Mutations of penicillin acylase residue B71 extend substrate specificity by decreasing steric constraints for substrate binding

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Two mutant forms of penicillin acylase from Escherichia coli strains, selected using directed evolution for the ability to use glutaryl-l-leucine for growth [Forney, Wong and Ferber (1989) Appl. Environ. Microbiol. 55, 2550–2555], are changed within one codon, replacing the B-chain residue PheB71 with either Cys or Leu. Increases of up to a factor of ten in $k_{cat}/K_m$ values for substrates possessing a phenylacetyl leaving group are consistent with a decrease in $K_m$. Values of $k_{cat}/K_m$ for glutaryl-l-leucine are increased at least 100-fold. A decrease in $k_{cat}/K_m$ for the CysB71 mutant with increased pH is consistent with binding of the uncharged glutaryl group. The mutant proteins are more resistant to urea denaturation monitored by protein fluorescence, to inactivation in the presence of substrate either in the presence of urea or at high pH, and to heat inactivation. The crystal structure of the LeuB71 mutant protein, solved to 2 Å resolution, shows a flip of the side chain of PheB726 into the periphery of the catalytic centre, associated with loss of the $\pi$-stacking interactions between PheB726 and PheB71. Molecular modelling demonstrates that glutaryl-l-leucine may bind with the uncharged glutaryl group in the $S_2$ subsite of either the wild-type or the LeuB71 mutant but with greater potential freedom of rotation of the substrate leucine moiety in the complex with the mutant protein. This implies a smaller decrease in the conformational entropy of the substrate on binding to the mutant proteins and consequently greater catalytic activity.

Key words: directed evolution, enzyme kinetics, three-dimensional structure.

INTRODUCTION

The mature molecule of penicillin acylase from Escherichia coli strain W comprises two polypeptide chains, A and B, that are formed by proteolytic cleavage of a precursor [1]. The mature enzyme catalyses the hydrolysis of penicillin G to 6-amino-6-deacetylpenicillanic acid and phenylacetic acid. Crystal structures of the free wild-type enzyme and complexes with the poor substrate penicillin G sulphoxide [2], the product phenylacetic acid and several of its analogues [3, 4], show that the preferred acyl group, phenylacetyl, is bound at a subsite ($S_2$) formed by a pocket lined with non-polar residues. With some exceptions, these compounds bind in a position favourable for nucleophilic attack by the hydroxyl group of the catalytic residue Ser303, assisted by its z-amino group, a relatively unusual catalytic arrangement [5]. Evidence for nucleophilic catalysis has come from site-directed chemical modification [6, 7] and the kinetics of accumulation of an acyl enzyme intermediate with non-specific 4-nitrophenyl ester substrates [8, 9]. Some analogues of phenylacetic acid bind in an alternative position that is apparently less favourable for catalysis and the phenylacetyl group of penicillin G binds in a similar position in the inactive AsnB71→Ala mutant enzyme [2, 10]. This complicates and limits the use of structural and comparative observations for intelligent engineering of substrate specificity.

A different approach has been used with the aim of extending the well-established pharmaceutical use of the enzyme to a wider range of substrates in the semi-synthesis of new $\beta$-lactam antibiotics, particularly cephalosporins, and to other hydrolytic and synthetic reactions. Directed evolution depends on the selective isolation of mutants expressing more effective catalysts [11] at a pH value similar to that of the growth medium when the enzyme is located in the periplasm, as is penicillin acylase. Selected growth on analogues of primary amides as the sole nitrogen source, for example [12], led to the isolation of enzyme with mutations in the $S_2$ binding pocket. Other experiments relied on the ability of the enzyme to accept a variety of substituents on the amine portion of the substrate $P_1^\alpha$, including the amino acid leucine, which gave the basis for selective isolation of cells expressing the enzyme in a leucine-auxotrophic strain [13]. Using this strategy, with the additional modification of the acyl moiety of phenylacetyl-l-leucine, mutants selected at pH 5–6 for the ability to hydrolyse the cephalosporin mimic, glutaryl-l-leucine, were found to express enzymes with substitutions at PheB71 [14,15]. This position contained Leu and Cys in first- and second-generation mutants designated PA251 and PA251-47.

Abbreviation used: NIPAB, N-[3-carboxy-4-nitrophenyl]phenylacetamide.

