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Biochemical studies on the activity of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase from *Streptomyces clavuligerus*

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The enzyme activity of purified δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) synthetase from *Streptomyces clavuligerus* was studied biochemically. The dependence of ACV synthetase activity on reaction parameters, including substrates, cofactors, temperature and pH, were determined, resulting in a substantially increased enzyme activity. The activity is very labile to high temperature and is also unstable at acidic pH. The enzyme specificity is strict towards L-α-aminoadipate, but rather loose with respect to L-valine; certain modifications of L-cysteine can also be tolerated. Some unnatural tripeptides synthesized by ACV synthetase can be converted into bioactive compounds by isopenicillin N synthase. The only nutrient found to negatively affect ACV synthetase activity is phosphate, but various compounds such as thiol-blocking reagents and ATP-utilization products (AMP and pyrophosphate) are inhibitory to the enzyme.

INTRODUCTION

δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine (ACV) synthetase is the first enzyme in the branched pathway leading to all natural penicillins and cephalosporins from amino acid precursors. Its activity plays a crucial role in the biosynthesis of these β-lactams by both prokaryotic and eukaryotic micro-organisms, and will be important for applications *in vitro* such as the enzymic synthesis of novel antibiotics. However, owing to its extreme instability and the rather recent establishment of a reliable assay (Banko *et al.*, 1986, 1987), many of its biochemical properties are unknown, and its activity has largely been unexplored.

We recently succeeded in partially stabilizing crude ACV synthetase, and achieved purification of the active enzyme to apparent electrophoretic homogeneity from both the fungus *Cephalosporium acremonium* and the bacterium *Streptomyces clavuligerus* (Zhang & Demain, 1990*a*,*b*). Baldwin *et al.* (1990) also reported on the purification of both enzymes. The availability of active purified ACV synthetase made possible the investigation of its biochemical properties in the absence of interfering activities and contaminating materials. Such studies are important in advancing our understanding of the biochemical aspects of ACV biosynthesis and in making use of ACV synthetase in enzyme technology. Baldwin *et al.* (1990) have carried out qualitative studies on substrate specificity of ACV synthetase. This paper describes our studies *in vitro* on various aspects of the purified ACV synthetase activity from *S. clavuligerus*.

MATERIALS AND METHODS

Organisms, media and culture conditions

The filamentous bacterium *S. clavuligerus* N.R.R.L. 3585 (A.T.C.C. 27064) was used. It is a typical prokaryotic producer of cephamycin C. *Micrococcus luteus* ATCC 381, which is sensitive to penicillin-type antibiotics, was used for the isopenicillin N synthase ('cyclase') assay.

Medium composition and culture conditions were as described earlier (Piret *et al.*, 1990).

Cell-free extract preparation and enzyme purification

Preparation of extracts and purification of ACV synthetase were carried out by procedures described earlier (Zhang & Demain, 1990*b*).

Assays

Protein. Protein contents were measured by the method of Bradford (1976), with BSA as standard.

ACV synthetase. This activity was measured by h.p.l.c. estimation of the ACV formed in the cell-free enzyme reaction, by using a method modified from Banko *et al.* (1986, 1987). Initially, reactions were carried out in 5 mM-$MgCl_2$/5 mM-dithiothreitol (DTT)/100 mM-Mops buffer, pH 7.5 (purification buffer), containing 5 mM-L-α-aminoadipate, 1 mM-L-cysteine, 5 mM-L-valine, 3 mM-DTT, 5 mM-$MgCl_2$, 10 mM-ATP, and purified enzyme. The mixture was incubated at 25 °C at 220 rev./min for 1–2 h, and quenched by adding 4% (w/v) trichloroacetic acid. Some of these conditions were modified during the course of the ACV synthetase characterization study, as specified below. A 20 μl portion of reaction supernatant (which could be stored at −20 °C) was neutralized with 0.27 M-KOH, buffered with 0.3 M-NH_4HCO_3, reduced with 5 mM-DTT for 15 min and allowed to react with 33 mM-monobromobimane in the dark for 15 min. After the unchanged monobromobimane had been extracted with water-saturated methylene dichloride three times, the mixture was acidified with 1 M-citrate/HCl buffer, pH 2.2, to approx. pH 4.5. Thus the thiol group of ACV was converted into the fluorescent derivative of monobromobimane, which was separated on a μBondapak C_{18} h.p.l.c. column (3.9 cm × 30 cm) and monitored with a fluorescence detector. The two mobile phases were solvent A (880 mg of pentane-1-sulphonic acid, 2.5 ml of acetic acid, and 1 litre of water) and solvent B (800 mg of pentane-1-sulphonic acid dissolved in 50 ml of water, 2.5 ml of acetic acid and 950 ml of acetonitrile). Elution was carried out with a linear gradient over 120 min (1.5 ml/min) from 10% solvent B in A to 100% solvent B. Under these conditions, authentic ACV was eluted with a retention time of about 20 min. Estimation of ACV concentration in reaction mixtures was

Abbreviations used: ACV, δ-(L-α-aminodipyl)-L-cysteinyl-D-valine; DTT, dithiothreitol.

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based on a comparison of the heights of peaks uniform in shape with those of ACV standards. One unit of enzyme activity was defined as the amount producing 1 μmol of ACV/min; specific activity was expressed as munits/mg of protein.

