Chemical modification of serine at the active site of penicillin acylase from *Kluyvera citrophila*

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The site of reaction of penicillin acylase from *Kluyvera citrophila* with the potent inhibitor phenylmethanesulphonyl fluoride was investigated by incubating the inactivated enzyme with thioacetic acid to convert the side chain of the putative active-site serine residue to that of cysteine. The protein product contained one thiol group, which was reactive towards 2,2′-dipyridyl disulphide and iodoacetic acid. Carboxymethylcysteine was identified as the *N*-terminal residue of the *β*-subunit of the carboxy[^{3}H]methylthiol-protein. No significant changes in tertiary structure were detected in the modified penicillin acylase using near-u.v. c.d. spectroscopy. However, the catalytic activity ($k_{cat.}$) with either an anilide or an ester substrate was decreased in the thiol-protein by a factor of more than 10^4. A comparison of sequences of apparently related acylases shows no other extensive regions of conserved sequence containing an invariant serine residue. The side chain of this residue is proposed as a candidate nucleophile in the formation of an acyl-enzyme during catalysis.

INTRODUCTION

Penicillin G acylase (penicillin amidohydrolase; EC 3.5.1.11) from *Kluyvera citrophila* (Barbero *et al.*, 1986), like that of *Escherichia coli* (Schumacher *et al.*, 1986), is a heterodimer derived from a precursor protein with loss of a signal peptide and an endopeptide during post-translational processing. The mature protein (M_r 85171) contains an *α*-subunit (M_r 23572) and a *β*-subunit (M_r 61599) derived from precursor protein residues 27–235 and 290–844 respectively. Similarities in sequence and subunit structure have been found in the penicillin G acylase from *Arthrobacter viscosus* (Ohashi *et al.*, 1988) and in two pseudomonad cephalosporin acylases (Matsuda & Komatsu, 1985; Matsuda *et al.*, 1987), supporting the view that these proteins are members of a sequence-related class of enzymes. However, other penicillin/cephalosporin acylases appear to be unrelated to this group (reviewed by Virden, 1990).

Phenylmethanesulphonyl fluoride (PMSF) is a potent inhibitor of the penicillin acylases from *E. coli* (Kutzbach & Rauenbusch, 1974) and *K. citrophila* (Martín *et al.*, 1990). The similarity of PMSF to a phenylacetyl substrate of the *E. coli* enzyme has been noted (Kutzbach & Rauenbusch, 1974) and progressive in-activation by PMSF in the presence of a substrate has been interpreted in terms of second-order reversible binding preceding first-order covalent modification (Shvyadas *et al.*, 1977). The enzyme from *Proteus rettgeri* is also inhibited by PMSF and, although no modified site has been located in the amino acid sequence, subunit complementation experiments have shown that sensitivity towards PMSF is exclusively associated with the larger *C*-terminal *β*-subunit (Daumy *et al.*, 1985). It is often assumed that PMSF reacts with the hydroxy group of a serine residue of penicillin acylase, but this has not been directly demonstrated. The absence of inhibition of the *E. coli* enzyme by di-isopropyl fluorophosphate (Kutzbach & Rauenbusch, 1974) might argue against the involvement of serine.

In the present paper we report the effects of incubating the PMSF-inactivated penicillin acylase with thioacetic acid. The results are comparable with those obtained using similar chemical procedures with serine proteinases, converting the active-site serine residue to cysteine by displacing the sulphonyl moiety with the readily hydrolysed thioacetyl group (Neet & Koshland, 1966; Polgár & Bender, 1967; Yokosawa *et al.*, 1977). Analysis of the modified penicillin acylase was simplified by the absence of cysteine residues in the normal mature enzyme (Barbero *et al.*, 1986).

EXPERIMENTAL

Materials

6-Nitro-3-phenylacetamidobenzoic acid and PMSF were from Sigma. Potassium thioacetate, 2,2′-dipyridyl disulphide, iodo-acetamide and iodoacetic acid were from Aldrich. Iodoacetic acid was washed with diethyl ether and dried in air before use. Iodo[2-^{3}H]acetic acid (175 Ci/mol) was from Amersham International. 4-Nitrophenyl phenylacetate was synthesized as described by Wang *et al.* (1986).

