The kinetics of release of 4-nitrophenol were followed by stopped-flow spectrophotometry with two 4-nitrophenyl ester substrates of penicillin G acylase from *Kluyvera citrophila*. With the ester of acetic acid, but not of propionic acid, there was a pre-steady-state exponential phase, the kinetics of which were inhibited by phenylacetic acid (a product of hydrolysis of specific substrates) to the extent predicted from $K_i$ values. This was interpreted as deriving from rapid formation (73 mM$^{-1}$s$^{-1}$) and slow hydrolysis (0.76 s$^{-1}$) of an acetyl derivative of the side chain of the catalytic centre residue Ser-290. With the mutant F360V, which differs from the wild-type enzyme in its ability to hydrolyse adipyl-l-leucine and has a $k_{cat}$ for 4-nitrophenyl acetate one-twentieth that of the wild-type enzyme, the corresponding values for the rates of formation and hydrolysis of the acetyl-enzyme were 11.1 mM$^{-1}$s$^{-1}$ and 0.051 s$^{-1}$ respectively. The ratio of these rate constants was three times that for the wild-type enzyme, suggesting that the mutant is less impaired in the rate of formation of an acetyl-enzyme than in its subsequent hydrolysis.

**INTRODUCTION**

There is circumstantial evidence in favour of an acyl-enzyme catalytic intermediate for penicillin G acylase (penicillin amido-hydrolase; EC 3.5.1.11). For the enzyme from *Kluyvera citrophila*, the non-linear Arrhenius plots for the temperature dependence of $k_{cat}$ and $k_{cat}/K_m$ for the good substrate benzylpenicillin have been analysed [1] in terms of the sequential release of an amine or alcohol and an acid product (P$_1$ and P$_2$ respectively) and the formation of an acyl-enzyme intermediate E (eqn. 1), assuming a different step to be partly rate-determining as a function of temperature:

$$
E + S \rightleftharpoons ES \rightarrow E' \rightarrow E + P_2 + P_1
$$

(eqn. 1)

Structural studies have provided stronger evidence for an acyl-enzyme catalytic intermediate. The side chain hydroxyl group of Ser-290 has been identified by site-directed chemical modification as a potential catalytic nucleophile both in this enzyme [2] and in the related enzyme from *Escherichia coli* [3]. High-resolution crystal structures of product and inhibitor complexes with the latter enzyme show the O-$\gamma$ atom of serine B1 (the N-terminal residue generated during post-translational processing of the pro-enzyme into non-covalently associated A and B chains) to be in an ideal position for nucleophilic attack on the acyl carbon of the substrate [4].

In spite of a preference for phenylacetyl esters and amides, penicillin acylase will catalyse the slow hydrolysis of 4-nitrophenyl acetate and other reactive esters with small acyl groups, suggesting the possibility of the accumulation of a slowly hydrolysed acyl-enzyme [5]. A small acyl group, and especially the acetyl group, could be expected to bind with lower affinity, being unable to occupy the hydrophobic pocket normally filled by the phenylacetyl group. However, by offering a minimal impediment to substrate binding, 4-nitrophenyl acetate should provide a useful probe of changes in the catalytic mechanism of an enzyme with altered substrate specificity. Here we demonstrate for the first time with any substrate of penicillin G acylase that the kinetics of approach to the steady-state hydrolysis of this substrate are characteristic of stoichiometric accumulation of an acetyl-enzyme both in the wild-type and in the mutant protein F360V [6], which has altered substrate specificity associated with a substitution close to the catalytic centre.

**EXPERIMENTAL**

**Buffers**

The 0.1 M phosphate buffer, pH 7.5, contained 16 mM NaH$_2$PO$_4$ and 84 mM Na$_2$HPO$_4$.

**Enzyme preparations**

Recombinant wild-type and mutant penicillin G acylases from *Kluyvera citrophila* were purified as previously described [6] from *E. coli* HB101 containing the plasmids pYKD59 and pADLA respectively. Purified preparations were homogeneous on SDS/PAGE [7]. With a colorimetric method [8] to determine the rate of hydrolysis of a saturating concentration [9] of 14 mM benzyl-penicillin, the specific enzyme activities of the wild-type and mutant proteins were 34.2 and 1.5 pmol·min$^{-1}$ per mg of protein respectively. The protein concentration was determined from the UV absorption spectrum [10] ($\varepsilon_{280}$ 2.24 × 10$^4$ M$^{-1}$·cm$^{-1}$).

