Penicillin G acylase catalysed the hydrolysis of 4-nitrophenyl acetate with a $k_{\text{cat}}$ of 0.8 s$^{-1}$ and a $K_m$ of 10 $\mu$M at pH 7.5 and 20 °C. Results from stopped-flow experiments fitted a dissociation constant of 0.16 mM for the Michaelis complex, formation of an acetyl enzyme with a rate constant of 32 s$^{-1}$ and a subsequent deacylation step with a rate constant of 0.81 s$^{-1}$. Non-linear Van’t Hoff and Arrhenius plots for these parameters, measured at pH 7.5, may be partly explained by a conformational transition affecting catalytic groups, but a linear Arrhenius plot for the ratio of the rate constant for acylation relative to deacylation step may be partly explained by a conformational transition affecting catalytic groups, but a linear Arrhenius plot for the ratio of the rate constant for acylation relative to $K_a$ was consistent with energy-compensation between the binding of the substrate and catalysis of the formation of the transition state. At 20 °C, the pH-dependence of $k_{\text{cat}}$ was similar to that of $k_{\text{cat}}/K_m$, indicating that formation of the acyl-enzyme did not affect the $pK_a$ values (6.5 and 9.0) of an acidic and basic group in the active enzyme. The heats of ionization deduced from values of $pK_a$ for $k_{\text{cat}}$, which measures the rate of deacylation, are consistent with $\alpha$-amino and guanidinium groups whose $pK_a$ values are decreased in a non-polar environment. It is proposed that, for catalytic activity, the $\alpha$-amino group of the catalytic Ser$^{61}$ and the guanidinium group of Arg$^{126}$ are required in neutral and protonated states respectively.

**Key words:** pre-steady-state kinetics, stopped-flow spectrophotometry.

### INTRODUCTION

Penicillin G acylase from *Escherichia coli* ATCC 11105, in common with several other acylases acting on penicillin or cephalosporin substrates, catalyses the hydrolysis of benzylpenicillin to release 6-aminopenicillanic acid and phenylacetic acid. The enzyme has long been of industrial interest in the synthesis of semi-synthetic $\beta$-lactam antibiotics but the catalytic pathway is still incompletely understood.

During post-translational membrane-translocation and protein assembly [1], there is excision of a 54-residue endopeptide and the mature enzyme contains an N-terminal A-chain, ($M_r$ 23800) and a B-chain ($M_r$ 62200) in which Ser$^{60}$ occupies position B1 [2]. Chemical and structural evidence implicate this serine residue as a catalytic nucleophile. The inhibitor PMSF [3] which has a structural resemblance to a specific substrate, uniquely modified the hydroxyl group of the Ser$^{61}$ residue in the enzyme from *E. coli* [4] and the corresponding group in the sequence-related enzyme from *Kluyvera citrophila* [5]. This provided the basis for chemical conversion of the modified serine residue to cysteine (Ser$\rightarrow$Cys) yielding an enzyme with no detectable catalytic activity, results that were underlined by the expression and processing of an inactive *E. coli* Ser$\rightarrow$Cys mutant protein [6]. Crystal structures of the native protein, the non-covalent complex with the product phenylacetic acid, and the covalent PMSF complex [7] showed the O$\gamma$ atom of Ser$^{61}$ to be appropriately placed to form an acyl-enzyme as an intermediate in the catalytic pathway. Similar folding motifs have been found in three different hydrolases with an N-terminal nucleophile other than serine [8]. This position of a putative catalytic nucleophile residue implies an unusual potential mobility which must be overcome by interactions with nearby groups in the protein.

Although these properties suggested the likelihood of an acylation step, they did not demonstrate its significance during catalysis. Accumulation of an acyl-enzyme during hydrolysis of a phenylacetyl substrate has not yet been demonstrated but several reactive esters of aliphatic acids have been shown to be substrates of the sequence-related penicillin acylase from *K. citrophila* [9] and the rapid pre-steady-state release of 4-nitrophenol from 4-nitrophenyl acetate provides evidence for the accumulation of an acetyl-enzyme [10]. Among several substrates tested, this was the only one for which the rate constants for both acylation and deacylation could be determined.