Buffers

Phosphate buffers (50 mM) had the following compositions: pH 7.5, 8 mM-NaH$_2$PO$_4$/42 mM-Na$_2$HPO$_4$; pH 7.25, 13 mM-NaH$_2$PO$_4$/37 mM-Na$_2$HPO$_4$; pH 7.0, 19.5 mM-NaH$_2$PO$_4$/30.5 mM-Na$_2$HPO$_4$; pH 6.0, 44 mM-NaH$_2$PO$_4$/6 mM-Na$_2$HPO$_4$.

Protein concentration

The concentration of penicillin acylase solutions was de-termined from measurements of A_{280}, assuming $A_{1cm}^{1\%}$ 22.1, a value estimated (Wetlaufer, 1962) from the amino acid com-position obtained from the amino acid sequence of the mature protein (Barbero *et al.*, 1986). Examination of absorption spectra (240–340 nm) confirmed the absence of significant protein aggregation.

Penicillin acylase

Penicillin acylase from *K. citrophila* was isolated and purified

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Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; PTH-amino acid, amino acid phenylthiohydantoin derivative; CM, carboxymethyl.
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as described by Barbero *et al.* (1986). Titration with PMSF (Shvyadas *et al.*, 1977; Martín *et al.*, 1990) showed that the purified penicillin acylase contained 0.96 mol of reactive sites/mol of protein.

Thiol-penicillin acylase

The procedure used was similar to that described for the preparation of thiol-subtilisin (Neet & Koshland, 1966). Penicillin acylase (85 μM) was incubated with 95 μM-PMSF in 50 mM-phosphate buffer, pH 7.25, for 15 min at 25 °C. Solid potassium thioacetate was added to give a 0.7 M solution. After adjustment to pH 5.3 with 0.2 M-HCl, the solution was kept under N$_2$ at 4 °C for 72 h and dialysed against 50 mM-phosphate buffer, pH 6.0, at 4 °C for 72 h.

SDS/PAGE

Electrophoresis was carried out at 10 °C in gels containing 0.1% (w/v) SDS, 12.5% (w/v) acrylamide and 0.34% (w/v) methylenebisacrylamide (Laemmli & Favre, 1973). The gel was fixed, stained with Coomassie Brilliant Blue R and, for subsequent fluorography, was de-stained (Steck *et al.*, 1979), treated with 1 M-sodium salicylate (Chamberlain, 1979) and exposed for 30 days at −70 °C to pre-flashed (Laskey & Mills, 1975) Fuji X-ray film. The M_r values of acylase subunits were determined by reference to marker proteins (M_r 14000–94000) from Pharmacia.

Peptide generation and isolation

Carboxy[^{3}H]methylthiol-protein was digested with pepsin [pepsin/substrate, 1:50 (w/w)] in 0.01 M-HCl for 4 h at 37 °C. Peptides were purified using a Beckman h.p.l.c. system fitted with a model 167 detector, monitoring at 214 and 280 nm. A reverse-phase C$_8$ chromatography column (4.6 mm × 75 mm) was used. Gradient elution with water/acetonitrile was from 0.1% (v/v) trifluoroacetic acid to 70% (v/v) acetonitrile containing 0.05% (v/v) trifluoroacetic acid. Gradient elution with water/methanol was from 0.1% (v/v) trifluoroacetic acid to 70% (v/v) methanol containing 0.05% (v/v) trifluoroacetic acid. The flow rate was 1 ml/min.

Automated sequence analysis

Sequence analysis was performed with Applied Biosystems 470A gas-phase and 477A pulsed liquid-phase peptide sequenators. Amino acid phenylthiohydantoin derivatives (PTH-amino acids) were analysed on-line with Applied Biosystems 120A analysers (Geisow & Aitken, 1989). Data collection and analysis were done with an Applied Biosystems 900A module calibrated with 25 pmol of PTH-amino acid standards.

C.d.

Measurements were made using a Jobin Yvon Dichrographe IV (10 mm light-path; semi-micro cuvette; 25 °C). Each spectrum was obtained as the difference between four averaged scans of the solution and four averaged scans of the solvent (Craig *et al.*, 1989).

Catalytic activity

The hydrolysis of 6-nitro-3-phenylacetamidobenzoic acid and of 4-nitrophenyl phenylacetate was measured from the increase in absorbance at 405 nm (Kutzbach & Rauenbusch, 1974) and 400 nm (Margolin *et al.*, 1980) respectively, using a Cary model 210 spectrophotometer. Kinetic parameters were determined for the normal enzyme by analysis of complete reaction curves (Koerber & Fink, 1987) and for the thiol-enzyme from initial-velocity measurements.