Biochemical studies

Unless otherwise specified, the enzyme preparations were always in purification buffer.

Reaction conditions for catalysis. For the investigation of improved conditions for the ACV synthetase reaction, only the studied parameter was varied, whereas all other conditions were kept constant during each experiment. The initial reaction conditions, as described above for the ACV synthetase assay, are termed the 'original reaction conditions'. As the characterization went on, some of these parameters were modified. Unless otherwise specified, the reactions were carried out under 'normal reaction conditions': 80–90 μl of enzyme preparation in purification buffer containing 5 mM-ATP, 10 mM-Mg^{2+}, 5 mM-L-α-aminoadipate, 5 mM-L-valine and 1 mM-L-cysteine along with an additional 3 mM-DTT, total volume 100 μl at 29–30 °C at 220 rev./min for 1–2 h.

Stability. The enzyme preparations (in purification buffer) were incubated at the given temperature or pH for specified times. The cofactors and substrates were added and the enzyme reactions were carried out to determine the residual activities, expressed as a percentage of the control.

Substrate specificity. For substrate-specificity studies, two of the substrate amino acids were kept unchanged while the third was replaced in the enzyme reaction. Activity was detected by the formation of a new peak in the tripeptide region on the h.p.l.c. profile. The identities of the new peaks were further confirmed when the authentic corresponding tripeptides were available (i.e. L-*S*-carboxymethylcysteinyl-CV, L-glutamyl-CV, AC-D-allo-isoleucine, AC-D-α-aminobutyrate and AC-D-isoleucine). Tripeptide formation was quantified as a percentage of the ACV peak height observed with the natural substrate. The amino acid replacements were used at the same concentration as the natural amino acids and were incubated for the same reaction time. The comparison was made on the basis of tripeptide formation rate rather than on conversion yield. Further conversion of a new tripeptide into a bioactive compound was examined by adding a cyclase preparation (from *C. acremonium* C-10) and cofactors (Fe^{2+}/Mg^{2+}/ascorbate) (Kupka *et al.*, 1983) to the tripeptide-forming reaction mixture which had been incubated for 2 h and the reaction terminated by heating. The bioactivity was qualitatively shown by the appearance of inhibition zones on *M. luteus* plates.

Effectors of ACV synthetase activity. Various potential effectors were added at different concentrations directly to the reaction mixtures just before the reactions were started. When the specific activity in the experimental case was lower than that of the control, it was concluded that there was direct or indirect inhibition of enzyme activity by the compound. In the phosphate inhibition study, KCl at the same concentration as used for phosphate was added to the control to normalize ionic strength.

RESULTS AND DISCUSSION

Reaction conditions for catalysis

ACV synthetase activity is affected by the concentrations of substrates and cofactors specific for the enzyme, and of compounds such as salts and buffers, as well as pH, ionic strength and temperature. It is necessary to study these factors in order to increase enzyme activity. Originally, we used the reaction conditions for crude *C. acremonium* ACV synthetase (Banko *et al.*, 1986, 1987) for the assay of *S. clavuligerus* ACV synthetase (the 'original reaction conditions' as stated above). Although Jensen *et al.* (1988) modified these conditions for crude *S. clavuligerus* ACV synthetase (i.e. they decreased ATP concentration to 1 mM and added an ATP-regenerating system), we did not find their conditions to be superior to the original conditions (results not shown). With purified *S. clavuligerus* ACV synthetase available, various parameters were studied.

Linearity with time. The enzyme reaction was linear with time for at least 3 h. This indicated that the enzyme is fairly stable under reaction conditions and that product inhibition is not obvious within this period of time. Normally the reactions were run for 1–2 h to ensure the measurement of a true initial rate.

Effect of cofactor concentrations. Like many bacterial non-ribosomal peptide synthetases, the purified enzyme requires ATP and Mg^{2+} for its activity (Fig. 1), i.e. for the activation of substrate amino acids and their binding to the enzyme, although in the absence of exogenous Mg^{2+}, the enzyme still retained marginal (approx. 5%) activity. This activity is possibly due to the incorporation of a trace amount of Mg^{2+} into the protein. It should be noted that Mg^{2+} was present in the purification buffer before it was removed by desalting immediately before the running of this reaction. Under the normal reaction conditions (Mg^{2+} at 10 mM), the apparent optimum concentration range of ATP was 1.5–5 mM. Lower concentrations were insufficient and higher concentrations inhibitory. When ATP was at 5 mM, the apparently optimum concentration for Mg^{2+} was 10 mM, with 15 mM giving slightly lower activity.