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respectively. In the present work, steady- and pre-steady-state kinetics provide evidence that glutaryl-l-leucine binds with a greater affinity to mutant enzymes. We explain the increased affinity by an increase in mobility of the remainder of the bound substrate, permitted by the changed structure near to position B71, as revealed by X-ray crystallography.

**MATERIALS AND METHODS**

**Materials**

4-Nitrophosphoryl phenylacetate was synthesized as in [6]. N-(3-Carboxy-4-nitrophosphoryl)phenylacetamid (NIPAP) and other 4-nitrophosphoryl esters were from Sigma-Aldrich. Acetonitrile was HPLC grade. Other reagents were of analytical grade.

Purified wild-type penicillin acylase, a gift from Boehringer Mannheim (Mannheim, Germany), was prepared and assayed as described previously [2]. Mutant genes were created by site-directed mutagenesis using the Sculptor kit (Amersham Biosciences, Little Chalfont, Bucks, U.K.), incorporating a silent BamHI restriction site to aid screening. The mutant gene fragment DraII/BglII was subcloned into the expression plasmid pA1 and expressed in E. coli BL21. The purified mutant protein was obtained with a yield of 4 mg/l of cell culture, approximately half that of the wild-type protein [2].

**Reaction kinetics**

The absorbance of 3-amino-6-nitrobenzoic acid-4-nitrophosphoryl phenylacetyl, released from NIPAP, 4-nitrophosphoryl ester substrates and phenylacetate, was monitored at 405, 400 and 270 nm respectively. To monitor the time course of hydrolysis of glutaryl-l-leucine, samples were withdrawn and incubated at 90 °C for 15 min to stop further enzymic catalysis. After conversion into the phenylisothiocarbamoyl derivative [16], with aspartic acid as an internal standard, the products were quantified by HPLC, monitoring at 200 nm.

To determine the rate constant for loss of enzyme activity, in the presence of substrate, the concentration of the product [P] was fitted to eqn 3 [18], with time (t) and total enzyme concentration [E] as the dependent variables, using two enzyme concentrations that differed by a factor of two. [S] and [I], are concentrations initially and at time t, respectively.

\[
P_{1} = k_{\text{cat}} [E]_{r} \left(1 - \exp\left(-k^{\text{inact}} \cdot t\right)\right) / \left(k^{\text{inact}} - K_{m} \ln([S]_{0}/[S])\right)
\]

\[\text{(3)}\]

**Crystallization and X-ray data collection**

Recombinant Leuβ71 penicillin acylase was purified as described previously for other mutant proteins [2]. Crystals of the Leuβ71 protein (those of the Cysγ71 protein were not of diffraction quality) were obtained using a combination of the hanging-drop vapour-diffusion method and streak-seeding techniques with a protein concentration of 11 mg/ml in 11% (w/v) polyethylene glycol MME 2K/50 mM Mops buffer, pH 7.2. After serial transfer (5% steps) to 30% (v/v) ethylene glycol, in mother liquor for cryoprotection, data were collected at 120 K on a RAXIS II image plate detector with a Rigaku RU200 rotating anode X-ray generator. The crystals, belonging to the triclinic space group P1 with unit cell dimensions a = 2.02 Å, b = 64.23 Å, c = 70.67 Å, α = 70.58°, β = 72.81° and γ = 73.84°, contained one molecule in the asymmetric unit. Data, measured to 2 Å resolution, were processed using DENZO [19]. The CCP4 suite [20] was used for all further crystallographic computing.

**Structure solution and refinement**

Crystal freezing caused a shrinkage in the c cell dimension by 5.6 Å; hence the structure of the mutant Leuβ71 protein was determined by molecular replacement with the program AMoRe [21], using the structure of wild-type penicillin acylase (PDB code 1pnk [3]) with water molecules removed. The reoriented model was refined using maximum likelihood as implemented in REFMAC [22] with bulk solvent correction and anisotropic scaling. Towards the end of refinement the contribution of hydrogen atoms in their riding positions was included in the model using HGEN. The addition of water molecules used the automated refinement programme ARP [23] in concert with REFMAC. The complete data set was used to refine the model to an Rmerge of 15.2%, and an Rfree of 19.4% [24]. Crystallographic statistics for the structure are reported in Table 4 (see below) and structural data have been deposited at the European Bioinformatics Institute under PDB code 1h2g.

