mechanism can only be made when more accurate data can be obtained since it requires precise measurement of small differences in curvature of the proton inventory. Given the influence of the viscosity and the additional measurements that were carried out to account for background hydrolysis and the influence of D_2O on the extinction coefficient of the hydrolysis product, more accurate data are needed to draw definitive conclusions about the number of protons being transferred in the transition state of NIPAOB hydrolysis.

The results of the proton inventories indicate that the mechanisms for the conversion of the substrates that were studied, differ significantly from each other. Substrate-dependent proton inventories have also been observed for the serine proteases elastase [31] and chymotrypsin [32]. The results of the proton inventories for NIPAB and NIPAOB suggest that also in PA-catalysed reactions the structure of the leaving group of the substrate has an effect on the details of the kinetic mechanism.

Deacylation by β-lactam nucleophiles

Since the rate-limiting step for the hydrolysis of phenylacetylated and phenylglycylated substrates is the acylation step, kinetic effects such as solvent KIEs on the steady-state parameters can be attributed to effects on events in the acylation step. For the deacylation such information is more difficult to obtain since the individual rate and binding constants cannot be measured separately. Some information may be obtained by performing nucleophile competition experiments [7]. The rate of aminolysis vs. hydrolysis can be expressed as the V_s/V_h ratio and according to Scheme 1, is given by [33]:

$$\frac{V_s}{V_h} = \frac{[N] \cdot k_s}{k_{h1} \cdot K_{EAcN} + k_{h2} \cdot [N]} \qquad (7)$$

The above kinetic experiments showed that the hydrolysis of the phenylacetylated enzyme (k_{h1}) proceeds with a rate of at least 1000 s^{-1}. To obtain significant rates of aminolysis at such a high rate of hydrolysis, it follows from Eqn (7) that either the reactivity of the β-lactam nucleophile with the acyl-enzyme (k_s) must be considerably higher than k_{h1}, or tight binding of the β-lactam nucleophile to the acyl-enzyme, giving a low value for K_{EacN}, must occur, in which case k_s must be higher than k_{h2}. Moreover, competition for nucleophilic attack between H_2O and a β-lactam nucleophile may be affected by the type of acyl-enzyme that is formed, i.e. whether phenylacetic acid or phenylglycine is the acyl moiety, and by the structure of the β-lactam nucleophile. To investigate the kinetic mechanism of deacylation and to assess the influence of the structure of the acyl donor and nucleophilic β-lactam on this reaction, we performed nucleophile competition experiments using two different acyl donors, phenylacetamide and phenylglycine amide and two different nucleophiles, 6-APA and 7-ADCA.

From Eqn (7) it follows that the relationship of V_s/V_h vs. [N] is hyperbolic and may be written as

$$\frac{V_s}{V_h} = \frac{\left(\frac{V_s}{V_h}\right)_{max} \cdot [N]}{K_{Napp} + [N]} \qquad (8)$$

in which

$$\left(\frac{V_s}{V_h}\right)_{max} = \frac{k_s}{k_{h2}} \qquad (9)$$

and K_{Napp}, at which half of the $(V_s/V_h)_{max}$ is reached, is

$$K_{Napp} = \frac{k_{h1}}{k_{h2}} \cdot K_{EAcN} \qquad (10)$$

Equation (10) shows that the apparent affinity of the enzyme for the nucleophile (K_{Napp}) is given by the binding constant of the nucleophile to the acyl-enzyme

(K_{EAcN}) and the ratio between the rates of hydrolysis of the acyl-enzyme with nucleophile bound (k_{h2}) and without nucleophile bound (k_{h1}). In other words, when binding of the β-lactam nucleophile leads to a reduction of the rate of deacylation by H_2O by lowering the nucleophilicity of H_2O or by displacement of water from the active site, the apparent affinity for the β-lactam nucleophile decreases (K_{Napp} increases) and the relationship between V_s/V_h and [N] will approach to a straight line, given by,

$$\frac{V_s}{V_h} = \frac{k_s}{k_{h1} \cdot K_{EAcN}} \cdot [N] \qquad (11)$$

For the synthesis of ampicillin, using phenylglycine amide as the acyl donor and 6-APA as the nucleophile, a saturation of V_s/V_h was observed at increasing 6-APA concentrations, indicating that when 6-APA is bound to the acyl-enzyme, H_2O is not excluded from the active site and still able to deacylate the enzyme (Fig. 5A). In contrast, a linear dependence was observed in the concentration range of 0–250 mM 6-APA when phenylacetamide was used as the acyl donor, producing penicillin G. Both the $(V_s/V_h)_{max}$ and the K_{Napp} for this reaction were higher than observed for synthesis of ampicillin.

