White Rose Research Online

eprints@whiterose.ac.uk
https://eprints.whiterose.ac.uk
Universities of Leeds, Sheffield and York

Deposited via The University of York.

White Rose Research Online URL for this paper:
https://eprints.whiterose.ac.uk/id/eprint/463/

Article:
Morillas, M, McVey, C E, Brannigan, J A et al. (2003) Mutations of penicillin acylase residue B71 extend substrate specificity by decreasing steric constraints for substrate binding. Biochemical journal. pp. 143-150. ISSN: 1470-8728

Reuse
Items deposited in White Rose Research Online are protected by copyright, with all rights reserved unless indicated otherwise. They may be downloaded and/or printed for private study, or other acts as permitted by national copyright laws. The publisher or other rights holders may allow further reproduction and re-use of the full text version. This is indicated by the licence information on the White Rose Research Online record for the item.

Takedown
If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.

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

Manuel MORILLAS*[1,2], Colin E. McVEY†[2,3], James A. BRANNIGAN†‡[4], Andreas G. LADURNER†[5], Larry J. FORNEY‡ and Richard VIRDEN*

*School of Cell and Molecular Biosciences, University of Newcastle upon Tyne, Framlington Place, Newcastle upon Tyne NE2 4HH, U.K., †Structural Biology Laboratory, Department of Chemistry, University of York, Heslington, York YO10 5YW, U.K., and ‡Department of Biological Sciences, University of Idaho, Moscow, ID 83844-3051, U.S.A.

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 Phe^{B71} 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_s. 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 Cys^{B71} 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 Leu^{B71} mutant protein, solved to 2 Å resolution, shows a flip of the side chain of Phe^{B256} into the periphery of the catalytic centre, associated with loss of the π-stacking interactions between Phe^{B256} and Phe^{B71}. Molecular modelling demonstrates that glutaryl-L-leucine may bind with the uncharged glutaryl group in the S_1 subsite of either the wild-type or the Leu^{B71} 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-penicillanic 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_1) 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 Ser^{B1}, assisted by its α-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 Asn^{B241} → 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 β-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_1 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, 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 Phe^{B71} [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.

[1] Present address: Institute for Molecular Biology and Biophysics ETHZ, ETH Hönggerberg, HPK Building, CH-8093 Zürich, Switzerland.

[2] These authors contributed equally to this work.

[3] Present address: Department of Virology, Wright-Fleming Institute, St Mary's Campus, Faculty of Medicine, Imperial College of Science and Medicine, Norfolk Place, London W2 1PG, U.K.

[4] To whom correspondence should be addressed (e-mail jab@ysbl.york.ac.uk).

[5] Present address: Howard Hughes Medical Institute, Department of Molecular and Cell Biology, University of California at Berkeley, 401 Barker Hall #3204, Berkeley, CA 94720, U.S.A.

© 2003 Biochemical Society

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-Nitrophenyl phenylacetate was synthesized as in [6]. *N*-(3-Carboxy-4-nitrophenyl)phenylacetamide (NIPAB) and other 4-nitrophenyl 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 *Bam*HI restriction site to aid screening. The mutant gene fragment *Dra*III/*Bgl*II 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-nitrophenol, released from NIPAB, 4-nitrophenyl 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 A_{254} (Apex column ODS 4M25310, 4.6 mm × 25 cm; Jones Chromatography, Hengoed, Mid Glamorgan, Wales, U.K.). The solvent was 0.5 % (v/v) trifluoroacetic acid with a linear gradient to 60 % (v/v) acetonitrile after 30 min (flow rate, 1 ml/min). The derivatives of aspartic acid and leucine were eluted after 15 and 21 min respectively.

Steady-state and pre-steady-state kinetic parameters, defined by eqn 1, were determined by fitting initial velocities to the Michaelis–Menten equation, using non-linear least-squares regression and instrumentation described previously [8].

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\leftrightarrow}} ES \xrightarrow{k_{+2}} P_1 + E' \xrightarrow{k_{+3}} E + P_2 \quad (1)$$

Protein unfolding in the presence of urea was monitored by protein fluorescence (λ_{ex}, 295 nm; λ_{em}, 320 nm). Fitted lines were calculated according to eqn 2 [17]:

$$F = (F_N + p[\text{urea}] + F_U \exp\alpha)/(1 + \exp\alpha)$$

$$\alpha = (-\Delta G^0_{N-U} + m_{UN}[\text{urea}])/(RT) \quad (2)$$

where F is the observed intensity of fluorescence. The fitted parameters were F_N, F_U, p, ΔG^0_{N-U} and m_{UN}. F_N and F_U represent the fluorescence of the native and unfolded states, N and U, respectively, p describes the effect of urea on the fluorescence in the pre-transition region and m_{UN} represents the dependence of the free energy on the denaturant concentration.