**Molecular modelling**

QUANTA software (Molecular Simulations, San Diego, CA, U.S.A.) was used with a Silicon Graphics Indigo workstation. Models were based on crystal structures of the wild type [3] and the Leuβ71 mutant. The substrate carbonyl carbon and oxygen atoms adjacent to the scissile bond were placed to correspond with those in the enzyme–phenylacetic acid complex (PDB code 1 pnk). Torsion angles in the substrate molecule were adjusted to minimize close contacts and the enzyme–substrate complex was subjected to at least 50 cycles of energy minimization (CHARMM) using the method of steepest descents. Inter-atomic distances were calculated using the Swiss-Pdb Viewer programme (http://www.expasy.ch/spdbv/mainpage.htm) [25].

**RESULTS AND DISCUSSION**

**Characterization of the altered specificity mutants**

In agreement with [26], the complete nucleotide sequence of the naïve (wild-type) E. coli strain W (ATCC 11105) pac gene encoding penicillin acylase from the plasmid pA1 [14] differed
Mutant CysB71
Mutant LeuB71

of the active-site pocket predicted to contribute to the S-
portion of benzylpenicillin, correlating with change in the region
analogue of ampicillin and cephalexin [28]) also displayed
isolation of mutations at the equivalent Phe
experiments using growth on adipyl-
T
for hydrolysis of
TGC Cys for codon B71. Surprisingly, a mutant (PA135) selected
independent isolate from the same experiment (PA324) also had a
generation mutant PA251-47 (the mutant showing the greatest
butions of widely distributed residues [32].
transversion in the codon specifying Val
mutation is to a synonymous codon.
The complete DNA sequence of the pac gene from the second-
generation mutant PA251-47 (the mutant showing the greatest
activity for glutaryl-\(\text{-}\)leucine hydrolysis [14]) differed only within
the codon specifying Phe[871] (TTC \(\rightarrow\) TGC, Cys[871]). The sequence
of this region from the first-generation parent plasmid (PA251)
also had an alteration at this codon (TTC \(\rightarrow\) CTC, Leu[871]). An
independent isolate from the same experiment (PA324) also had a
TGC Cys for codon B71. Surprisingly, a mutant (PA135) selected
for hydrolysis of \(\alpha\)-aminophenylacetyl-\(\text{-}\)leucine (a substrate
anologe of ampicillin and cephalexin [28]) also displayed
replacement of Phe[871] by leucine. Similar directed-evolution
experiments using growth on adipyl-\(\text{-}\)leucine resulted in the
isolation of mutations at the equivalent Phe[871] to Val in Klyvera
\(\text{citrophila}\) penicillin acylase [29] and growth on phthalyl-\(\text{-}\)leucine
led to the selection of the mutation Gly[871] \(\rightarrow\) Asp [30,31]. In each
of these selective substrates leucine replaced the \(\beta\)-lactam amide
portion of benzylpenicillin, correlating with change in the region
of the active-site pocket predicted to contribute to the S\(_s\) subsite,
which suggests that this region plays a dominant role in deter-
mining substrate specificity, in contrast with cumulative contribu-
tions of widely distributed residues [32].
To confirm that a single mutation was the unique cause of the
change in substrate specificity, the mutants Cys[871] and Leu[871]
were reproduced by site-directed mutagenesis in an otherwise
wild-type gene.

**Kinetic parameters**

Steady-state kinetic parameters for the hydrolysis of amide and
ester substrates at pH 7.5 (Table 1) include results for the wild-
type enzyme with 4-nitrophenylacetate that agree with published
values [8]. Because NIPAB and 4-nitrophenyl phenylacetate
produce the same phenylacetyl-\(\text{-}\)enzyme intermediate E' (eqn 1),
the value of \(k_{cat}\) for 4-nitrophenyl phenylacetate is a lower limit
of \(k_{cat}\) for both substrates. For each protein, \(k_{cat}\) was less for the
anilde than for the ester substrate, consistent with a \(k_{cat}\) step that
is close to rate-determining for NIPAB hydrolysis. In agreement
with preliminary results [15], the efficiency of hydrolysis of the
anilde substrate was significantly greater in the mutant proteins;
\(k_{cat}/K_m\) was greater by a factor of up to 10, mainly because \(K_m\)
was smaller. These effects are equivalent to a greater value of
\(k_{cat}/K_m\) but, because there is no large difference in \(k_{cat}\), it is unlikely that there is a large difference in \(k_{cat}\). The main effect
of mutation is therefore a smaller \(K_m\), reflecting greater affinity
for the substrate in the ground state.
The similar pH-dependence of \(k_{cat}\) and \(k_{cat}/K_m\) for wild-type
and mutant enzymes (Figure 1) is consistent with ionizable