The fact that the apparent optimum concentration of Mg^{2+} is higher than that of ATP could be due to two reasons: (i) at

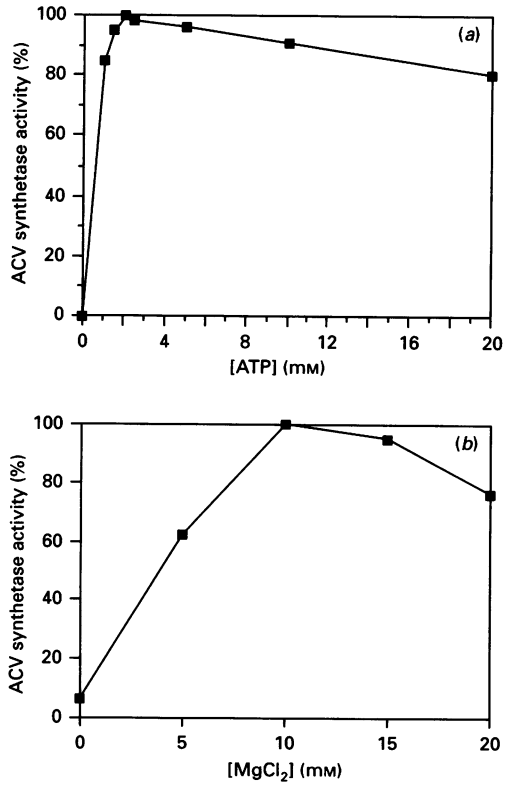

Fig. 1. Dependence of ACV synthetase activity on cofactor [(*a*) ATP and (*b*) Mg^{2+}] concentrations

In (*a*) the $MgCl_2$ concentration was fixed at 10 mM and in (*b*) the ATP concentration was fixed at 5 mM.

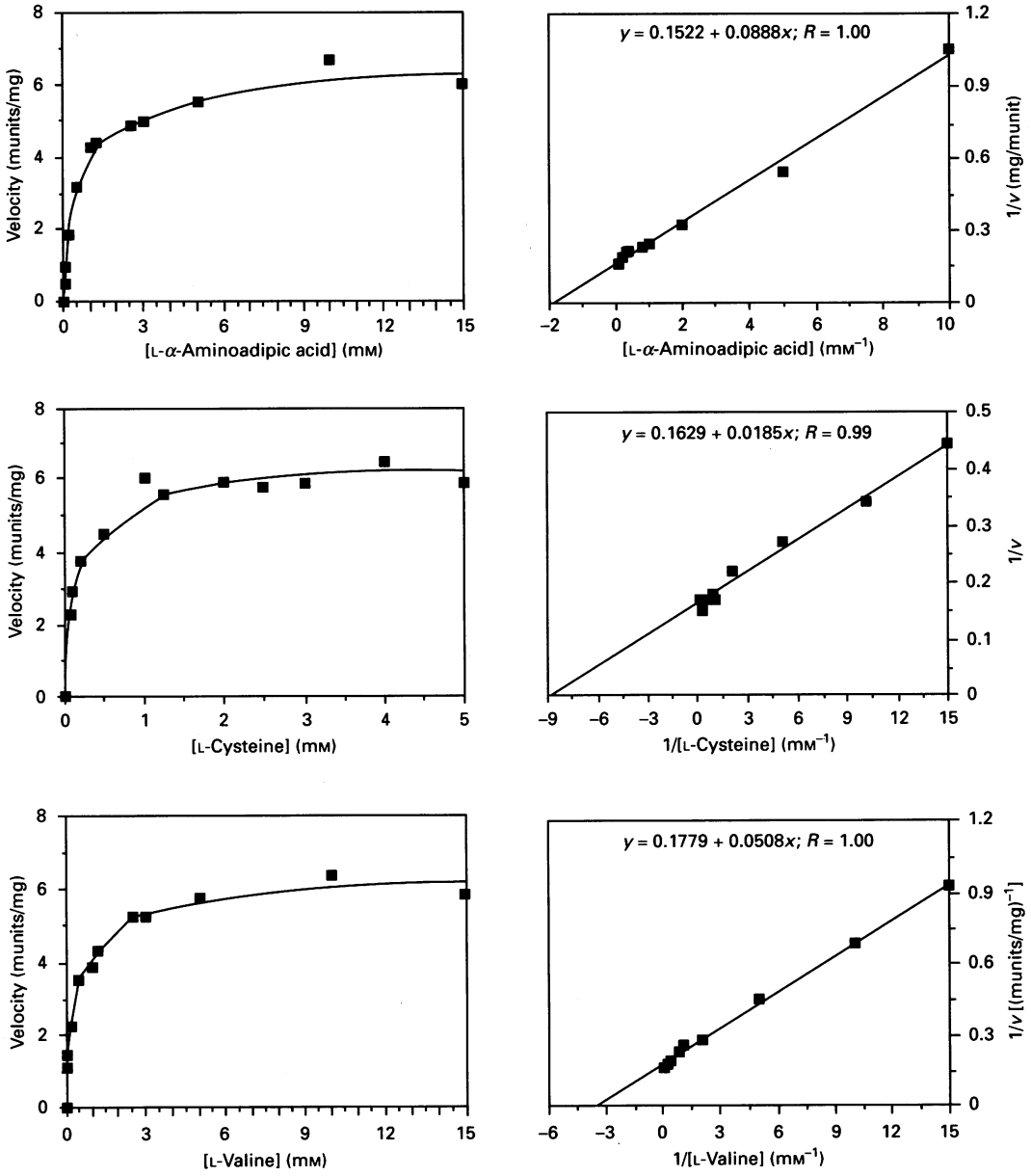

Fig. 2. Dependence of ACV synthetase activity on substrate amino acid concentrations plus the Lineweaver–Burk plots

neutral pH and in the presence of Mg^{2+}, ATP exists in several forms, i.e. ATP^{4-}, $HATP^{3-}$, H_2ATP^{2-}, $MgATP^{2-}$ and $MgHATP^{1-}$ (Langer, 1974). Mg^{2+} will shift the equilibrium

$$HATP^{3-} \rightleftharpoons H^+ + ATP^{4-}$$

to the right because of its much greater affinity for the tetra-negative anionic form of the nucleotide. Thus if the Mg^{2+}/ATP ratio is high, the active species for the nucleotide-dependent enzyme reaction, $MgATP^{2-}$, will predominate (Fromm, 1975); (ii) the products of ATP utilization, i.e. PP_i and AMP (or $MgPP_i^{2-}$ and MgAMP when Mg^{2+} is present), are inhibitory to ACV synthetase (see below).