Values of 47 ± 8 mM for K_{Napp} and of 3.6 ± 0.22 for $(V_s/V_h)_{max}$ for the synthesis of ampicillin were obtained from fitting Eqn (8) to the data. For synthesis of penicillin G, a value of 0.018 mM^{-1} for the slope of the curve was obtained, but due to the almost linear dependence of V_s/V_h on the concentration of 6-APA no reliable values for $(V_s/V_h)_{max}$ and a K_{Napp} for penicillin G synthesis could be obtained.

These results show that the competition between H_2O and 6-APA for the acyl-enzyme is strongly dependent on the type of acyl group and that binding of 6-APA to the phenylacetylated enzyme suppresses hydrolysis more than binding of 6-APA to the phenylglycylated enzyme.

When 7-ADCA was used as the deacylating nucleophile instead of 6-APA and phenylglycine amide as the acyl donor, a higher V_s/V_h was observed at all concentrations of 7-ADCA. The dependence of V_s/V_h on [7-ADCA] was linear, with a value of 0.33 mM^{-1} for the slope, in contrast to the saturation at a lower V_s/V_h value that was found with 6-APA as the nucleophile, indicating that the enzyme has a lower apparent affinity for 7-ADCA (Fig. 5B).

Yousko *et al.* (2002) found similar constants for the V_s/V_h for deacylation with 7-ADCA and 6-APA using PAA and PGA. However they observed in all cases a saturation of the $(V_s/V_h)_{max}$, whereas our data indicate a linear relation for the combinations of 7-ADCA/PGA and 6-APA/PAA. Since their measurements were carried out at a different pH, this indicates that competition between water and the nucleophilic β-lactam at the active-site may be pH-dependent.

From Eqn (10) it follows that the higher K_{Napp} for 7-ADCA may be caused by a lower affinity of the enzyme for 7-ADCA compared to 6-APA or it could be that binding of 7-ADCA to the acyl-enzyme lowers the k_{h2} more than binding of 6-APA. To discriminate between these two phenomena we studied binding of 6-APA and 7-ADCA to the enzyme by measuring the inhibition of the hydrolysis of