**Table 1** Steady-state kinetic parameters for substrates of wild-type and mutant enzymes

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td></td>
<td>(k_{cat}) (s(^{-1}))</td>
<td>(K_m) ((\mu)M)</td>
<td>(k_{cat}/K_m) (s(^{-1}) (\mu)M(^{-1}))</td>
</tr>
<tr>
<td>NIPAB (7)</td>
<td>20 ± 3</td>
<td>18 ± 2</td>
<td>1.1 ± 0.3 (10^6)</td>
</tr>
<tr>
<td>4-Nitrophenyl phenylacetate (6)</td>
<td>140 ± 20</td>
<td>12 ± 2</td>
<td>1.1 ± 0.4 (10^7)</td>
</tr>
<tr>
<td>Phenyl acetate (8)</td>
<td>0.84 ± 0.09</td>
<td>4.3 ± 0.7</td>
<td>2.0 ± 0.2 (10^6)</td>
</tr>
<tr>
<td>4-Nitrophenyl acetate (8)</td>
<td>0.71 ± 0.06</td>
<td>9 ± 1</td>
<td>8 ± 1 (10^7)</td>
</tr>
<tr>
<td>2-Nitrophenyl acetate (6)</td>
<td>–</td>
<td>–</td>
<td>(3.5 ± 1.6 (10^8))</td>
</tr>
</tbody>
</table>

Results are means ± S.E.M.

**Figure 1** pH-dependence of kinetic parameters for wild-type and mutant Cys[871] enzymes

Solutions contained 28 nM enzyme, 3.3 mM–0.33 mM substrate, 0.5% (v/v) acetonitrile and buffer: 0.2 M sodium acetate/acidic acid at pH 4.0–6.8; 0.1 M phosphate, sodium salts at pH 5.8–8.0; 50 mM Tris/HCl at pH 7.5–9.1 and 50 mM glycine/NaOH at pH 8.8–10.6. The temperature was 20 °C. Wild type: ●: Cys[871]; ○: (a) \(k_{cat}\); (b) \(k_{cat}/K_m\).
groups in similar environments in the free and bound forms of the enzyme and substrate. Values of $pK_a$ for $k_{cat}$ and $k_{cat}/K_m$ in the wild-type and mutant proteins were 4.7 and 4.3 respectively and the corresponding values for $pK_a$ were 9.7 and 10.1 for $k_{cat}$ and 9.2 and 9.4 for $k_{cat}/K_m$ (S.E.M. for each estimate, $\leq 0.1$).

The effects of mutation were therefore small. In the hydrolysis of the acetyl enzyme, a value of 6.5 for $pK_a$ for $k_{cat}$ has been proposed to reflect the ionization of the $\alpha$-amino group of Ser$^{\text{B71}}$, required in its base form [8]. For the specific substrate NIPAB, the lower value of $pK_a$ implies a greater perturbation of the $pK_a$ of the amino group in the free enzyme and in the formation of the phenylacetyl enzyme.

Mutation resulted in only small effects on $k_{cat}/K_m$ for the hydrolysis of substrates forming an acetyl enzyme. The hydrolysis of 4-nitrophenylacetate is rate-limited by the deacylation step in both the wild-type [2] and mutant Cys$^{\text{B71}}$ and Leu$^{\text{B71}}$ enzymes (Table 2). Because 4-nitrophenol is a better leaving group than phenol, $k_{cat}$ might be expected to be less for phenylacetate than for 4-nitrophenylacetate. However, the values of $k_{cat}$ for each enzyme are similar and the efficiency of formation of the acetyl enzyme, as measured by $k_{cat}/K_m$, is greater for phenylacetate. Because it remains likely that $k_{cat}/K_m \approx k_{cat}/K_m$, it may be deduced that wild-type and mutant enzymes have a greater affinity for phenylacetyl than for 4-nitrophenylacetate. Similarly, the smaller value of $k_{cat}/K_m$ for 2-nitrophenylacetate is interpreted as a smaller affinity for this substrate.