This substrate has therefore been used to demonstrate a similar accumulation of an acetyl-derivative of the enzyme from *E. coli* and to show that the steady state value of $k_{\text{cat}}$ was equal to the rate constant for the deacylation step. The results provide evidence for lowered $pK_a$ values of ionizable groups in the region of the catalytic centre.

### MATERIALS AND METHODS

#### Materials and solutions

*N-(3-Carboxy-4-nitrophenyl)phenylacetamide* (NIPAB) and 4-nitrophenyl esters were from Sigma-Aldrich. Acetonitrile was HPLC grade. Other reagents were of analytical grade.

Purified penicillin acylase from Boehringer Mannheim (Mannheim, Germany) was stored as a suspension in 3.2 M ammonium sulphate. The specific enzyme activity (0.4 mM NIPAB as substrate in 100 mM phosphate buffer, pH 7.5, 20 °C), determined as described by Slade et al. [4], was 16–20 kat (mol of protein)$^{-1}$.

The 100 mM phosphate buffer, pH 7.5 contained 82.8 mM Na$_2$HPO$_4$, and 17.2 mM NaH$_2$PO$_4$; other 100 mM phosphate buffers, pH 6.6–8.0, contained a total of 100 mM phosphate (sodium salts); 50 mM Tris buffers, pH 7.2–9.0, contained the

### Abbreviation used: NIPAB, "N-(3-carboxy-4-nitrophenyl)phenylacetamide."
required amount of HCl. Values of pH were corrected for the pH, temperature and ionic strength of the solution [11].

Substrate solutions in acetonitrile were diluted to give a final acetonitrile concentration of 0.5% (v/v) or 5% (v/v) where this was necessary to maintain an ester in solution.

Spectrophotometric methods

A Cary model 4E spectrophotometer was used with a 10 mm light-path cuvette in a cell holder thermostatically controlled at 20 ± 0.1 °C. The protein concentration of penicillin acylase was determined from measurement of $A_{280}$ assuming $\epsilon_{280}$ to be 1.97 × 10$^4$ M$^{-1}$ cm$^{-1}$ [12].

Kinetic methods

For the determination of $K_m$ and $k_{cat}$ in the steady-state, solutions (1.0 ml) contained 0.1–0.5 μM penicillin acylase and 10–120 μM 4-nitrophenyl acetate in 100 mM phosphate buffer, pH 7.5. The value of the molar absorption coefficient of the 4-nitrophenoxide anion, 1.83 × 10$^4$ M$^{-1}$ cm$^{-1}$ [13], was adjusted for the effects of pH, temperature and ionic strength [11], using a value of $pK'$ determined by spectrophotometric titration at each temperature. Initial velocity was determined, using linear least-squares regression, from the steady-state part of progress curves and kinetic parameters were calculated from plots of initial velocity against substrate concentration [14]. Unless otherwise stated, standard errors are shown for the indicated number of different substrate concentrations in a single experiment.

A stopped-flow spectrophotometer (Applied Photophysics model SX-17MV), with the flow-path thermostatically controlled, was used with a 10 mm light path to monitor the formation of the product 4-nitrophenol ($A_{405}$). Equal volumes (62.5 μl) of solutions, mixed with a dead-time of 1.8 ms, contained 0.1–0.5 μM penicillin acylase and 10–800 μM 4-nitrophenyl acetate, propionate, butyrate or valerate in 100 mM phosphate buffer, pH 7.5, with a concentration of acetonitrile twice that of the final mixture. The results were analysed according to relationships defining steady-state kinetic parameters (eqns. 1–5) and the burst amplitude and kinetics of approach to the steady state (eqns. 6–8) [15]:

$$ E+S \rightarrow ES \rightarrow P_e + E \rightarrow E + P_{2} \quad (1) $$

$$ k_{cat} = k_{cat}^{0} k_{cat}/(k_{cat} + k_{cat}^{0}) \quad (2) $$

$$ K_{m} = k_{cat}^{0} k_{cat}/((k_{cat} + k_{cat}^{0})k_{cat}^{+}) \quad (3) $$

$$ K_{m}^{rev} = (k_{cat} + k_{cat}^{0})/k_{cat} \quad (4) $$

$$ K_{m} = k_{cat}^{0} / k_{cat} \quad (5) $$

$$ [P_{1}] = A + B(1 - e^{-kt}) \quad (6) $$

$$ B = [E]_{0} k_{cat}^{0} k_{cat}^{+}/(1 + K_{m}^{rev} + [S]_{0}) \quad (7) $$

$$ k' = (k_{cat} + k_{cat}^{0})[S]_{0} + k_{cat}^{0} k_{cat}^{+}/(K_{m}^{rev} + [S]_{0}) \quad (8) $$