To determine the rate constant for loss of enzyme activity, k^{inact}, in the presence of substrate, the concentration of the product $[P_1]$ was fitted to eqn 3 [18], with time (t) and total enzyme concentration $[E]_T$ as the dependent variables, using two enzyme concentrations that differed by a factor of two. $[S]_0$ and $[S]_t$ are concentrations initially and at time t, respectively.

$$[P_1]_t = k_{cat}\,[E]_T\,\{1 - \exp(-k^{inact} \cdot t)\}/k^{inact} - K_m \ln([S]_0/[S]_t) \quad (3)$$

Crystallization and X-ray data collection

Recombinant Leu^{B71} penicillin acylase was purified as described previously for other mutant proteins [2]. Crystals of the Leu^{B71} protein (those of the Cys^{B71} 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, $\alpha = 70.58°$, $\beta = 72.81°$ and $\gamma = 73.84°$, 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 Leu^{B71} 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 R_{factor} of 15.2 % and an R_{free} 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 Leu^{B71} 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 pnl). 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

© 2003 Biochemical Society

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

Solutions contained 0.1 M phosphate buffer, pH 7.5, and 0.5% (v/v) acetonitrile. The temperature was 20 °C. The numbers of different substrate concentrations used are shown in parentheses. Results are means ± S.E.M.

	Wild-type Phe^{B71}			Mutant Cys^{B71}			Mutant Leu^{B71}		
Substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)
NIPAB (7)	20 ± 3	18 ± 2	$(1.1 \pm 0.3) \times 10^6$	19 ± 2	3.1 ± 0.4	$(6.1 \pm 0.7) \times 10^6$	37 ± 4	3.4 ± 0.4	$(1.1 \pm 0.3) \times 10^7$
4-Nitrophenyl phenylacetate (6)	140 ± 20	12 ± 2	$(1.1 \pm 0.4) \times 10^7$	72 ± 15	3.6 ± 0.5	$(2.0 \pm 0.6) \times 10^7$	135 ± 25	3.9 ± 0.6	$(3.4 \pm 0.7) \times 10^7$
Phenyl acetate (8)	0.84 ± 0.09	4.3 ± 0.7	$(2.0 \pm 0.2) \times 10^5$	0.09 ± 0.01	0.73 ± 0.08	$(1.2 \pm 0.3) \times 10^5$	0.18 ± 0.02	0.75 ± 0.09	$(2.4 \pm 0.3) \times 10^5$
4-Nitrophenyl acetate (8)	0.71 ± 0.06	9 ± 1	$(8 \pm 1) \times 10^4$	0.09 ± 0.01	3.9 ± 0.5	$(2.3 \pm 0.6) \times 10^4$	0.19 ± 0.09	3.5 ± 0.5	$(5.4 \pm 0.5) \times 10^4$
2-Nitrophenyl acetate (6)	–	–	$(3.5 \pm 1.6) \times 10^2$	–	–	$(4.4 \pm 1.4) \times 10^2$	–	–	$(5.0 \pm 2.8) \times 10^2$

from the published sequence [27] at two sites, with a T → G transversion in the codon specifying Val^{B5} (GTT → GTG) and a T → C transition in the codon for Val^{35} of the linker peptide (Val GTT → Ala GCT). Neither sequence variation should affect the reactivity of the native protein because the linker peptide is removed during processing to the mature protein form, and the Val^{B5} 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-L-leucine hydrolysis [14]) differed only within the codon specifying Phe^{B71} (TTC → TGC, Cys^{B71}). The sequence of this region from the first-generation parent plasmid (PA251) also had an alteration at this codon (TTC → CTC, Leu^{B71}). An independent isolate from the same experiment (PA324) also had a TGC Cys for codon B71. Surprisingly, a mutant (PA135) selected for hydrolysis of α-aminophenylacetyl-L-leucine (a substrate analogue of ampicillin and cephalexin [28]) also displayed replacement of Phe^{B71} by leucine. Similar directed-evolution experiments using growth on adipyl-L-leucine resulted in the isolation of mutations at the equivalent Phe^{B71} to Val in *Kluyvera citrophila* penicillin acylase [29] and growth on phthalyl-L-leucine led to the selection of the mutation Gly^{B70} → Asp [30,31]. In each of these selective substrates leucine replaced the β-lactam amide portion of benzylpenicillin, correlating with change in the region of the active-site pocket predicted to contribute to the $S_{1'}$ subsite, which suggests that this region plays a dominant role in determining substrate specificity, in contrast with cumulative contributions of widely distributed residues [32].