RESULTS

Thiol content and reactivity in thiol-acylase

Treatment of the PMSF-inactivated protein with thioacetic acid yielded a protein containing 1.1 mol of thiol/mol of protein, determined by monitoring the increase in A_{343} during incubation of 2 μM-protein with 1 mM-2,2'-dipyridyl disulphide in 50 mM-phosphate buffer, pH 7.5, at 25 °C (Brocklehurst & Little, 1973).

To carboxymethylate the thiol-protein, a solution (1 ml) containing 62 nmol of penicillin acylase and 5.7 μmol of iodo[2-^{3}H]acetic acid in 50 mM-phosphate buffer, pH 7.0, was incubated at 25 °C for 4 h. Unlabelled solid iodoacetic acid was added to give a final concentration of 5 mM; the solution was incubated at 25 °C for 3 h and dialysed against water at 4 °C for 72 h. The protein product contained no thiol detectable by titration with 2,2'-dipyridyl disulphide. The radioactivity incorporated was 26 Ci/mol of protein, equivalent to an average incorporation of 0.15 labelled carboxymethyl group before the addition of unlabelled iodoacetic acid.

SDS/PAGE

The results of SDS/PAGE and fluorography of the carboxy[^{3}H]methylthiol-acylase (Fig. 1) showed that radioactivity was associated with the β-subunit (apparent M_r 70000), but not with the α-subunit (apparent M_r 23000). Radioactivity was also detected in a band accounting for less than 10% of the total protein (in terms of bound dye) and migrating to a position intermediate between the α- and β-subunits (apparent M_r 39000). This would be consistent with limited cleavage of a small proportion of β-subunit chains.

N-Terminal-sequence analysis

In an initial experiment, a sample (20 nmol) of carboxy[^{3}H]methylthiol-acylase was digested with pepsin and fractionated by reverse-phase h.p.l.c. A labelled peptide was eluted at 16% acetonitrile with 58% recovery of radioactivity, and this fraction was further purified by h.p.l.c. A radioactive fraction eluted at 23% methanol with 93% recovery of radioactivity was associated with a peak at 214 nm, but not at 280 nm.
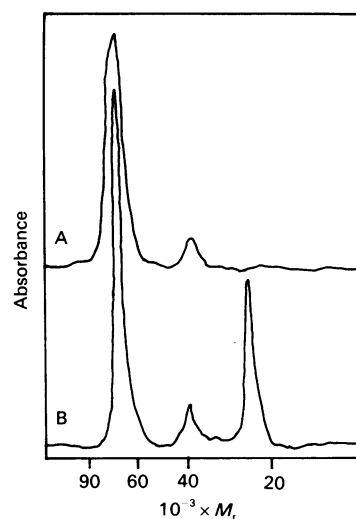

Fig. 1. SDS/PAGE of carboxy[^{3}H]methylthiol-acylase and normal penicillin acylase

Trace A shows the densitometry scan of a fluorograph of a track containing 60 μg of labelled protein. Trace B shows the scan of an adjacent track containing 10 μg of untreated enzyme and stained with Coomassie Brilliant Blue R.

Table 1. Recoveries of PTH-amino acids from the N-terminus of the β-subunit in automated Edman sequencing of carboxy[³H]methylthiol-acylase

A sample of carboxy[³H]methylthiol-enzyme (100 pmol) containing 4000 c.p.m. of radioactivity was loaded. In each cycle, 42% of the sample was analysed by h.p.l.c. and 33% was counted for radioactivity. Except for residue 8, which is identical in the α- and β-subunit, the yields of PTH-amino acids are shown for the β-subunit only; radioactivity counts are corrected for background and normalized to relate to the original sample. Values shown are averages for three runs.