At pH 6, slow hydrolysis of glutaryl-leucine was catalysed by the mutant enzymes (Table 3), with markedly greater values of $K_m$ than observed with other substrates. It was not possible to determine accurate kinetic parameters for the slower hydrolysis catalysed by the wild-type enzyme. However, it is likely that $k_{cat}$ for an N-acyl substrate is limited by $k_{cat}$ and therefore it is reasonable to assume that $K_m$ is not increased in the mutant enzymes. On this basis, the rate of hydrolysis of 53 mM glutaryl-leucine gave an upper limit for $k_{cat}/K_m$ of 0.1–0.4 s$^{-1} \cdot$M$^{-1}$, implying increased values for the mutant enzymes by factors of at least 100. At pH 8, the value of $k_{cat}/K_m$ for the mutant Cys$^{\text{B71}}$ was 1.5±0.1 s$^{-1} \cdot$M$^{-1}$, less than that at pH 6 by a factor of approx. 100. Assuming that the pH-dependence of $k_{cat}/K_m$ reflects the ionization of free substrate, this is consistent with exclusive binding of the charged form of the glutaryl chain side in the $S_1$ subsite.

Table 2 Pre-steady-state kinetic parameters for the hydrolysis of 4-nitrophenyl acetate

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_a$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (s$^{-1} \cdot$M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>173 ± 44</td>
<td>33 ± 4</td>
<td>0.79 ± 0.09</td>
</tr>
<tr>
<td>Mutant Cys$^{\text{B71}}$</td>
<td>446 ± 142</td>
<td>21 ± 6</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>Mutant Leu$^{\text{B71}}$</td>
<td>311 ± 78</td>
<td>19 ± 3</td>
<td>0.20 ± 0.02</td>
</tr>
</tbody>
</table>

Table 3 Steady-state kinetic parameters for hydrolysis of glutaryl-leucine by mutant enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}/K_m$ (s$^{-1} \cdot$M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys$^{\text{B71}}$</td>
<td>0.8 ± 0.1</td>
<td>18 ± 4</td>
<td>46 ± 15</td>
</tr>
<tr>
<td>Leu$^{\text{B71}}$</td>
<td>0.9 ± 0.1</td>
<td>16 ± 6</td>
<td>56 ± 17</td>
</tr>
</tbody>
</table>

Figure 2 Urea-denaturation of wild-type and mutant Cys$^{\text{B71}}$ enzymes

The fitted values of the urea concentration at the transition midpoint were 4.2±0.1 and 4.6±0.1 M respectively. This evidence of greater stability of the mutant Cys$^{\text{B71}}$ protein is supported by the kinetics of denaturation, measured in the presence of substrate and at a concentration of urea close to the transition midpoint, illustrated in Figure 3. The wild-type enzyme (Figure 3, traces 1 and 2) ceased to release product after using less than 20% of the available substrate. This is interpreted as progressive inactivation over a period of time when the release of product by the more stable mutant enzyme (Figure 3, traces 3 and 4) continued with an almost linear dependence on time. Values of the first-order rate constant for loss of enzyme activity for the wild-type and Cys$^{\text{B71}}$ enzymes (eqn 3) were $(1.70±0.06) \times 10^{-3}$ and $(8.3 ± 0.1) \times 10^{-5} \text{s}^{-1}$ respectively. Similarly, the mutant enzymes were more stable than the wild-type enzyme at high pH. Using 12.3 µM NIPAB in 50 mM glycine/NaOH buffer, pH 10.8, the rate constants were $(2.3±0.1) \times 10^{-3}$, $(3.3±0.8) \times 10^{-5}$ and $(1.3±0.4) \times 10^{-4} \text{s}^{-1}$ for wild-type, Cys$^{\text{B71}}$ and Leu$^{\text{B71}}$ respectively. This accounts for an apparent change in the alkaline limb of the pH profile [15].

Both mutant proteins showed an increase by a factor of 2 in $t_1/2$ for heat inactivation at 54 °C (pH 8.0), assayed with 0.4 mM NIPAB. The site of mutation is near the Ca$^{2+}$-binding loop, including residues at positions B73, B75 and B76, but there is no evidence of increased affinity for Ca$^{2+}$ in the mutant protein, as found with subtilisin S41 mutants [34]. Similar effects have been observed with other enzymes modified at sites remote from the catalytic centre [35].
Mutations extending substrate specificity of penicillin acylase

Other mutations of residues close to the surface of the penicillin acylase molecule result in increased stability. The mutant protein Trp**B146**→ Arg, changing a residue 32 Å from the catalytic centre [36], showed a 2-fold increase in *t*<sub>1/2</sub> at pH 8.5 [37], perhaps deriving from a hydrogen bond between an arginine and a glutamine residue on a neighbouring helix. Increased thermal stability has also been shown for other altered specificity mutants [30] including Ala**A146**→ Asp [38].