To confirm that a single mutation was the unique cause of the change in substrate specificity, the mutants Cys^{B71} and Leu^{B71} 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–enzyme intermediate E′ (eqn 1), the value of k_{cat} for 4-nitrophenyl phenylacetate is a lower limit of k_{+3} for both substrates. For each protein, k_{cat} was less for the anilide than for the ester substrate, consistent with a k_{+2} step that is close to rate-determining for NIPAB hydrolysis. In agreement with preliminary results [15], the efficiency of hydrolysis of the anilide 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 $k_{+1}k_{+2}/(k_{+2}+k_{-1})$ but, because there is no large difference in k_{cat}, it is unlikely that there is a large difference in k_{+2}. The main effect of mutation is therefore a smaller K_s, reflecting greater affinity for the substrate in the ground state.

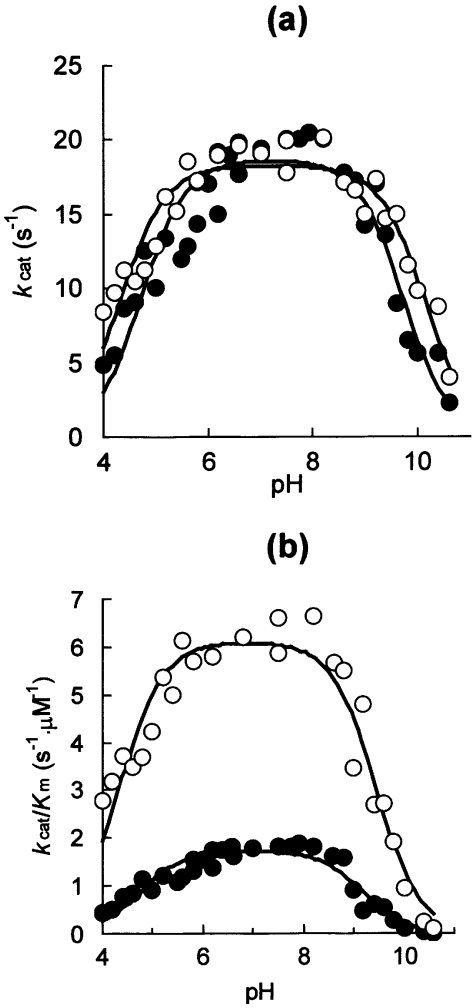

Figure 1 pH-dependence of kinetic parameters for wild-type and mutant Cys^{B71} enzymes

Solutions contained 28 nM enzyme, 3.3 μM–0.33 mM substrate, 0.5% (v/v) acetonitrile and buffer: 0.2 M sodium acetate/acetic acid at pH 4.0–5.8; 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^{B71}, ○. (**a**) k_{cat}; (**b**) k_{cat}/K_m.

The similar pH-dependence of k_{cat} and k_{cat}/K_m for wild-type and mutant enzymes (Figure 1) is consistent with ionizable

© 2003 Biochemical Society

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

Kinetic parameters were determined from progress curves obtained using stopped-flow spectrophotometry. Solutions contained 2.2 μM enzyme, 0.5% (v/v) acetonitrile and 0.1 M phosphate buffer, pH 7.5. Eight substrate concentrations were used, of 30–456 μM (wild-type), 97–784 μM (mutant Cys^{B71}) and 63–679 μM (mutant Leu^{B71}). The temperature was 20 °C. Values are means ± S.E.M.

Enzyme	K_s (μM)	k_{+2} (s^{-1})	k_{+3} (s^{-1})
Wild-type (Phe^{B71})	173 ± 44	33 ± 4	0.79 ± 0.09
Mutant Cys^{B71}	446 ± 142	21 ± 6	0.09 ± 0.02
Mutant Leu^{B71}	311 ± 78	19 ± 3	0.20 ± 0.02

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

Results (mean ± S.E.M.) are shown for seven different substrate concentrations (0.42–127 mM). The enzyme concentration was 0.1–2.5 μM. Solutions contained 0.01 M phosphate buffer, pH 6.0. The temperature was 37 °C.

Enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)
Cys^{B71}	0.8 ± 0.1	18 ± 4	46 ± 15
Leu^{B71}	0.9 ± 0.1	16 ± 6	56 ± 17

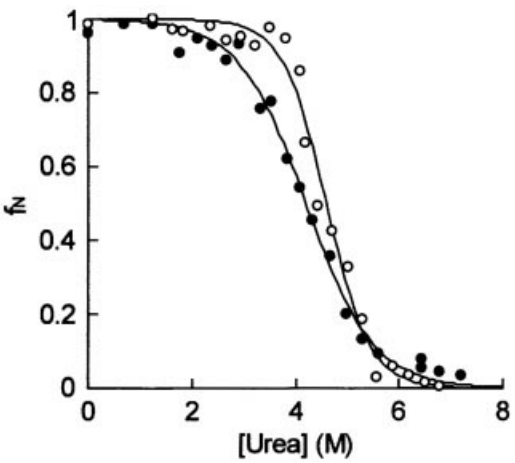

Figure 2 Urea-denaturation of wild-type and mutant Cys^{B71} enzymes

Solutions containing 0.76 μM enzyme, 50 mM NaCl, 20 mM phosphate buffer, pH 7.5, and urea, as indicated, were incubated for 24 h at 22 °C. Results of measurement of protein fluorescence are shown as a fraction of the native state, f_N, calculated using the fitted parameters of eqn 3. Wild type, ●; Cys^{B71}, ○.