**Steady-state kinetics**

Initial velocity was determined as previously described [5] from the increase in $A_{340}$ over not more than the first 10% of the total reaction, by linear least-squares regression [11]. Data were fitted to the Michaelis–Menten equation by using weighted non-linear least-squares regression [12].

**Stopped-flow experiments**

The release of 4-nitrophenol was monitored as $A_{400}$ ($\varepsilon_{400}$ 1.3 × 10$^4$ M$^{-1}$·cm$^{-1}$, pH 7.5) with a Hi-Tech stopped-flow spectro-
photometer (model SF-3L/SU-40) with a 10 mm optical path, thermostatically controlled at 22 °C. Solutions contained lysozyme (1 mg/ml), to minimize losses of penicillin G acylase in the flow path, and 1 % (v/v) acetonitrile in 0.1 M phosphate buffer, pH 7.5. Equal volumes (150 µl) of 0.25–0.1 mM 4-nitrophenyl acetate and 1–4 µM protein were mixed (dead time 1.1 ms). Eqns. (2)–(8), corresponding to those applied to the acylation of chymotrypsin [13], were used in the determination of kinetic parameters:

\[
k_{\text{cat}} = \frac{k_{+2} k_{-3}}{k_{+3} + k_{-3}}
\]

(2)

\[
K_a = \frac{k_{-3} + k_{+3}}{k_{+2} + k_{-2}}
\]

(3)

\[
K_m^{\text{NIPAB}} = \frac{k_{-3} + k_{+3}}{k_{+2} + k_{-2}}
\]

(4)

\[
K_a = \frac{k_{-1}}{k_{+1}}
\]

(5)

\[
[P_i] = A + (1 - e^{-t})
\]

(6)

\[
B = \frac{[E_i] \left(\frac{k_{+2} + k_{+3}}{k_{+2} + k_{-2}}\right)^2}{1 + K_{m^{\text{NIPAB}}}}
\]

(7)

\[
k^* = \frac{(k_{+2} + k_{+3}) [S]_0 + k_{-2} K_m^{\text{NIPAB}}}{K_m^{\text{NIPAB}} + [S]_0}
\]

(8)

Progress curves were fitted to eqn. (6) by using non-linear least-squares regression [12] to obtain the amplitude and the first-order rate constant for the burst phase and the velocity of the steady-state reaction. Noting that \( A \) in eqn. (6) corresponds to the steady-state velocity defined by the Michaelis-Menten equation and expressing \( A \) and \( B \) in terms of eqns. (2) and (7), it may be shown that the intercept of the graph of \( A/B \) against \( 1/[\text{substrate}] \) corresponds to \( k_{+2}(k_{+2} + k_{+3})/k_{+3} \), which approaches \( k_{+2} \) when \( k_{+3} \gg k_{+2} \). The experimentally derived value of this parameter was used as a constraint in non-linear least-squares analysis of the dependence of \( k^* \) on substrate concentration. Appropriate weighting was used in linear regression and in calculating standard errors of derived parameters [14].