**Structure of the altered specificity mutant Phe**<sup>B71</sup> → Leu**

The overall structure of the altered specificity mutant Phe**<sup>B71</sup>**→ Leu, refined to a resolution of 2.0 Å (Table 4), was the same as that of the native protein (root-mean-square deviation, 0.28 Å) from the catalytic centre. The electron density clearly confirmed the substitution by leucine at position B71 (Figure 4, top panel) and indicated changes in the positions of the side chains of the neighbouring residues Leu**<sup>B71</sup>** and Phe**<sup>B91</sup>**, which adopt a different rotamer and a pronounced side-chain movement respectively. In the native wild-type enzyme, the phenylalanine aromatic side chains of residues B71 and B256 are aligned with each other in an off-centre parallel fashion through π-stacking forces [39]. In the mutant Leu**<sup>B71</sup>**, loss of these aromatic stacking interactions leads to a flip of the hydrophobic side chain of Phe**<sup>B91</sup>** into the periphery of the catalytic centre (Figure 4, bottom panel). The C<sub>α</sub> atom of Phe**<sup>B91</sup>** moves 4.9 Å, leaving the side chain pinned between Leu**<sup>B71</sup>** and Leu**<sup>B92</sup>**. In addition, there are minor shifts in the positions of the side chains of Phe**<sup>B92</sup>** and Phe**<sup>A146</sup>**. The rearranged side-chain positions alter the substrate-binding environment, in particular the surface that contributes to the S<sub>1</sub> subsite, and narrows the entrance to the catalytic centre. Similar loss of aromatic stacking interactions and remodelling of the hydrophobic surface in the substrate-binding site would be predicted for the Cys**<sup>B71</sup>** mutant protein.

The importance of the phenylalanine residues at positions A146 and B71 in substrate binding and catalytic efficiency has been illuminated by crystal structures of penicillin acylase in complex with substrates [2,10]. The close proximity of the mutation to the main-chain oxygen atom residue Ala**<sub>169</sub>** may be a contributing factor to the modest increase in *k*<sub>cat</sub> and *k*<sub>cat</sub>/*K*<sub>m</sub> for NIPAB hydrolysis, bearing in mind effects such as perturbed electrostatic fields and vibrational modes that are not revealed by the static crystal structure.

**Table 4 Crystallographic data for Leu**<sup>B71</sup> altered-specificity mutant**

<table>
<thead>
<tr>
<th>Data collection</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>P1</td>
</tr>
<tr>
<td>No. of reflections</td>
<td>102,704</td>
</tr>
<tr>
<td>No. of unique reflections</td>
<td>50,677</td>
</tr>
<tr>
<td>Completeness</td>
<td>93% (80%)</td>
</tr>
<tr>
<td>R&lt;sub&gt;merge&lt;/sub&gt;</td>
<td>0.0152</td>
</tr>
<tr>
<td>R&lt;sub&gt;free&lt;/sub&gt;</td>
<td>0.0194</td>
</tr>
<tr>
<td>R&lt;sub&gt;free&lt;/sub&gt; test set size</td>
<td>5%</td>
</tr>
<tr>
<td>No. of protein atoms</td>
<td>6078</td>
</tr>
<tr>
<td>No. of water molecules</td>
<td>596</td>
</tr>
<tr>
<td>No. of calcium ions</td>
<td>1</td>
</tr>
<tr>
<td>No. of ethylene glycol molecules</td>
<td>4</td>
</tr>
<tr>
<td>r.m.s.d. from ideal geometry</td>
<td></td>
</tr>
<tr>
<td>Bond lengths</td>
<td>0.013 Å</td>
</tr>
<tr>
<td>Bond angles</td>
<td>1.7°</td>
</tr>
<tr>
<td>Mean σ value (all atoms)</td>
<td>18.9 Å</td>
</tr>
</tbody>
</table>