groups in similar environments in the free and bound forms of the enzyme and substrate. Values of pK_1 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_2 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, $\leqslant 0.1$). The effects of mutation were therefore small. In the hydrolysis of the acetyl enzyme, a value of 6.5 for pK_1 for k_{cat} has been proposed to reflect the ionization of the α-amino group of Ser^{B1}, required in its base form [8]. For the specific substrate NIPAB, the lower value of pK_1 implies a greater perturbation of the pK 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^{B71} and Leu^{B71} enzymes (Table 2). Because 4-nitrophenol is a better leaving group than phenol, k_{+2} 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 $k_{cat}/K_m \approx k_{+2}/K_s$, it may be deduced that wild-type and mutant enzymes have a greater affinity for phenylacetate 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-L-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_2 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-L-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^{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 uncharged form of the glutaryl chain side in the S_1 subsite.

Protein stability

Urea-unfolding curves (Figure 2), which are not fully reversible [33], gave apparent values for the mutant Cys^{B71} and wild-type proteins of 27 ± 3 and 16 ± 3 kJ · mol^{-1} respectively for ΔG^0_{N-U} and 5.9 ± 0.7 and 3.8 ± 0.5 kJ · mol^{-1} · M^{-1} respectively for m_{UN}. 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^{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^{B71} enzymes (eqn 3) were $(1.70 \pm 0.06) \times 10^{-3}$ and $(8.3 \pm 0.1) \times 10^{-5}$ 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 $(2.3 \pm 0.1) \times 10^{-3}$, $(3.3 \pm 0.8) \times 10^{-5}$ and $(1.3 \pm 0.4) \times 10^{-4}$ s^{-1} for wild-type, Cys^{B71} and Leu^{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 $t_{\frac{1}{2}}$ 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].

© 2003 Biochemical Society

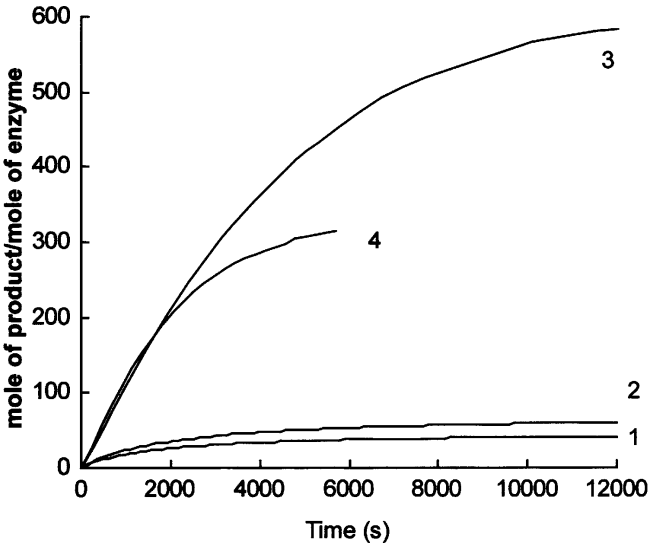

Figure 3 Catalytic activity of wild-type and Cys^{B71} mutant enzymes in 4.5 M urea

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^{B71}, 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.

Table 4 Crystallographic data for Leu^{B71} altered-specificity mutant

$R_{merge} = \Sigma_h\Sigma_i|I_{h,i} - \langle I_h\rangle|/\Sigma_h\Sigma_i|I_{h,i}|$, where I is the intensity of reflection. $I/\sigma(I)$ is the ratio of the mean observed intensity to the mean standard uncertainty of the observed intensity. Values in the outer resolution shell (2.1–2.0 Å) are given in parentheses. r.m.s.d., root-mean-square deviation.

Data collection	
Space group	*P*1
No. of reflections	102704
No. of unique reflections	50677
Completeness	93% (80%)
R_{merge}	4% (10.1%)
Overall $I/\sigma(I)$	14.8 (7.1)
Refinement	
Resolution range	19.8–2.0 Å
Number of reflections	48181
R_{factor}	0.0152
R_{free}	0.0194
R_{free} test set size	5%
No. of protein atoms	6078
No. of water molecules	596
No. of calcium ions	1
No. of ethylene glycol molecules	4
r.m.s.d. from ideal geometry	
Bond lengths	0.013 Å
Bond angles	1.7°
Mean *B* value (all atoms)	18.9 Å^2

Other mutations of residues close to the surface of the penicillin acylase molecule result in increased stability. The mutant protein Trp^{B431} → Arg, changing a residue 32 Å from the catalytic centre [36], showed a 2-fold increase in $t_{1/2}$ 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^{A168} [38].