Another additive to the reaction system is DTT. DTT was present in the substrate stock solution and was added together with the substrates to the reaction system (at a 3:1 molar ratio to L-cysteine) to keep L-cysteine reduced. DTT was constantly present at a concentration of 5 mM to stabilize the enzyme. Thus under the original and normal reaction conditions where 1 mM-L-cysteine was used, the final DTT concentration was 8 mM. It was found that 8 mM-DTT led to a 10% higher enzyme activity than did 3 mM; there was no further increase in activity when the DTT concentration was increased to 13 mM.

Dependence on substrate amino acid concentrations. The effect of substrate concentrations on ACV synthetase activity is shown in Fig. 2. The enzyme seemed to obey Michaelis–Menten kinetics with respect to the individual amino acids. There was no obvious substrate activation or inhibition. The substrate concentrations under the original conditions (L-α-aminoadipate, 5 mM; L-cysteine, 1 mM; L-valine, 5 mM) appear to be insufficient for the enzyme to achieve its maximum activity. The Lineweaver–Burk plots (Fig. 2) gave the following K_m values for each substrate under the normal reaction conditions: L-α-aminoadipate, 0.63 mM; L-cysteine, 0.12 mM; L-valine; 0.30 mM. This pattern of K_m values is different from the estimates of Jensen *et al.* (1988) for crude *S. clavuligerus* ACV synthetase (L-α-aminoadipate, 0.56 mM; L-cysteine, 0.07 mM; L-valine, 1.14 mM). However, both

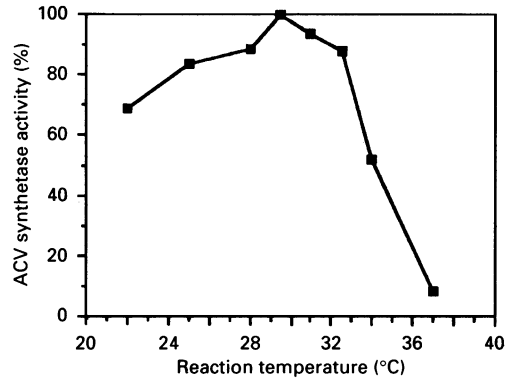

Fig. 3. Dependence of ACV synthetase activity on reaction temperature

The reactions were conducted for 1.5 h.

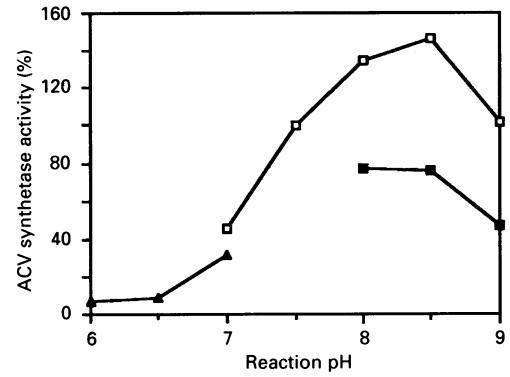

Fig. 4. Dependence of ACV synthetase activity on reaction pH and buffer system

The duration of the reaction was 2 h. All buffers were at 100 mM, containing 5 mM-DTT and 5 mM-Mg^{2+}. ▲, Mes; □, Mops; ■, Tris.

studies show that L-cysteine has the highest affinity with ACV synthetase among the three substrates. The maximum activity under the normal conditions is 6.3 munits/mg. The purified *S. clavuligerus* ACV synthetase obtained by Baldwin *et al.* (1990) had a specific activity of 610 nmol ACV/h per mg, which equals 10 munits/mg as defined by us. It should be noted that their assay differed from ours in that it was based on radioactivity.

It should be pointed out that we have found that the ACV synthetase activities reported in our previous publications (Zhang & Demain, 1990*a*,*b*) were not correct due to calibration problems with the h.p.l.c. detector. To obtain true activity values in those two reports, the values should be multiplied by 6.8 and 7.4 respectively.

Dependence on reaction temperature. The enzyme reaction took place even at 4 °C (results not shown). The reaction rate increased with temperature from 22 °C to 29.5 °C (Fig. 3), matching the typical 1.8-fold increase in rate for every 10 °C increase (Scopes, 1987). At higher temperatures, the ACV synthetase activity fell as the result of an increasing rate of thermal denaturation, but the fall was slow until above 32 °C. The best temperature under the conditions used (normal reaction conditions, 1.5 h) was 29.5 °C, which is significantly higher than the 25 °C found to be optimal for crude *S. clavuligerus* ACV synthetase (Jensen *et al.*, 1988).