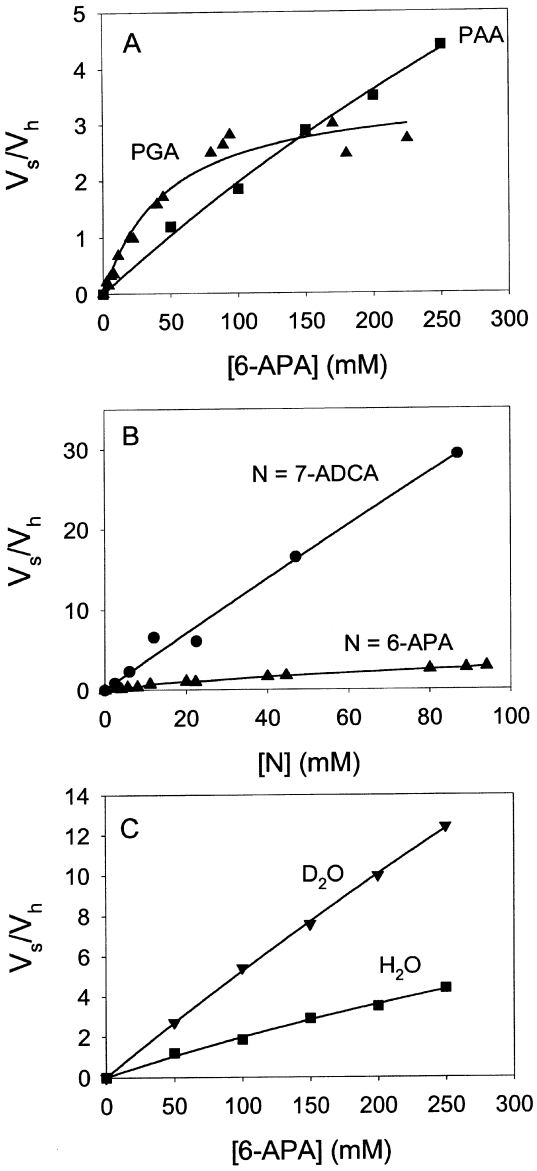

Fig. 5. Nucleophile competition experiments with penicillin acylase. (A) Dependence of V_s/V_h on [6-APA], using phenylacetamide (PAA) or phenylglycine amide (PGA) as the acyl donor. (B) Dependence of V_s/V_h on [N], using 7-ADCA or 6-APA as the nucleophiles and phenylglycine amide as the acyl donor. (C) Dependence of V_s/V_h on [APA], using phenylacetamide as the acyl donor in H_2O and 100% D_2O. The symbols represent experimental data, the lines are the best fit to the data using Eqn (8).

NIPGB. Both 6-APA (data not shown) and 7-ADCA competitively inhibit the hydrolysis of NIPGB as indicated by the Lineweaver-Burk plots that intersected on the *y*-axis (Fig. 6).

The nucleophile with the highest reactivity, 7-ADCA, has a K_i of 7 mM whereas for 6-APA a K_i of 50 mM was measured. The values for K_i represent binding of the nucleophile to the free enzyme and were used as an estimation for the K_{EacN}, which represents binding of the nucleophile to the acyl-enzyme. Substituting the values of K_i

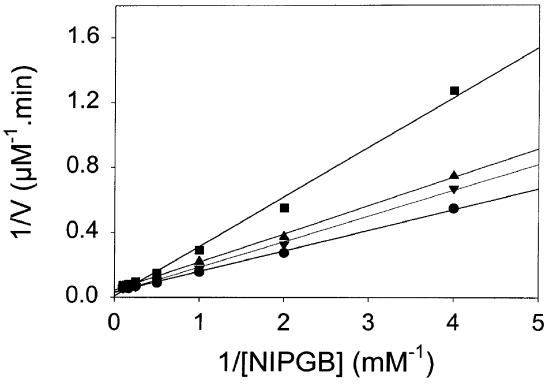

Fig. 6. Inhibition of NIPGB hydrolysis by 7-ADCA. Lineweaver–Burk plot of the effect of various concentrations of 7-ADCA on the conversion of NIPGB. ●, 0 mM 7-ADCA; ▼, 10 mM 7-ADCA; ▲, 15 mM 7-ADCA; ■, 20 mM 7-ADCA. The lines represent linear regressions through the data points.

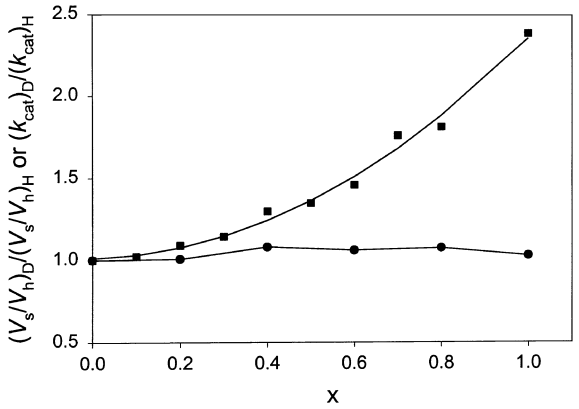

Fig. 7. Proton inventories for the deacylation of phenylacetylated PA and acetylated PA. ■, Dependence of V_s/V_h on the molar fraction D_2O (×) using phenylacetamide as the acyl donor and 6-APA as the nucleophile. The symbols represent the experimental data and the line is the best fit to the data using Eqn (5). ●, Dependence of the k_{cat} for hydrolysis of *p*-NPA on the molar fraction D_2O. For this substrate, acylation is faster than deacylation and the k_{cat} thus represents the rate of breakdown of the acyl-enzyme intermediate.

for K_{EAcN} in Eqn (10) revealed that the reduced apparent affinity for 7-ADCA is not caused by weaker binding of 7-ADCA but by a lower k_{h2}, which represents hydrolysis of the acyl-enzyme to which 7-ADCA is bound. The increased slope of the curve of V_s/V_h vs. [N] obtained for 7-ADCA can be fully explained by the fivefold higher affinity of the enzyme for 7-ADCA compared to 6-APA, which indicates that the rate constant k_s for 7-ADCA is equal to the k_s for 6-APA.