Structure of the altered specificity mutant Phe^{B71} → Leu

The overall structure of the altered specificity mutant Phe^{B71} → 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 based on superposition of all main-chain Cα atoms). Initial difference maps showed regions of both positive and negative density near 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^{B253} and Phe^{B256}, 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^{B71}, loss of these aromatic stacking interactions leads to a flip of the hydrophobic side chain of Phe^{B256} into the periphery of the catalytic centre (Figure 4, bottom panel). The Cζ atom of Phe^{B256} moves 4.9 Å, leaving the side chain pinned between Leu^{B71} and Leu^{B253}. In addition, there are minor shifts in the positions of the side chains of Phe^{B254} and Phe^{A146}. The rearranged side-chain positions alter the substrate-binding environment, in particular the surface that contributes to the $S_{1'}$ 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^{B71} 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 oxyanion hole residue Ala^{B69} may be a contributing factor to the modest increase in k_{cat} and k_{cat}/K_m for NIPAB hydrolysis, bearing in mind effects such as perturbed electrostatic fields and vibrational modes that are not revealed by the static crystal structure.

Molecular modelling

A model of the Michaelis complex of the wild-type enzyme with penicillin G resembled that observed in the crystal structure of the complex with penicillin G sulphoxide [2]. The distance from the phenylacetyl carbonyl oxygen of penicillin G to the Oγ atom of Ser^{B1} was 2.5 Å (2.9 Å in the crystal structure), with a possible hydrogen bond. The atoms of the phenylacetyl group were in positions close to those found in the complex with phenylacetic acid [3]. There were possible hydrogen bonds to the substrate carbonyl oxygen from the side chain of Asn^{B241}, and from the main-chain nitrogen of Ala^{B69}. These features were found in models of complexes with all of the substrates shown in Table 1, consistent with the proposed catalytic function of Ser^{B1} [3,8,9]. Two further possible interactions with penicillin G are not feasible with some other substrates: first, there was a possible hydrogen bond from the α-amino group of Ser^{B1} to the β-lactam carbonyl oxygen; secondly, an oxygen atom of the penicillin carboxylate group was 4.1 Å from atom Nη2 of Arg^{B263}, with a possible hydrogen bond to a water molecule. In the model complex with NIPAB, there was no group equivalent to the β-lactam carbonyl oxygen but the carboxylate group of the