Dependence on reaction pH, buffer system and ionic strength. The enzyme preparation was placed in different buffers at different pH values and reactions were immediately carried out. The ACV synthetase activity was low at pH values below 7.5, but high at pH 7.5–9.0 (Fig. 4). This pointed to the possibility that the maximum enzyme activity might require deprotonization of certain groups in the active centre. Since there exist thiol groups essential for ACV synthetase catalysis (see below), and the activated substrate amino acids were shown to be bound to *Aspergillus nidulans* ACV synthetase through thioester bonds (van Liempt *et al.*, 1989), it is possible that the thiol group of one or more cysteine residues (pK_a 8 at free state) needs to be ionized in order to facilitate binding of the activated substrates and thus enzyme catalysis. The lower activity at pH 9.0 than at 8.5 might be due to the lower stability of the enzyme during the reaction at this pH (see below). The apparent optimum pH for ACV synthetase activity under the conditions used (normal reaction conditions, in Mops or Tris) was pH 8.0–8.5, which is higher than the normal pH in the cytoplasm of cells (approx. 7.2) (Darnell *et al.*, 1986). A pH of 8.5 supported an approx. 50% higher activity than did pH 7.5, the reported optimum pH for crude *S. clavuligerus* ACV synthetase (Jensen *et al.*, 1988). Doubling the substrate concentrations did not affect the relationship between the enzyme activities at pH 8 and pH 7.5 (results not shown).

As can be seen in Fig. 4, Mops is a better buffer than Mes and Tris for ACV synthetase activity. It should be noted that Mes can bind Mg^{2+} (Good *et al.*, 1966), and the stability constant of $MgATP^{2-}$ in Tris is low (Fromm, 1975). Phosphate would not be a suitable buffer since it is an inhibitor of ACV synthetase activity (see below).

Increasing the ionic strength in the reaction mixture slightly inhibited ACV synthetase activity. Addition of 100 mM-KCl caused 20% inhibition, even though the total ionic strength was still within the physiological range of 0.1–0.2 M (Scopes, 1987).

Effect of enzyme concentration. Below about 325 μg of protein/ml, the reaction velocity was proportional to ACV synthetase concentration. This suggests the absence of subunit association or dissociation (which is in agreement with the monomeric character of *S. clavuligerus* ACV synthetase; Zhang & Demain, 1990*b*), of activators or inhibitors in the enzyme preparation, and of limitation of the assay method in this range. The enzyme reactions for characterization studies were carried out in this range of protein concentration.

Table 1 summarizes the best reaction conditions for catalysis by purified *S. clavuligerus* ACV synthetase. Under these improved conditions, the maximum activity reached 11.3 munits/mg, which is 2.4 times that of the same preparation achieved under the original reaction conditions, and 1.4 times that under the normal reaction conditions. Based on our estimate of the molecular mass of ACV synthetase (i.e. 350–400 kDa; Zhang & Demain, 1990*b*), the $k_{cat.}$ under our improved conditions is 4–4.5/min.

Stability

Stability at low and moderate temperatures. The purified enzyme preparation in purification buffer was incubated at 25 °C and 4 °C for various times, and the residual activities were assayed under normal reaction conditions. The purified enzyme lost half of its activity in about 10 h at 25 °C and in about 70 h at 4 °C. The stability at 4 °C under these conditions was similar to that of the crude enzyme (results not shown), indicating the major contribution for the enzyme instability was not from factors present in crude extracts.

Thermal stability. Incubating the enzyme at different temperatures for 10 min followed by assaying the remaining activity under normal reaction conditions revealed that the purified ACV synthetase activity is quite thermally unstable (Fig. 5), losing half of its activity in 10 min at only 32 °C. Below 28 °C,

Table 1. Reaction parameters for optimal catalysis by ACV synthetase

The maximum velocity was measured in a reaction carried out in a mixture containing 100 mM-Mops buffer, pH 8.0, 5 mM-ATP, 10 mM-$MgCl_2$, 10 mM-L-α-aminoadipate (A), 2 mM-L-cysteine (C), 10 mM-L-valine (V) and 11 mM-DTT at 29.5 °C. The same enzyme sample gave 40% activity (4.71 munits/mg) under the original reaction conditions and 70% activity (7.96 munits/mg) under the normal reaction conditions.

K_m (mM)			Optimum cofactor concn. (mM)		Optimum reaction		Suitable reaction buffer	Maximum velocity (munits/mg)
A	C	V	ATP	Mg^{2+}	Temperature (°C)	pH		
0.63	0.12	0.30	1.5–5	10	29–30	8.0–8.5	Mops	11.32

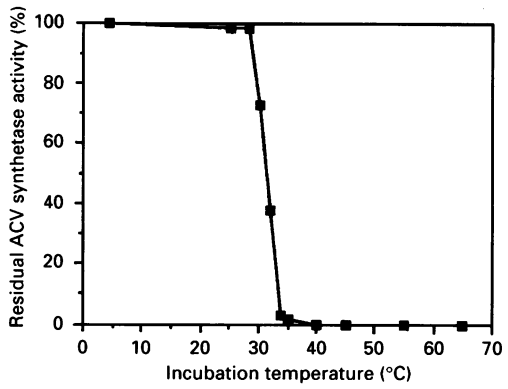

Fig. 5. Thermal stability of ACV synthetase activity

Enzyme preparations were in purification buffer during a 10 min incubation at various temperatures.