where $E'$, $P_{1}$ and $P_{2}$ represent the acyl-enzyme, 4-nitrophenol and the acid product respectively.

Molecular graphics

Distances between atoms, the degree of exposure of amino acid side chains to solvent, and the positions of possible hydrogen bonds were calculated using QUANTA software (Molecular Simulations, San Diego, CA, U.S.A.) and a Silicon Graphics Indigo workstation.

RESULTS AND DISCUSSION

Steady-state kinetic parameters

Steady-state parameters for four 4-nitrophenyl esters are shown in Table 1. As was observed with the enzyme from K. citrophila [9], there was significant catalysis of hydrolysis of each of these esters and the values of $k_{cat}$ and $K_m$ for 4-nitrophenyl acetate were decreased and increased respectively when the concentration of acetonitrile was increased by a factor of 10. The value of $k_{cat}$ for 4-nitrophenyl acetate in 0.5% (v/v) acetonitrile was small compared with the values, measured under similar conditions, with derivatives of phenylacetic acid, being less than 5% of the value (20 s$^{-1}$) with the anilide NPRP and less than 1% of the value (140 s$^{-1}$) with the ester 4-nitrophenyl phenylacetate [4]. Since the enzyme shows a marked specificity for amide and ester substrates containing the phenylacetyl or a similar group, the value of $K_m$, which was similar to that previously reported for other amide and ester substrates [3,16], is consistent with a value considerably less than $K_m$, as expected if an acyl-enzyme accumulates. Values of $k_{cat}$ and $k_{cat}/K_m$ were less with other esters of 4-nitrophenol, consistent with less efficient acylation by these substrates. Stereocchemical factors presumably account for an absence of catalytic activity with 2,4-dinitrophenyl acetate.

Pre-steady-state kinetics

There was a biphasic time-course of release of 4-nitrophenol, illustrated in Figure 1, after mixing 4-nitrophenyl acetate and penicillin acylase under conditions such that the spontaneous rate of substrate hydrolysis was less than 1% of the enzyme-catalysed steady-state rate. Both the burst amplitude and the steady-state rate of release of product showed little dependence on substrate concentration, as expected for a range of substrate concentrations more than 3-fold greater than $K_m$. By contrast, the rate constant for the burst phase, $k'$, was strongly dependent on substrate concentration in a range much greater than $K_m$, consistent with $K_m^{rev}/K_m > K_m$. The extrapolated value of $(k_{cat} + k_{cat}^{0})$ at substrate saturation was greater than the separately determined steady-state value of $K_m$ by a factor of approximately 50. These results imply that $K_m$ is rate-limited by $k_{cat}$.$^0$. This was confirmed by a method of calculation independent of knowledge of the enzyme concentration [10]: the dependence of $B/A$ on [substrate] provides an estimate of $k_{cat}(k_{cat} + k_{cat}^{0})/k_{cat}^{+}$ and in those experiments where $k_{cat} > k_{cat}^{0}$, this parameter is almost equal to $k_{cat}$.$^0$. This estimate was used as a constraint in the non-linear least-squares analysis to obtain a ratio $k_{cat}/k_{cat}^{0}$ of 38 at 20 °C. The