© 2003 Biochemical Society

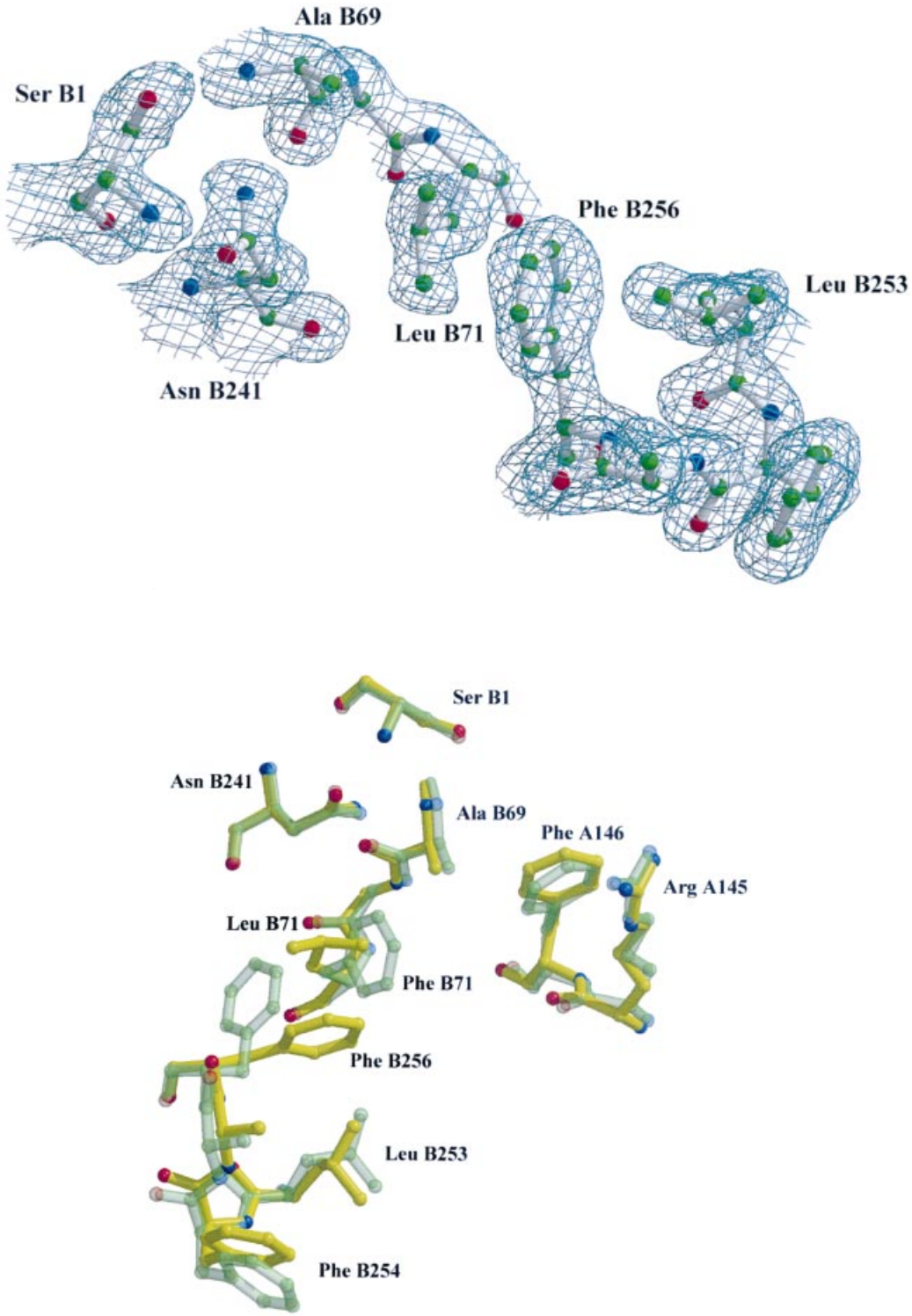

Figure 4 Structure of the Leu^{B71} mutant protein

Top panel: the $2F_o$-F_c electron density map around the mutation site, contoured at the 1 σ level. Atoms are coloured by type: carbon, green; oxygen, red; nitrogen, blue. Bottom panel: superposition of the native (green) and mutant (yellow) protein structures in the region of the catalytic centre. The loss of aromatic interactions owing to replacement of the residue Phe^{B71} results in a shift of the Phe^{B256} side chain into the substrate-binding site.

substrate occupied a comparable position to the carboxylate group of penicillin G, 4.3 Å from Arg^{B263}. Neutral ester substrates lack groups capable of making these interactions. The 3-carboxy-4-nitrophenyl ring was less than 3 Å from Cϵ2 of Phe^{B71} in the wild-type enzyme but 3.6 Å from Cγ of Leu^{B71} in the mutant. Similarly, the distances from Phe^{B256} were 3.5 Å from Cϵ in the

© 2003 Biochemical Society

wild-type and 3.9 Å from Cζ 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 glutaryl moiety was 3.3 Å from Cε of Met^{A142}. 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 Ser^{B1} 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 Ser^{B1}. In the model of the wild-type structure, the leucyl side chain of the substrate was 3.1 Å from the side chains of Phe^{A146} and Phe^{B71} and the substrate carboxylate group was 3.4 Å from Arg^{B263}. In the mutant Leu^{B71} crystal structure, the substrate side chain was located between Phe^{A146}, Leu^{B71} and Phe^{B256} 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_s.

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_1 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 Phe^{B71}, 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-acyl 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 phenylacetate with both wild-type and mutant proteins. In models, the 2-nitro group occupied a position between the side chains of Gln^{B23} and Phe^{A146} with short contacts including one of 2.7 Å between an oxygen atom of the nitro group and Cε2 of Phe^{A146}. The 4-nitrophenyl group extended towards the side chains of Phe^{B256} and Asn^{B388}, with a larger range of plausible conformations in the direction of Phe^{B71} and Asn^{B241}. 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 Cys^{B71} 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]. Cys^{B71} might either be changed into one of several amino acid analogues, altering specificity in the $S_{1'}$ pocket, or provide the site of attachment of an environmentally sensitive probe.

The research was supported by the Biotechnology and Biological Sciences Research Council and funding for stopped-flow equipment was from the Wellcome Trust. We thank Dr H. Burtscher (Boehringer Mannheim, Penzberg, Germany) for the gift of penicillin acylase and Dr C. Kell (SmithKline Beecham, Worthing, West Sussex, U.K.) for the gift of glutaryl-L-leucine.