The dependence of V_s/V_h on the nucleophile concentration [N] and the results of the inhibition studies show that, despite of the high rate of hydrolysis of the acyl-enzyme, tight binding of the nucleophile and displacement of the catalytic H_2O molecule ensure that significant deacylation by β-lactam nucleophiles can occur. The results obtained with 7-ADCA and 6-APA showed that differences in structure of the nucleophiles far from the nucleophilic amino function exert a large influence on the competition of the nucleophiles with H_2O for the acyl-enzyme. The 6-APA moiety of penicillin G binds with the thiazolidine ring to the enzyme via hydrophobic interactions between the 2β-methyl group and αF146 and βF71 and hydrogen bonding between its carboxylate group and αR145:NH_2 [4,9,34]. In 7-ADCA, which has a dihydrothiazine ring instead of a thiazolidine ring, the 2β-methyl group is not present and due to the more planar character of the dihydrothiazine ring, the carboxylate group may be in a different position than in 6-APA. Consequently, it is conceivable that binding of 7-ADCA to the enzyme is mediated via different interactions than binding of 6-APA, which may explain the higher affinity of the enzyme for 7-ADCA and the higher nucleophilicity of 7-ADCA compared to 6-APA.

Proton transfer in the deacylation reaction

To study whether proton transfer would be important in the deacylation of phenylacetylated acyl-enzyme, we measured the initial V_s/V_h in D_2O at several concentrations of 6-APA (Fig. 5C). The V_s/V_h showed an inverse isotope effect of 2.6 at all concentrations of 6-APA, indicating that aminolysis is less sensitive to an increasing concentration of D_2O than hydrolysis. A curved proton inventory for V_s/V_h was observed that was best fitted with a second order polynomial (Fig. 7).

The V_s/V_h ratio is the ratio of the reaction rates of aminolysis and hydrolysis of the acyl-enzyme. The curvature of the plot of V_s/V_h vs. the mole fraction of D_2O indicates therefore that the deuterium KIE arises from either multiple protons being transferred in one of the transition states of the deacylation reaction or from separate effects of D_2O on the transition states of both the hydrolysis and aminolysis reaction. The latter possibility seems more likely, in view of the one-proton transfer mechanism suggested for deacylation of PA [3,35].

Conversion of *p*-NPA leads to an acyl-enzyme in which an acetyl group is attached to the active-site serine. Deacylation of this acetyl-enzyme is the rate-limiting step in the catalytic cycle. No solvent KIE was observed on the k_{cat} of *p*-NPA suggesting that in the transition state of this hydrolytic reaction no protons are transferred (Fig. 7). This indicates that in this deacylation reaction a chemical reaction involving proton transfer is not the rate-limiting step, in contrast to deacylation of the phenylacetylated enzyme. The rate constant for deacylation of the acetyl-enzyme may reflect another step, such as a conformational change of the enzyme prior to chemical hydrolysis of the acyl-enzyme by water. A conformational change in the deacylation of *p*-NPA is not unlikely in view of structural results obtained by Done *et al.* [36]. In this work it was shown that *p*-nitrophenylacetic acid may bind in the acyl-binding site of PA. Hydrolysis of *p*-NPA may thus proceed via reversed binding of the substrate, in which the leaving group, *p*-nitrophenol, occupies the acyl binding site and the acetyl group binds at the leaving group binding site, sterically hindering the deacylating H_2O molecule for efficient nucleophilic attack. A conformational change may be necessary for release of *p*-nitrophenol from the active site and subsequent deacylation of the acetyl-enzyme.