Table 1 Steady-state kinetic parameters with esters of aliphatic acids

<table>
<thead>
<tr>
<th>Ester</th>
<th>[Acetonitrile] (%)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Nitrophenyl acetate</td>
<td>0.5</td>
<td>0.80 ± 0.10</td>
<td>10 ± 6</td>
<td>(8.4 ± 4.2) × 10$^4$</td>
</tr>
<tr>
<td>4-Nitrophenyl acetate</td>
<td>5</td>
<td>0.80 ± 0.03</td>
<td>65 ± 18</td>
<td>(5.5 ± 1.2) × 10$^3$</td>
</tr>
<tr>
<td>4-Nitrophenyl propionate</td>
<td>5</td>
<td>0.39 ± 0.03</td>
<td>318 ± 52</td>
<td>(1.2 ± 0.1) × 10$^3$</td>
</tr>
<tr>
<td>4-Nitrophenyl butyrate</td>
<td>0.15 ± 0.01</td>
<td>99 ± 20</td>
<td>(1.5 ± 0.2) × 10$^3$</td>
<td></td>
</tr>
<tr>
<td>4-Nitrophenyl valerate</td>
<td>0.27 ± 0.12</td>
<td>551 ± 380</td>
<td>(4.9 ± 1.3) × 10$^2$</td>
<td></td>
</tr>
</tbody>
</table>
values of \((k_{cat} + k_{cat})/K_m^{\text{avg}}\) at 5, 20 and 43 °C, obtained by fitting the data to eqn. (8), were \(0.10 \pm 0.01, 0.20 \pm 0.01\) and \(0.51 \pm 0.07\) s\(^{-1}\) M\(^{-1}\) respectively. These values reflect relatively inefficient catalysis which, together with the evidently large value of \(K_m^{\text{avg}}\) and the assumption of a diffusion-limited value for \(k_{cat}\), implies that \(k_{cat} \ll k_{cat}\) and that \(K_m^{\text{avg}}\) is therefore closely similar to \(K_m\). Kinetic parameters, measured at 20 °C and derived from progress curves recorded at 9 different substrate concentrations, gave values for \(k_{cat}, k_{cat}\) and \(K_m\) of \(31.7 \pm 4.7\) s\(^{-1}\), \(0.81 \pm 0.03\) s\(^{-1}\) and \(156 \pm 20\) μM respectively. Values of steady-state parameters, calculated from these results using eqn. (2) and (3), were \(k_{cat} = 0.79 \pm 0.03\) s\(^{-1}\) and \(K_m = 3.9 \pm 0.6\) μM, values which are in reasonable agreement with those determined by steady-state measurements after manual mixing (Table 1). Eqn. (7) was used to estimate the catalytic-centre concentration of enzyme, assuming the value of \(k_{cat}/K_m\) to be large: a weighted plot [17] of \(1/\sqrt{B}\) against \(1/\text{substrate}\) yielded a value of \(0.78 \pm 0.23\) of catalytic centres relative to total protein for the data shown in Figure 1. The mean value (± S.E.M.) for three independent experiments was \(0.90 \pm 0.07\). Use of this fraction to correct the apparent \(k_{cat}\) determined in a separate steady-state experiment gave a value of \(0.83\) s\(^{-1}\), in good agreement with the value calculated from the pre-steady-state parameters. This procedure therefore provides a more direct measure of the content of catalytic centres than titration of the decrease in catalytic activity after site-directed covalent chemical modification by PMSF [18].

Arrhenius plots of \(k_{cat}, k_{cat}\) and \(K_m\) and the Van’t Hoff plot of \(K_m\) determined at pH 7.5 for the temperature range 3–43 °C, deviated from a straight line with a decreased slope at temperatures greater than 20 °C equivalent to decreased values of \(\Delta H\) of 40.1 ± 2.3, 44 ± 5.6, 43.2 ± 9.0 and 33.3 ± 4.3 kJ mol\(^{-1}\) respectively. As expected from the similarity in these values, the Arrhenius plot of \(k_{cat}/K_m\) shown in Figure 2 was linear, with an apparent value of \(\Delta H\) of 33.6 ± 1.0 kJ mol\(^{-1}\). These results are consistent with energy compensation between binding and the rate of formation of the acetyl-enzyme [19] and with common features in the catalytic processes determining \(k_{cat}\) and \(k_{cat}\).