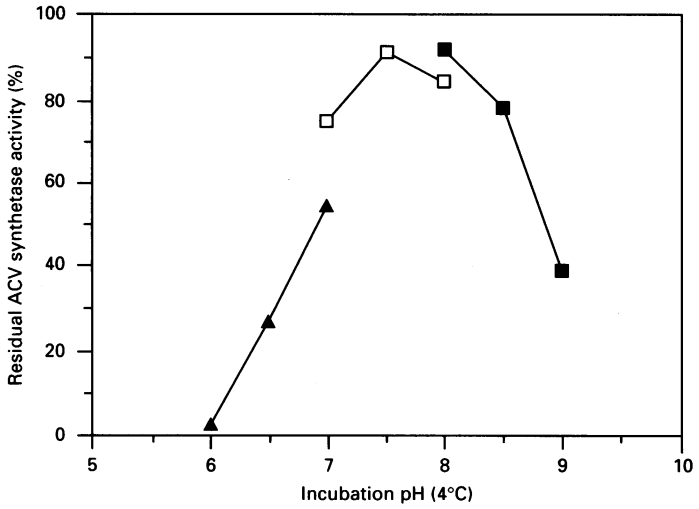

Fig. 6. pH stability of ACV synthetase activity

All buffers were 100 mM, with 5 mM-DTT and 5 mM-Mg^{2+}. The same enzyme sample, after determination of the initial enzyme activity, was transferred into different buffer systems as specified, incubated at 4 °C for 2 h; then the preparations were transferred back into purification buffer and reactions were carried out immediately. ▲, Mes; □, Mops; ■, Tris.

the enzyme was stable, but at 34 °C the activity was almost completely lost in 10 min. The result was quite surprising in that the best temperature for a 90 min reaction had been found to be 29.5 °C (Fig. 3), and a 32 °C reaction exhibited nearly 90% of activity. Even at 34 °C, the 90 min reaction still gave 50% activity. These results indicated that the enzyme activity was stabilized by a reaction component(s).

pH stability. pH affects the ionization of proteins which influences their stability. After the determination of the initial activity, the purified enzyme was transferred from purification buffer into various buffer systems by gel filtration, and incubated at 4 °C for 2 h. Then all the samples were placed back into the purification buffer, and the residual activities were assayed. Fig. 6 demonstrates that ACV synthetase activity is very unstable above pH 8.5 and below 7.0, especially in the acidic range; in between the activity is fairly stable.

Substrate specificity of ACV synthetase activity

It is known that cyclase can cyclize many unnatural tripeptides, and deacetoxycephalosporin C synthetase ('expandase') can expand some unnatural penicillins to cephalosporins (for review, see Baldwin & Bradley, 1990). It is important to study substrate specificity of purified ACV synthetase because of the possibility of producing ACV analogues with the enzyme, which could result in total enzymic synthesis of novel penicillins and cephalosporins. In addition, knowledge of substrate specificity of ACV synthetase could be useful for future studies on the enzymic mechanism and other aspects of this enzyme.

Replacements for L-α-aminoadipate, L-cysteine and L-valine. Under normal reaction conditions, one of the three substrate amino acids was replaced by various analogues which were close in structure to the natural substrate and thus had the potential to be accepted by ACV synthetase for synthesizing an ACV analogue. The results are expressed as tripeptide formation rate (% of ACV formation rate) and are shown in Table 2. It can be seen that ACV synthetase is very strict with respect to its specificity towards L-α-aminoadipate in terms of chain length, substitution groups and steric configuration. Only the change within the chain, i.e. L-*S*-carboxymethylcysteine (γ-CH_2 in L-α-aminoadipate replaced by S), was accepted by the enzyme, at about half the normal rate. On the other hand, the enzyme is quite broad in its specificity towards L-valine, and a number of substitutes with different alkyl group sizes were tolerated. The analogues that are closest in structure to L-valine were the best substitutes as substrates. Although the final tripeptide product is LLD-ACV, D-valine is nevertheless not a substrate, indicating the importance of the correct steric configuration for a substitute to be accepted by the enzyme. This was also seen by comparing L-*allo*-isoleucine and L-isoleucine, the latter being a much poorer replacement. L-*allo*-Isoleucine and L-α-aminobutyrate were good substitutes (40% of L-valine rate), followed by L-norvaline and L-allylglycine (27% and 20%).

Since the ACV synthetase assay used is based on the thiol group of the L-cysteine residue, any substitute for L-cysteine without a free thiol group would not be assayable by our h.p.l.c.

Table 2. Substrate-specificity of ACV synthetase

Tripeptides were quantified by measuring peak heights on h.p.l.c. profiles.

Replacement (5 mM)	Tripeptide formation rate (%)
For L-α-aminoadipic acid	
L-α-Aminoadipic acid	100*
L-*S*-Carboxymethylcysteine	52*
L-Glutamic acid	0
DL-α-Aminopimelic acid	0
D-α-Aminoadipic acid	0
L-Adipic acid	0
L-Lysine	0
L-Aspartic acid	0
Phenylacetic acid	0
For L-cysteine	
L-Cysteine	100*
DL-Homocysteine	73*
L-*S*-Methylcysteine	+*†
For L-valine	
L-Valine	100*
L-*allo*-Isoleucine	44
L-α-Aminobutyric acid	41*
L-Norvaline	27
L-Allylglycine	20
L-Leucine	13
L-Isoleucine	11
Glycine	10
L-Norleucine	8
D-Valine	0

* These tripeptides were observed to form bioactive compounds by adding cyclase and its cofactors. Others were not detectable by such an assay.