Table 2. Kinetic constants for synthesis and hydrolysis of β-lactam antibiotics by penicillin acylase. The rate and binding constants refer to the constants as depicted in Fig. 1. The values were obtained by setting the k_2 to the value obtained for the k_{cat} and setting k_{h1} to the lower limit obtained by stopped flow experiments. The other parameters were calculated from the nucleophile competition and nucleophile inhibition experiments, using equations 8–11, as described in the text.

Reaction	Phenylglycin + 7-ADCA → cephalexin	Phenylglycin + 6-APA → ampicillin	Phenylacetamide + 6-APA → penicillin G
K_{AD} (mM)	40	40	0.2
k_2 (s^{-1})	30	30	50
K_Q (mM)	1.2	2.5	0.005
k_{hQ} (s^{-1})	50	30	40
k_{h1} (s^{-1})	> 75	> 75	> 1000
k_{h2} (s^{-1})	< k_s/35	k_s/3	< k_s/4
k_s (s^{-1})	> 308	> 150	> 4000
K_{EacN} (mM)	10	50	50

Structural analysis of an enzyme-substrate complex could confirm the existence of such a reversed binding mode [4,5].

Kinetic constants for PA catalyzed synthesis and hydrolysis of β-lactam antibiotics

The kinetic properties of PA are important with respect to the yield that can be obtained in a kinetically controlled synthesis of β-lactam antibiotics. Combining the data from the steady-state and presteady state experiments and the data from nucleophile competition and inhibition experiments, the kinetic constants for the synthesis and hydrolysis of β-lactam antibiotics can be calculated (Table 2). Exact values can be determined for the rate constants of acylation, whereas only relative rates can be obtained for the deacylation by various nucleophiles. The values for the hydrolysis and synthesis of ampicillin are close to the numbers obtained by Yousko and Svedas [15]. Eqn (1) shows that for the application of the enzyme in synthesis two kinetic properties are important. First, the enzyme should have a low activity for the antibiotic compared to the acyl donor. The relative specificity of the enzyme for both substrates may be expressed by the factor α, given by [7],

$$\alpha = \frac{(k_{cat}/K_m)_Q}{(k_{cat}/K_m)_{AD}} = \frac{k_{hQ}/K_Q}{k_2/K_{AD}} \quad (12)$$

The data in Table 1 show that α for the synthesis of ampicillin cannot be decreased by using the chemically more reactive phenylglycine ester instead of phenylglycine amide. The chemical reactivity of the ester bond cannot be fully exploited by the enzyme to increase the rate of acylation. This may be caused by the fact that the enzyme is probably evolutionary optimized for phenylacetylated rather than phenylglycylated compounds [37,38]. Furthermore the K_m for the product ampicillin is much lower than the K_m values for the acyl donors phenylglycine amide and phenylglycine methyl ester. Both the low activity of PA for ester substrates compared to amides and the high affinity of the enzyme for the product of synthesis increase α and hence reduce the yield in synthesis reactions.

A second requirement for efficient synthesis is a high rate of aminolysis compared to hydrolysis. The experiments with the most important nucleophiles in β-lactam antibiotic synthesis, 7-ADCA and 6-APA, showed that tight binding of the nucleophile to the acyl-enzyme and displacement of the hydrolytic water molecule are the two most important factors in determining the reactivity of the nucleophile. The fact that the acyl-enzyme to which 7-ADCA is bound cannot be hydrolysed, signifies that the V_s/V_h ratio in a synthesis reaction can be increased by adding more 7-ADCA. In contrast, when using 6-APA as the nucleophile, the V_s/V_h ratio levels off to a maximum at increasing 6-APA concentrations. Moreover, the affinity of the enzyme for 6-APA is in the order of 50 mM, which means that significant hydrolysis takes place at relatively high concentrations of this nucleophile.

The above analysis indicates that several properties of the enzyme can be optimized to accomplish higher yields in synthesis reactions. It has been shown that site-directed mutagenesis of residues in the active site indeed leads to mutant enzymes in which hydrolysis of the acyl-enzyme is suppressed leading to mutants with improved biocatalytic properties [4,9,10].

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