Parameter	Cycle...	1	2	3	4	5	6	7	8
PTH-amino acid		CM-Cys	Asn	Met	Trp	Val	Ile	Gly	Lys
Yield of PTH-amino acid (pmol)		12	24	36	29	29	36	29	36
Radioactivity (c.p.m.)		1860	306	132	174	108	78	84	80

K. citrophila	290 S N H W V I G K N K	A Q D A K A I H V N	G P Q F G W Y A P A
E. coli	290 S N H W V I G K S K	A Q D A K A I H V N	G P Q F G W Y A P A
A. viscosus	S N A A I V G S E K	S A T G N A L L F S	G P Q V G F V A P G
GL-7ACA	199 S N S W A V A P G K	T A N G N A L L L Q	N P H L S W T T D Y
acyII protein	240 S N N W A V A P G R	T A T G R P I L A G	D P H R V F E I P G
Consensus	S N . w . v g . . k	. a . g . a i l . .	g P q . g w . a p .

Fig. 2. Sequences of the β-subunit N-terminal region of sequence-related acylases

The β-subunit N-terminal sequences shown are of penicillin acylases from *K. citrophila* (Barbero *et al.*, 1986), *E. coli* (Schumacher *et al.*, 1986) and *A. viscosus* (Ohashi *et al.*, 1988) and pseudomonad cephalosporin acylases from the gene encoding GL-7ACA acylase (Matsuda & Komatsu, 1985) and the gene *acyII* (Matsuda *et al.*, 1987). The N-terminal residue number is shown in relation to each published precursor sequence. In the consensus sequence, residues occurring in a majority of sequences and invariant residues are shown in lower case and capital letters respectively.

Table 2. Kinetic parameters for native penicillin acylase and thiol-acylase

K_m and $k_{cat.}$ values are shown for ester (4-nitrophenyl phenylacetate) and anilide (6-nitro-3-phenylacetamidobenzoic acid) substrates. The conditions were 50 mM-phosphate buffer, pH 7.5, 25 °C.

Enzyme and substrate	Parameter...	$k_{cat.}$ (s⁻¹)	K_m (μM)
Normal enzyme			
Anilide		23	30
Ester		495	91
Thiol-enzyme			
Anilide		1.7×10^{-3}	43
Ester		2.9×10^{-2}	92

This fraction was subjected to N-terminal-sequence analysis, and the results suggested the presence of an incompletely homogeneous carboxymethyl (CM)-tripeptide with the sequence CM-Cys-Asn-Met. Assuming CM-Cys to be derived from Ser, this sequence can only be obtained from the N-terminus of the β-subunit. Further detailed evidence was obtained by analysis of
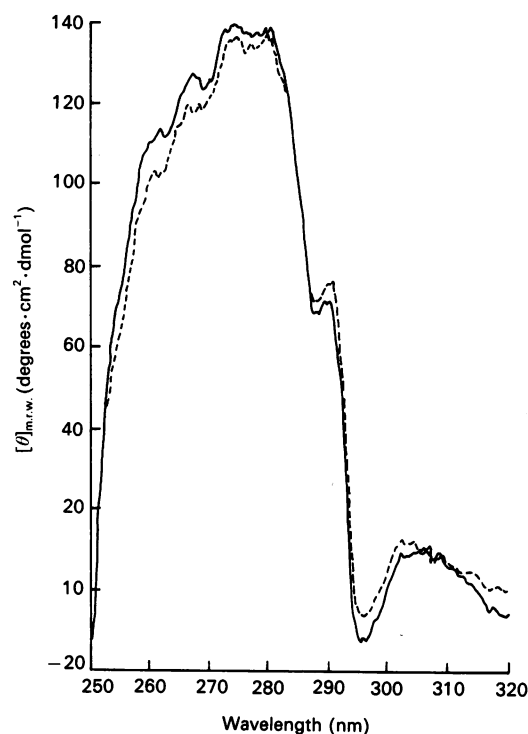
Fig. 3. C.d. spectra of penicillin acylase and thiol-penicillin acylase

Conditions were: 50 mM-phosphate buffer, pH 7.5, 25 °C, 3.15 μM-protein. ————, Penicillin acylase; ———, thiol-penicillin acylase.