Similar effects of temperature on steady-state kinetic parameters were reported for the hydrolysis of the specific substrate benzylpenicillin by the sequence-related penicillin G acylase from K. citrophila [20] and these results were interpreted by assuming a linear Arrhenius temperature-dependence of \(k_{cat}\) and \(k_{cat}\); the non-linearity was suggested to be caused by change in the relative contributions of the individual rate constants to steady-state kinetic parameters as a function of temperature. However, this explanation does not apply to the present results. Here, part of the effect may be explained by a change in the optimum pH as a function of temperature; there may also be a rapidly established equilibrium between two states of the enzyme comparable to that proposed for chymotrypsin [21], favouring a state making less effective catalytic use of binding energy at higher temperature.

**pH dependence of kinetic parameters**

The pH-dependence of steady-state kinetic parameters is shown in Figure 3, where the fitted lines were calculated according to eqn. (9):

\[
k_{\text{obs}} = k_{\text{lim}}/(1+[H^+]/K_a + [H^+]/[H^+])
\]

(9)

The values of \(pK_a\) and \(pK_b\) for \(k_{\text{obs}}\) were 6.5 and 9.0 respectively and the corresponding \(pK_a\) values from the plot of \(k_{\text{cat}}/K_m\) were 6.1 and 8.6 respectively, with values of S. E. M. < 0.2. These results are similar to the effects of pH observed with this enzyme [3] and that from K. citrophila [22] acting on substrates containing the phenylacetyl group. Interpreting the pH dependence of \(k_{\text{cat}}\) and \(k_{\text{cat}}/K_m\) in terms of acidic and basic groups in the catalytic centre of the acyl-enzyme and the free enzyme respectively, these results imply little change in the states of ionization of catalytic centre groups on formation of the acetyl-enzyme. Within the range of pH 7.1–8.5, there was a stoichiometric pre-steady-state burst similar to that observed at pH 7.5, implying that \(k_{cat} \gg k_{cat}\) and that \(k_{cat}\) was rate-limited by \(k_{cat}\). Together with the observation that there was no significant pH-dependence of \(K_m\),
noting that 6.3, 7.6 and 8.0, each using 7 substrate concentrations), and formation and hydrolysis of the acyl-enzyme [7].

proposed involvement of the same acidic and basic groups in the heats of ionization for $p_{k,\text{cat}}$.

For determination of $k_{\text{cat}}/K_m$, the pseudo-first-order kinetics of hydrolysis of 4-nitrophenyl acetate were followed using 0.74 $\mu$M 4-nitrophenyl acetate and 20 nM penicillin acylase. The lines were fitted to eqn. (9): $k_{\text{cat}}/K_m$ (---); $k_{\text{cat}}$ (----). Error bars show S.D. for 2 experiments.

Figure 3 pH-dependence of steady-state kinetic parameters at 20 °C

For determination of $k_{\text{cat}}/K_m$, the pseudo-first-order kinetics of hydrolysis of 4-nitrophenyl acetate were followed using 0.74 $\mu$M 4-nitrophenyl acetate and 20 nM penicillin acylase. The lines were fitted to eqn. (9): $k_{\text{cat}}/K_m$ (---); $k_{\text{cat}}$ (----). Error bars show S.D. for 2 experiments.

Figure 4 Effect of temperature on $p_{K_a}$ values derived from the pH-dependence of $k_{\text{cat}}$

Values of $k_{\text{cat}}$ were determined at 11–15 different pH values in the range 4–10. Error bars show the standard errors of the mean value obtained by weighted non-linear least squares regression.

with a value 10±4 $\mu$M (mean ± S.D. for experiments at pH 5.7, 6.3, 7.6 and 8.0, each using 7 substrate concentrations), and noting that $k_{\text{cat}}/K_m = k_{\text{cat}}/K_m$ for $p_{K_a}$, these results imply a similar pH-dependence for $k_{\text{cat}}$ and $k_{\text{cat}}$. This is consistent with the proposed involvement of the same acidic and basic groups in the formation and hydrolysis of the acyl-enzyme [7].

The apparent $p_{K_a}$ values obtained from the pH-dependence of $k_{\text{cat}}$ and hence $k_{\text{cat}}$, were affected by temperature (Figure 4) with heats of ionization for $p_{K_a}$ and $p_{K_g}$ of 47±11 and 70±14 kJ·mol⁻¹ respectively. Similar heats of ionization have been reported for $p_{K_a}$ and $p_{K_g}$ determined from the pH-dependence of $k_{\text{cat}}$.

steady-state kinetic parameters in the hydrolysis of benzylpenicillin catalysed by the enzyme from K. citrophila [22] but those results were less clearly interpretable in terms of individual rate constants.