REFERENCES

1 Hewitt, L., Kasche, V., Lummer, K., Lewis, R. J., Murshudov, G. N., Verma, C. S., Dodson, G. G. and Wilson, K. S. (2000) Structure of a slow processing precursor penicillin acylase from *Escherichia coli* reveals the linker peptide blocking the active-site cleft. J. Mol. Biol. **302**, 887–898
2 McVey, C., Walsh, M. A., Dodson, G. G., Wilson, K. S. and Brannigan, J. A. (2001) Crystal structures of penicillin acylase enzyme-substrate complexes: structural insights into the catalytic mechanism. J. Mol. Biol. **313**, 139–150
3 Duggleby, H. J., Tolley, S. P., Hill, C. P., Dodson, E. J., Dodson, G. and Moody, P. C. E. (1995) Penicillin acylase has a single-amino-acid catalytic centre. Nature (London) **373**, 264–268
4 Done, S. H., Brannigan, J. A., Moody, P. C. E. and Hubbard, R. E. (1998) Ligand-induced conformational change in penicillin acylase. J. Mol. Biol. **284**, 463–475
5 Brannigan, J. A., Dodson, G., Duggleby, H. J., Moody, P. C. E., Smith, J. L., Tomchick, D. R. and Murzin, A. G. (1995) A new catalytic framework acting through an N-terminal nucleophile and capable of autocatalytic processing. Nature (London) **378**, 416–419
6 Slade, A., Horrocks, A. J., Lindsay, C. D., Dunbar, B. and Virden, R. (1991) Site-directed chemical conversion of serine to cysteine in penicillin acylase from *Escherichia coli* ATCC 11105: effect on conformation and catalytic activity. Eur. J. Biochem. **197**, 75–80
7 Martín, J., Slade, A., Aitken, A., Arche, R. and Virden, R. (1991) Chemical modification of serine at the active site of penicillin acylase from *Kluyvera citrophila*. Biochem. J. **280**, 659–662
8 Morillas, M., Goble, M. L. and Virden, R. (1999) The kinetics of acylation and deacylation of penicillin acylase from *Escherichia coli* ATCC 11105: evidence for lowered pK_a values of groups near the catalytic centre. Biochem. J. **338**, 235–239
9 Roa, A., Goble, M. L., Garcia, J. L., Acebal, C. and Virden, R. (1996) Rapid burst kinetics in the hydrolysis of 4-nitrophenyl acetate by penicillin G acylase from *Kluyvera citrophila*. Biochem. J. **316**, 409–412
10 Alkema, W. B. L., Hensgens, C. M. H., Kroezinga, E. H., de Vries, E., Floris, R., van der Laan, J. M., Dijkstra, B. W. and Janssen, D. B. (2000) Characterization of the β-lactam binding site of penicillin acylase of *Escherichia coli* by structural and site-directed mutagenesis studies. Protein Eng. **13**, 857–863
11 Powell, K. A., Ramer, S. W., delCardayre, S. B., Stemmer, W. P. C., Tobin, M. B., Longchamp, P. F. and Huisman, G. W. (2001) Directed evolution and biocatalysis. Angew. Chem. **40**, 3948–3959
12 Daumy, G. O., Danley, D., McColl, A., Apostolakos, D. and Vinick, F. J. (1985) Experimental evolution of penicillin G acylases from *Escherichia coli* and *Proteus rettgeri*. J. Bacteriol. **163**, 925–932
13 Garcia, J. L. and Buesa, J. M. (1986) An improved method to clone penicillin acylase genes: cloning and expression in *Escherichia coli* of penicillin G acylase from *Kluyvera citrophila*. J. Biotechnol. **3**, 187–195
14 Forney, L. J., Wong, D. C. L. and Ferber, D. M. (1989) Selection of amidases with novel substrate specificities from penicillin amidase of *Escherichia coli*. Appl. Environ. Microbiol. **55**, 2550–2555
15 Brannigan, J., Ladurner, A., McVey, C. and Forney, L. (1995) Altered specificity mutants of penicillin acylase. In Perspectives on Protein Engineering (Geisow, M. and Epton, R., eds.), pp. 124–126, Mayflower Worldwide, Kingswinford
16 Heinrikson, R. L. and Meredith, S. C. (1984) Amino acid analysis by reverse-phase high-performance liquid chromatography: precolumn derivatization with phenylisothiocyanate. Anal. Biochem. **136**, 65–74
17 Santoro, M. M. and Bolen, D. W. (1988) Unfolding free energy changes determined by the linear extrapolation method. 1. Unfolding of phenylmethanesulfonyl α-chymotrypsin using different denaturants. Biochemistry **27**, 8063–8068
18 Duggleby, R. N. (1986) Progress curves of reactions catalyzed by unstable enzymes. A theoretical approach. J. Theor. Biol. **123**, 67–80
19 Otwinowski, Z. and Minor, W. (1997) Processing of X-ray diffraction data collected in oscillation mode. Methods Enzymol. **276**, 307–325
20 Collaborative Computational Project, no. 4 (1994) The CCP4 Suite: programs for protein crystallography. Acta Crystallogr. D **50**, 760–763
21 Navaza, J. (1994) *AMoRe*: an automated package for molecular replacement. Acta Crystallogr. A **50**, 157–163