the intact carboxy[³H]methylthiol-acylase, and Table 1 shows the recovery of radioactivity and PTH-amino acids after N-terminal sequencing. The recovery of radioactivity in the first cycle of the Edman degradation is consistent with carboxymethylation essentially exclusively at N-terminal Cys arising from the stoichiometric transformation of the corresponding N-terminal Ser. The relatively low yield of PTH-CM-Cys in the first cycle may be associated with an S-to-N acyl shift, and the presence of PTH-alanine suggests the occurrence of β-elimination and reduction. The CM-Cys was confirmed in a separate experiment where the h.p.l.c. separation was carried out using a pH 3.8 (in place of pH 4.0) buffer. The elution of PTH-CM-cysteine in the first Edman cycle was shifted away from a position close to that of PTH-Ser to a position close to that of PTH-Gly, confirming that CM-Cys was not being confused with Ser. In cycles 2–7 there was a uniform recovery of PTH-amino acids, in agreement with the sequence of the corresponding positions in the β-subunit (Fig. 2). The expected residues from the α-subunit were also found in cycles 1–7 (results not shown), whereas, in cycle 8, the presence of PTH-Lys was in accord with the occurrence of Lys in position 8 in both α- and β-subunits (Barbero *et al.*, 1986). As a further confirmation of the identification of CM-Cys at the N-terminal position, 30 μM-thiol-enzyme was incubated with 10 mM-iodoacetamide in 50 mM-phosphate buffer, pH 7.0, for 7 h at 20 °C and the solution was dialysed against water. The first cycle of N-terminal-sequence analysis of the protein product yielded a PTH-derivative with the same elution position as that of carboxyamidomethyl-Cys.

Conformation of thiol-acylase

The near-u.v. c.d. spectrum of the thiol-acylase was not significantly different from that of the normal enzyme (Fig. 3), showing substantial contributions from aromatic residues in a well-defined tertiary structure. Any effect of chemical

modification on protein conformation was therefore too small or too localized to affect the asymmetry of the environment of aromatic side chains, indicating that the folding of the modified protein was very similar to that of the normal enzyme.

Kinetic parameters of thiol-acylase

Table 2 shows the kinetic parameters for anilide and ester derivatives of phenylacetic acid. In each case the predominant effect of chemical modification was on the $k_{cat.}$, with a decrease which was essentially identical for both substrates (factors of 1.4×10^4 and 1.5×10^4 respectively). The values of K_m were not significantly altered in the modified protein.

DISCUSSION

Comparison of amino acid sequences

Common features in the catalytic mechanism are suggested by similarities in the sequences of several acylases, as illustrated by the N-terminal sequences of the β-subunit for three enzymes (Fig. 2). Using the numbering of the K. citrophila precursor sequence, Ser-290, Asn-291 and Pro-311 are invariant and several nearby residues are substituted conservatively. For example, there is Val or Ile at position 295, Gly or Ala at position 296 and Lys or Arg at position 299. Some weak sequence similarities can be proposed on the C-terminal side of this region, so that Ser-692 and Ser-795 might be considered to be conserved in the three related acylases for which complete sequences are available (Barbero et al., 1986; Schumacher et al., 1986; Matsuda et al., 1987). There is a region of sequence similarity in the N-terminal region of the α-subunit, but no additional invariant Ser residue is evident.

Catalytic activity of thiol-acylase

It is possible that the thiol-acylase possesses a very low level of residual catalytic activity. However, the absence of significant effects on the K_m values suggests that some or all of the observed activity may be associated with the presence of a small residue of unmodified enzyme. The small stereochemical effect of replacing an oxygen atom with a sulphur atom in the conversion of Ser to Cys is associated with a substantial effect on catalysis in other enzymes: little or no activity towards specific substrates was found with semi-synthetic thiol variants of serine proteinases in which, as here, any disruption to the tertiary structure was apparently small (Neet & Koshland, 1966; Neet et al., 1968; Polgár & Bender, 1967; Yokosawa et al., 1977).

Ser-290 as a candidate active-site nucleophile

The present study shows that the hydroxy group of Ser-290 is sufficiently nucleophilic to undergo site-directed modification. Similarly, the equivalent serine residue is modified in the E. coli enzyme, yielding a thiol-acylase with similar properties to those reported here, except for the greater stability of the E. coli acetyl-thiol enzyme (Slade et al., 1991). The reactivity of an acetyl-thiol intermediate formed during chemical modification raises the possibility of group migration from an initially substituted nucleophile to Ser-290, but the available sequence evidence does not offer strong candidates from among α- and β-subunit Ser

residues. There is no evidence for migration from a nucleophile other than a Ser residue, although this cannot be ruled out. In summary, the simplest interpretation of the present results is that Ser-290 is the site modified by reaction with PMSF. The kinetics of transacylation reactions catalysed by penicillin acylase (Konecny, 1981) support an acyl-enzyme mechanism, and we suggest Ser-290 as a candidate site for this acylation.

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