† This tripeptide was not assayable by the h.p.l.c. procedure used.

procedure for its effectiveness as a substrate. Bioassay (i.e. further conversion of the resultant tripeptide into the corresponding penicillin by addition of cyclase plus its cofactors and detection of antibiotic activity) was useful for certain replacements, but it was unable to provide conclusions on such L-cysteine substitutes as L-methionine, L-serine, L-glycine or L-alanine. It should be noted that those compounds that did not show bioactivity under the conditions used (Table 2) might still have been accepted by cyclase, but failed to elicit an antibiotic zone either because the bioassay was not sensitive enough or the cyclized product was biologically inactive. One available analogue with a free thiol group, L-homocysteine, emerged as a good replacement for L-cysteine (> 70% of the rate), indicating that ACV synthetase is not strict with regard to the chain length of the L-cysteine substitute. In fact, homocysteine is a strong competitive inhibitor of ACV-forming activity (results not shown). Also, an analogue with a methylated thiol group, L-*S*-methylcysteine, was shown by bioassay to be a positive substitute. This implies that the enzyme tolerates modifications on the -SH group at least to some extent and that the thiol group is not very important for tripeptide formation.

Knowledge of the substrate specificity of ACV synthetase opens up the possibility of total enzymic synthesis of novel penicillins, or even novel cephalosporins. One such example is the use of L-*S*-carboxymethylcysteine to replace L-α-aminoadipate. ACV synthetase accepts this analogue (Table 2), as does cyclase (Wolfe *et al.*, 1984), and it has already been shown that extracts of *S. clavuligerus* can convert L-*S*-carboxymethylcysteinyl-L-cysteinyl-D-valine all the way into the cephalosporin stage, producing a novel cephalosporin containing D-*S*-carboxymethylcysteine as its side chain (Bowers *et al.*, 1984). Interestingly, this antibiotic is considerably more active than cephalosporin C itself (Wolfe *et al.*, 1985). For L-valine, ACV synthetase has a rather broad specificity, similar to cyclase which can accept many substitutes for the D-valine residue in ACV, yielding new β-lactams with five-, six- and seven-numbered sulphur-containing rings (Baldwin *et al.*, 1989). Most of the good L-valine substitutes for ACV synthetase, i.e. L-*allo*-isoleucine, L-α-aminobutyrate, L-norvaline and L-allyglycine, are also good substitutes for the D-valine residue of ACV with regard to the cyclase reaction (Kupka *et al.*, 1983; Wolfe *et al.*, 1984; Baldwin *et al.* 1986*a,b*, 1987). These cyclase products include antibiotically active and inactive penems, cephams, hydroxymethylcephams, homocephams and hydroxyhomocephams. However, ACV synthetase cannot accept D-form replacements, whereas cyclase does not accept L-form substitutes for D-valine in ACV; a valine racemization process is involved during ACV synthesis. As for L-cysteine, both tripeptides obtained from homocysteine and *S*-methylcysteine replacing L-cysteine were cyclized by cyclase to bioactive compounds (Table 2). Baldwin *et al.* (1988) have shown the products of cyclase action on A-L-homocysteinyl-V to be epimeric 5-hydroxy-γ-lactams rather than conventional bicyclic β-lactam antibiotics. It would be interesting to know the structure of the A-L-S-methylcysteinyl-V cyclization product, since there is no free thiol group present.

Independently of our work, Baldwin *et al.* (1990) have reported preliminary qualitative results on the acceptance of L-α-aminobutyrate, but not D-valine, as an L-valine substitute by *S. clavuligerus* ACV synthetase. They also reported that ACV synthetase does not catalyse the synthesis of ACV from LL-AC plus L-valine.

The absence of the reverse reaction. Incubation of ACV for 2 h with purified ACV synthetase under normal reaction conditions with or without cofactors (ATP and Mg^{2+}) resulted in no decrease in the ACV concentration (results not shown), indicating that ACV is not a substrate and there is no reverse reaction (ACV → A + C + V) catalysed by ACV synthetase. Also, this result shows that ACV is stable under reaction conditions.

The absence of glycyl-ACV and GSH formation. Although glycyl-ACV was isolated from the β-lactam-producing *Paecilomyces persicinus* (Enriquez & Pisano, 1979), addition of glycine to the normal reaction mixture, or incubation of glycine with ACV and ACV synthetase under normal reaction conditions without substrate amino acids, did not yield any peptide peak on the h.p.l.c. profile other than ACV, indicating again that ACV is not a substrate of ACV synthetase and there is no glycyl-ACV tetrapeptide synthesized by ACV synthetase.

Lara *et al.* (1982) proposed that in *Penicillium chrysogenum*, one single enzyme produces both ACV and GSH. However, no peak was found at the glutathione position on the h.p.l.c. profile when L-glutamate, L-cysteine and glycine were incubated with *S. clavuligerus* ACV synthetase, under normal reaction conditions but lacking L-α-aminoadipate and L-valine. This means that ACV synthetase is not a glutathione synthetase.