The simplest interpretation of the present results involves ionizable amino-acid side-chain groups close to the catalytic centre although indirect effects cannot be wholly excluded. Crystal structures have provided a plausible candidate for a catalytic base acting in both acylation and deacylation steps [7]. The $\alpha$-amino group of Ser⁷¹ is sufficiently close to the $O_Y$ atom of the same residue to accept a proton, either directly or through a bridging water molecule, so fulfilling the necessary function of increasing the nucleophilicity of Ser⁷¹, and there are no other groups close enough to act either as a general base or as a general acid. It has therefore been proposed that the $\alpha$-amino group is required to be present in the neutral base form. Considering groups expected to possess $p_{K_a}$ values closest to $p_{K_a}$, the observed heat of ionization for $p_{K_a}$ is greater than the value of zero expected for a carboxy group and is consistent with the expected values of approximately 40 and 50 kJ·mol⁻¹ for peptide and protein $\alpha$- and $\epsilon$-amino groups respectively and approximately 30 kJ·mol⁻¹ for imidazolium [23]. Although the value of $p_{K_a}$ is somewhat less than that expected for an $\alpha$-amino group, a decreased value is plausible in a protein environment that is less polar than that of the aqueous solvent. Additionally, the ionization of a carboxylic acid, lysine or histidine side chain is unlikely to account for $p_{K_a}$ because the shortest distances from the $O_Y$ atom of Ser⁷¹ to aspartic acid or glutamic acid, histidine and lysine side chains are 10, 12.5 and 14 Å respectively.

Although no other basic groups are close to the $O_Y$ atom of Ser⁷¹, the $N_{\gamma}$ atom of Arg⁹⁸² is capable of forming a hydrogen bond with the $O_{\delta}$ atom of Asn⁹⁴¹ and this residue is placed to form a hydrogen-bond with the $\alpha$-amino nitrogen of Ser⁷¹ [7]. Figure 5 shows possible hydrogen bonds in the region of Arg⁹⁸³.

Possible hydrogen-bonds in the region of Arg⁹⁸³

Positions of non-hydrogen atoms are from the Brookhaven protein database entry 1pnk and the broken lines show possible positions of hydrogen-bonds. Labels identify B-chain residues and atoms potentially involved in hydrogen-bonding. Wat360 represents a structural water molecule which may be hydrogen-bonded to the $\alpha$-amino group of Ser⁹¹, to the hydroxy group of the Ser⁷⁸⁶ side chain and the $N_{\gamma}$ group of Arg⁹⁸².

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an exposed arginine side chain, is consistent with the side chain being in an environment of low polarity. The heat of ionization of the arginine side chain has been reported to be 60 kJ mol⁻¹ [24], a value similar to that found here. The side chains of eight A-chain and nineteen B-chain arginine residues occupy an abnormal environment with an average fractional exposure to solvent of less than 0.5 but only ArgB¹⁰,A is located nearer than 9 Å from the Oγ atom of SerB¹⁰. The heat of ionization of an exposed phenolic group, 25 kJ mol⁻¹, is smaller than that observed for pKₐ and the nearest phenolic oxygen is more than 8.5 Å from the catalytic Oγ atom. It is unlikely that either pKₐ or pK₇ reflects the ionization of the SerB¹⁰ hydroxy group which, in an aqueous environment, may be expected to have a value of pKₐ ≈ 14, rather greater than that for a typical solvent-exposed guanidinium group. Furthermore, if the pKₐ of the hydroxy group corresponded to pK₇, only a small fraction of the enzyme molecules would contain the reactive species at the optimum pH; the greater perturbation needed for pKₐ to correspond to pK₇ would represent an unfavourable Gibbs energy greater than 40 kJ mol⁻¹. The presence of the protonated guanidinium group of ArgB¹⁰,A is therefore proposed to be obligatory for catalysis, in the orientation of the hydrogen-bonding network, orienting the N-terminal catalytic serine residue and contributing to a decrease in the pKₐ of the α-amino group.

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