© 2003 Biochemical Society

22 Murshudov, G. N., Vagin, A. A. and Dodson, E. J. (1997) Refinement of macromolecular structures by the maximum-likelihood method. Acta Crystallogr. D **53**, 240–255
23 Lamzin, V. S. and Wilson, K. S. (1993) Automated refinement of protein models. Acta Crystallogr. D **49**, 129–147
24 Brünger, A. T. (1992) Free *R* value: A novel statistical quantity for assessing the accuracy of crystal structures. Nature (London) **355**, 472–475
25 Guex, N. and Peitsch, M. C. (1997) SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis **18**, 2714–2723
26 Oh, S.-J., Kim, Y.-C., Park, Y.-W., Min, S.-Y., Kim, I.-S. and Kang, H.-S. (1987) Complete nucleotide sequence of the penicillin G acylase gene and the flanking regions, and its expression in *Escherichia coli*. Gene **56**, 87–97
27 Schumacher, G., Sizmann, D., Haug, H., Buckel, P. and Böck, A. (1986) Penicillin acylase from *E. coli*: unique gene-protein relation. Nucleic Acids Res. **14**, 5713–5727
28 Forney, L. J. and Wong, D. C. L. (1989) Alteration of the catalytic efficiency of penicillin amidase from *Escherichia coli*. Appl. Environ. Microbiol. **55**, 2556–2560
29 Roa, A., Garcia, J. L., Salto, F. and Cortes, E. (1994) Changing the substrate specificity of penicillin G acylase from *Kluyvera citrophila* through selective pressure. Biochem. J. **303**, 869–875
30 Niersbach, H., Kuhne, A., Tischer, W., Weber, M., Wedekind, F. and Plapp, R. (1995) Improvement of the catalytic properties of penicillin G acylase from *Escherichia coli* ATCC 11105 by selection of a new substrate specificity. Appl. Microbiol. Biotechnol. **43**, 679–684
31 Niersbach, H., Tischer, W., Weber, M., Wedekind, F. and Plapp, R. (1995) Isolation and mapping of a mutant penicillin G acylase with altered substrate specificity from *Escherichia coli*. Biotechnol. Lett. **17**, 19–24
32 Oue, S., Okamoto, A., Yano, T. and Kagamiyama, H. (1999) Redesigning the substrate specificity of an enzyme by cumulative effects of the mutations of non-active site residues. J. Biol. Chem. **274**, 2344–2349
33 Lindsay, C. D. and Pain, R. H. (1990) The folding and solution conformation of penicillin G acylase. Eur. J. Biochem. **192**, 133–141
34 Miyazaki, K., Wintrode, P. L., Grayling, R. A., Rubingh, D. N. and Arnold, F. H. (2000) Directed evolution study of temperature adaptation in a psychrophilic enzyme. J. Mol. Biol. **297**, 1015–1026
35 Forstner, M., Muller, A., Rognan, D., Kriechbaum, M. and Wallimann, T. (1998) Mutation of cis-proline 207 in mitochondrial creatine kinase to alanine leads to increased acid stability. Protein Eng. **11**, 563–568
36 Del Río, G., Lopez-Munguía, A. and Soberon, X. (1996) An engineered penicillin acylase with altered surface charge is more stable in alkaline pH. Ann. N.Y. Acad. Sci. **799**, 61–64
37 Del Río, G., Rodríguez, M., Munguía, M., Lopez-Munguía, A. and Soberon, X. (1995) Mutant *Escherichia coli* penicillin acylase with enhanced stability at alkaline pH. Biotechnol. Bioeng. **48**, 141–148
38 Martín, J., Prieto, I., Barbero, J. L., Pérez-Gil, J., Mancheño, J. M. and Arche, R. (1990) Thermodynamic profiles of penicillin G hydrolysis catalysed by wild-type and Met → Ala168 mutant penicillin acylases from *Kluyvera citrophila*. Biochim. Biophys. Acta **1037**, 133–139
39 McGaughey, G. B., Gagne, M. and Rappe, A. K. (1998) π-Stacking interactions. J. Biol. Chem. **273**, 15458–15463
40 McDonough, M. A., Klei, H. E. and Kelly, J. A. (1999) Crystal structure of penicillin G acylase from the Bro1 mutant strain of *Providencia rettgeri*. Protein Sci. **8**, 1971–1981
41 Xie, H., Bolam, D. N., Nagy, T., Szabó, L., Cooper, A., Simpson, P. J., Lakey, J. H., Williamson, M. P. and Gilbert, H. J. (2001) Role of hydrogen bonding in the interaction between a xylan binding module and xylan. Biochemistry **40**, 5700–5707
42 Qi, D. F., Tann, C. M., Haring, D. and Distefano, M. D. (2001) Generation of new enzymes via covalent modification of existing proteins. Chem. Rev. **101**, 3081–3111

Received 3 September 2002/20 December 2002; accepted 3 January 2003
Published as BJ Immediate Publication 3 January 2003, DOI 10.1042/BJ20021383

© 2003 Biochemical Society