**RESULTS AND DISCUSSION**

**Steady-state kinetics**

The F360V enzyme catalyses the hydrolysis of adipyl-L-leucine at a significant rate, whereas the wild-type enzyme shows no such activity, but other kinetic differences are less marked [6]. The steady-state kinetic parameters for the mutant enzyme with two different 4-nitrophenyl esters were therefore measured (Table 1) and compared with those for the wild-type enzyme [5] as a basis for subsequent stopped-flow experiments. With 4-nitrophenyl acetate and 4-nitrophenyl propionate, \( k_{\text{cat}} \) was less than that for the wild-type enzyme [5] by factors of 20 and 8 respectively, but values of \( K_a \) were little different, and neither the wild-type nor the mutant enzyme showed detectable activity towards 4-nitrophenyl trimethylacetate. These effects imply relatively unspecific interactions with small aliphatic esters of 4-nitrophenol, because other work [6] has shown the mutant enzyme to be at least 30-fold less active in the hydrolysis of benzylpenicillin, but to be equally active in the hydrolysis of \( N\)-(3-carboxy-4-nitrophenyl)-phenylacetamide (NIPAB). The latter two substances possess a phenylacetyl group; the difference in specificity presumably depends on interactions with the amine moiety of the substrate, which are likely to be relatively sparse compared with those made by the phenylacetyl moiety [4]. The implied similarity of the wild-type and mutant enzymes in their interaction with phenylacetic acid was demonstrated directly by measurement of the inhibition of NIPAB hydrolysis by phenylacetic acid [22 °C; 0.6 % (v/v) acetonitrile] both with the mutant enzyme and with the wild-type enzyme, for which a value of \( K_i \) of 70 µM had previously been incorrectly reported as 700 µM [5]. The present results fitted a model for simple competitive inhibition, with values (means ± S.E.M.) of \( K_i \) of 85 ± 7 and 88 ± 3 µM for the mutant and the wild-type enzymes respectively. Assuming simple mixed inhibition, no convergence was found for the wild-type, and, for the mutant enzyme, values of \( K_i \) of 86 ± 11 and 40 ± 260 mM were obtained for binding to the free enzyme and the enzyme-substrate complex respectively. Competitive inhibition is the expected type of inhibition [15] by the second product of the ordered Uni-Bi kinetic pathway implied by eqn. (1), and such inhibition has also been observed with the sequence-related penicillin acylase from *E. coli* [16].

**Table 1** Steady-state kinetics of hydrolysis of 4-nitrophenyl aliphatic esters catalysed by the F360V enzyme

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Ester</th>
<th>( k_{\text{cat}} ) (s(^{-1}))</th>
<th>( K_a ) (µM)</th>
<th>( k_{-3}/k_{+3} ) (s(^{-1})-mM(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>Acetate</td>
<td>0.91 ± 0.08</td>
<td>21 ± 1</td>
<td>43 ± 4</td>
</tr>
<tr>
<td>Wild-type</td>
<td>Propionate</td>
<td>3.9 ± 0.1</td>
<td>17 ± 6</td>
<td>23 ± 10</td>
</tr>
<tr>
<td>Mutant</td>
<td>Acetate</td>
<td>0.052 ± 0.008</td>
<td>10 ± 3</td>
<td>5.2 ± 0.6</td>
</tr>
<tr>
<td>Mutant</td>
<td>Propionate</td>
<td>0.46 ± 0.09</td>
<td>184 ± 50</td>
<td>2.5 ± 0.2</td>
</tr>
</tbody>
</table>

**Figure 1** Time-course of release of 4-nitrophenol from 4-nitrophenyl acetate catalysed by wild-type penicillin acylase

The stopped-flow cell contained 1.7 µM enzyme and 4-nitrophenyl acetate (0.125 mM, 0.175 mM, 0.25 mM and 0.5 mM; lines from bottom to top respectively). The broken lines show the extrapolated steady-state reactions, obtained by fitting the data to eqn. (6).
Figure 2 Effect of enzyme concentration on pre-steady-state burst amplitude

The amplitude of the pre-steady-state exponential phase was determined by fitting the data to eqn. (6). The substrate concentration was 0.2 mM. Symbols: ●, wild-type protein; ○, mutant protein. Error bars show S.D. for the means of five progress curves. Linear regressions: solid line, wild-type; broken line, mutant.

directly proportional to and apparently equivalent to 70% of the enzyme (total protein) concentration (Figure 2) and, like the steady-state velocity, was little affected by changing the substrate concentration, as expected in a range 10-40-fold greater than $K_m$. According to eqn. (7), this amplitude would be consistent with $k_{cat}$ being greater than $k_{cat}$. However, the steady-state velocity observed in stopped-flow experiments was less than that observed after manual mixing, presumably because of losses of active enzyme in the flow path. Allowing for this by taking the steady-state rate as a measure of the enzyme concentration, the burst amplitude was closely similar to the estimated concentration of active enzyme, implying that $k_{cat}$ was considerably greater than the ordinate intercept ($k_{cat}/k_d$), defining the apparent $K_{m}^{*}$ in eqn. (8) (solid line) was identical with that obtained by the combined fit to all of the data, including those where phenylacetic acid was present (broken lines), defining the apparent $K_{m}^{*}$ in eqn. (8) as $k_{cat}^{*} (1 + [phenylacetic acid]/K_i)$.