The hydrolysis of 16.7 μM NIPAB was monitored in solutions containing 4.5 M urea and 0.1 M phosphate buffer, pH 7.5. The temperature was 20 °C. Progress curves were normalized with respect to enzyme concentration: wild-type, 22 nM (trace 1) and 44 nM (trace 2); Cys**<sup>B71</sup>**, 22 nM (trace 3) and 44 nM (trace 4). The expected ordinate values of traces 3 and 4 at infinite time (complete use of substrate) are 760 and 380 mol/mol respectively.
substrate occupied a comparable position to the carboxylate group of penicillin G, 4.3 Å from ArgB146. Neutral ester substrates lack groups capable of making these interactions. The 3-carboxy-4-nitrophenyl ring was less than 3 Å from Cα2 of PheB256 in the wild-type enzyme but 3.6 Å from Cγ of LeuB253 in the mutant. Similarly, the distances from PheB254 were 3.5 Å from Cα in the
wild-type and 3.9 Å from Cε in the mutant. These differences imply a greater freedom of rotation of this part of the substrate molecule in the complex with the mutant protein.

The undissociated glutaric acid moiety of glutaryl-L-leucine was readily accommodated in the phenylacetyl-group-binding pocket of the wild-type and mutant enzyme and there was no significant effect on the positions of the twelve nearest protein residues (root-mean-square deviation, 0.1 Å). The carboxyl group oxygen of the glutaric moiety was 3.3 Å from Cε of Met142. Alteration of the corresponding residue has been identified among mutants of a closely related penicillin acylase with an altered substrate specificity [40]. Other distances from neighbouring groups were in the range 3.0–3.7 Å, somewhat less than the 3.2–4.3 Å found in the crystal structure of the complex with phenylacetic acid. The position of the α-amino group of Ser39 was unaffected, with a possible hydrogen bond between the carboxyl group of the leucyl moiety of glutaryl-L-leucine and the α-amino group of Ser39. In the model of the wild-type structure, the leucyl side chain of the substrate was 3.1 Å from the side chains of Phe64 and Phe87 and the substrate carboxylate group was 3.4 Å from Arg152. In the mutant Leu87 crystal structure, the substrate side chain was located between Phe81, Leu87, Leu147 and Phe153 with distances of 3.2, 3.4 and 3.9 Å respectively. The last value is considerably less than the 6.1 Å found in the wild-type model structure. There was little difference in the accessibility to solvent of the leucine side chain of the bound substrate, ruling out one otherwise plausible explanation of the difference in $K'_m$.

It has been proposed that flexibility in a ligand bound to xylanase compensates for loss of specific interactions by allowing binding with limited loss of conformational entropy [41]. The present results show similar features when the structural constraints on bound substrates are considered in relation to values of $k_{cat}/K_m$ as an index of substrate specificity. The phenyl group of a bound substrate is less constrained than the glutaryl group. This may contribute to weak binding of glutaryl-L-leucine to both wild-type and mutant proteins. Mutation led to no significant structural effects on the S$_S$ subsite, so that the effects on $k_{cat}/K_m$ for glutaryl-L-leucine and NIPAB probably result from changes in interactions with the leaving-group. Rotation of the leucine side chain of glutaryl-L-leucine and of the 3-carboxy-4-nitrophenyl group of NIPAB is limited in the wild-type protein by the proximity of the side chain of Phe87, requiring a decrease in conformational entropy on binding. Mutation at this site, by imposing less constraint, implies a smaller decrease in conformational entropy in the bound substrate. This is reinforced by the observation that the same mutations have been generated by a selection procedure based on a different N-acetyl derivative of leucine [28], a coincidence that is not easily explained by effects on specific enzyme–substrate interactions.

Similar factors may account for the smaller value of $k_{cat}/K_m$ observed for 2-nitrophenylacetate relative to 4-nitrophenylacetate and phenylacetyl with both wild-type and mutant proteins. In models, the 2-nitro group occupied a position between the side chains of Gln135 and Phe145 with short contacts including one of 2.7 Å between an oxygen atom of the nitro group and Cε2 of Phe145. The 4-nitrophenyl group extended towards the side chains of Phe135 and Asn138, with a larger range of plausible conformations in the direction of Phe87 and Asn131. Phenylacetate, lacking a nitro group, is least constrained, consistent with a larger value of $k_{cat}/K_m$ than for either of the inherently more reactive esters.

There is no other cysteine residue in the mature sequence of the Cys87 specificity variant. This solvent-accessible site is therefore uniquely reactive and a potential target for chemical modification as a tool to study substrate binding [42]. Cys87 might either be changed into one of several amino acid analogues, altering specificity in the S$_s$ pocket, or provide the site of attachment of an environmentally sensitive probe.

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