Figure 3 Effect of substrate concentration and phenylacetic acid on transient kinetics

The first-order rate constant for the approach to the steady state ($k'$) was determined for wild-type (upper panel) and mutant enzyme (lower panel). Symbols show S.D. for the means of 6-22 and 2 progress curves for the upper and lower panels respectively. Phenylacetic acid: ○, 0.2 mM; ■, 0.5 mM; ●, not present. The curve fitted to eqn. (8) (solid line) was identical with that obtained by the combined fit to all of the data, including those where phenylacetic acid was present (broken lines), defining the apparent $K_{m}^{*}$ in eqn. (8) as $k_{cat}^{*} (1 + [phenylacetic acid]/K_i)$.

Effect of phenylacetic acid on rapid burst kinetics

As shown by the results of Figure 3, the first-order rate constant $k'$ for both the wild-type and mutant enzymes was affected by phenylacetic acid in the manner expected for binding to the free enzyme in competition with the formation of an acyl-enzyme from the reaction with 4-nitrophenyl acetate. The values of $K_i$ were marginally greater than those found for competitive inhibition of NIPAB hydrolysis; this may reflect a difference in the concentration of acetonitrile, which has been shown to affect steady-state kinetic parameters for 4-nitrophenyl esters [5].
Table 2  Kinetic parameters for the hydrolysis of 4-nitrophenyl acetate determined from the kinetics of approach to the steady state

<table>
<thead>
<tr>
<th></th>
<th>$k^\text{cat}/K^\text{cat}$</th>
<th>$K_\text{M}^\text{cat}$</th>
<th>$k_\text{cat}$</th>
<th>$K_\text{i}$</th>
<th>$K_\text{in}$</th>
<th>$K_\text{m}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>73 ± 2</td>
<td>887 ± 118</td>
<td>65 ± 7</td>
<td>0.76 ± 0.07</td>
<td>0.75 ± 0.07</td>
<td>10.3 ± 1.5</td>
</tr>
<tr>
<td>Mutant</td>
<td>11 ± 1</td>
<td>820 ± 182</td>
<td>9.1 ± 1.3</td>
<td>0.051 ± 0.009</td>
<td>0.051 ± 0.009</td>
<td>4.6 ± 2.3</td>
</tr>
</tbody>
</table>

Stopped-flow experiments with 4-nitrophenyl propionate

With the wild-type enzyme, experiments with 10–75 µM 4-nitrophenyl propionate gave no evidence of a rapid burst of release of 4-nitrophenol. If $k_\text{cat}$ were equal to that for 4-nitrophenyl acetate, a greater value of $k_\text{cat}$ for 4-nitrophenyl propionate would be required to explain the 4-fold greater $k_\text{cat}$ and 8.5-fold greater $K_\text{M}$ [5]. The smaller ratio $k_\text{cat}/k_\text{cat}$ would give a burst amplitude close to the limit of detection with accessible substrate concentrations.

Effects of mutation on catalysis

One effect of the F360V mutation is on $k_\text{cat}/K_\text{cat}$, which is decreased to one-seventh, apparently primarily through a decrease in $k_\text{cat}$, confirming the expectation that the binding of the substrate is little affected. Additionally, there is a decrease to just over one-twentieth in $k_\text{cat}/K_\text{cat}$, implying a reduced reactivity of the acetyl enzyme in the hydrolysis step. Residue Phe-360 corresponds in the E. coli enzyme to B-chain Phe-71, which lies in the region of the catalytic cavity and, although not in close proximity to the bound substrate, is part of a conserved sequence including Ala-69, the backbone NH of which forms part of the proposed oxyanion hole stabilizing a tetrahedral intermediate in both acylation and de-acylation steps [4]. The approximately similar factors by which this mutation affects $k_\text{cat}$ and $k_\text{cat}$ are broadly consistent with an impaired ability to stabilize the transition states of both steps, although the effect appears to be slightly greater on the latter step.

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REFERENCES


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