Effectors of ACV synthetase activity

Effects of potential regulatory compounds. Nutrients used in the medium for fermentation were tested for their effects on the purified ACV synthetase activity (Table 3). Glycerol and NH_4^+ ions were not inhibitory; however, inorganic phosphate is an inhibitor, with 100 mM inhibiting the activity by 30%. The mechanism is unknown. Scopes (1987) suggested that phosphate or other multiply charged anionic ions may compete with a negatively charged substrate by binding weakly to the positively charged active site. Another possible reason could be the binding

Table 3. Effects of potential regulatory compounds on ACV synthetase activity

Additive	Concentration (mM)	ACV synthetase activity (%)
None	—	100
Glycerol	50	99*
Maltose	10	93
NH_4Cl	25	102
Phosphate	25	83†
	100	68†
ACV	0.035	112
Penicillin N	1	92
Cephamycin C	1	107
Glutathione	0.2	115

* Glycerol effect was tested with crude ACV synthetase preparations.
† The control contained KCl at the same concentrations as phosphate.

Table 4. Effects of various compounds on ACV synthetase activity

Additive	Concentration (mM)	ACV synthetase activity (%)
None	–	100
Pantothenic acid	5	103
Hydroxylamine	5	93
D-Valine	5	98
Glycine	5	89
L-Glutamate	10	103
DL-Methionine	12.5	103
EDTA	5	91
	10	67
Pyrophosphate	5	21
AMP	5	49
Pyridoxal 5′-phosphate	2	0
Iodoacetamide	1	14
5,5′-Dithiobis-2-nitrobenzoate	1	6*
N-Ethylmaleimide	1	0*

* The enzyme preparations in these two cases were desalted to eliminate DTT originating from the original purification buffer, which would react with 5,5′-dithiobis-2-nitrobenzoate or *N*-ethylmaleimide.

of phosphate with most multivalent cations (Good *et al.*, 1966), e.g. Mg^{2+}, which would decrease the concentration of $MgATP^{2-}$, the effective cofactor species for the enzyme activity. The pathway intermediates ACV and penicillin N and the final product cephamycin C did not show any feedback inhibition at the concentrations tested (Table 3). GSH, also a tripeptide containing L-cysteine and present in most types of cell, had no inhibitory effect on ACV synthetase activity. It should be noted that certain carbon source metabolites, e.g. glyceraldehyde 3-phosphate, strongly inhibit ACV synthetase activity due to a chemical reaction with the enzyme substrate L-cysteine (Zhang & Demain, 1992).

Effects of other compounds. Table 4 summarizes the effects of various additional compounds on ACV synthetase activity. The products of ATP utilization by ACV synthetase, pyrophosphate and AMP, were very inhibitory to the enzyme activity; this might explain the existence of an optimum ATP concentration. Thiol-blocking reagents such as *N*-ethylmaleimide, 5,5′-dithiobis-2-nitrobenzoate and iodoacetamide almost totally inhibited the activity at only 1 mM, indicating that the thiol group may play an important role in catalysis. In fact, ACV synthetase resembles most *Bacillus* non-ribosomal peptide synthetases which use the thiotemplate mechanism (Lipmann, 1980). The chelating agent EDTA slightly affected enzyme activity, most probably by sequestering Mg^{2+} which is required for catalysis. Pyridoxal 5′-phosphate was very inhibitory, and hydroxylamine, pantothenic acid, glycine, glutamate, methionine and D-valine had little or no effect. We have no explanation for the inhibition by pyridoxal 5′-phosphate.

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REFERENCES

Baldwin, J. E. & Bradley, M. (1990) Chem. Rev. **90**, 1079–1088
Baldwin, J. E., Adlington, R. M., Basak, A., Flitsch, S. L., Forrest, A. K. & Ting, H.-H. (1986*a*) J. Chem. Soc. Chem. Commun., 273–275
Baldwin, J. E., Adlington, R. M., Basak, A., Flitsch, S. L., Petursson, S., Turner, N. J. & Ting, H.-H. (1986*b*) J. Chem. Soc. Chem. Commun., 975–976
Baldwin, J. E., Killin, S. J., Pratt, A. J., Sutherland, J. D., Turner, N. J., Crabbe, M. J. C., Abraham, E. P. & Willis, A. C. (1987) J. Antibiot. **40**, 652–659
Baldwin, J. E., Norris, W. J., Freeman, R. T., Bradley, M., Adlington, R. M., Long-Fox, S. & Schofield, C. J. (1988) J. Chem. Soc. Chem. Commun., 1128–1130
Baldwin, J. E., Coates, J. B., Moloney, M. G., Shuttleworth, W. A. & Pratt, A. J. (1989) in Genetics and Molecular Biology of Industrial Microorganisms (Hershberger, C. L., Queener, S. W. & Hegeman, G., eds.), pp. 270–278, American Society for Microbiology, Washington
Baldwin, J. E., Bird, J. W., Field, R. A., O'Callaghan, N. M. & Schofield, C. J. (1990) J. Antibiot. **43**, 1055–1057
Banko, G., Wolfe, S. & Demain, A. L. (1986) Biochem. Biophys. Res. Commun. **137**, 528–535
Banko, G., Demain, A. L. & Wolfe, S. (1987) J. Am. Chem. Soc. **109**, 2858–2860
Bowers, R. J., Jensen, S. E., Lyubechansky, L., Westlake, D. W. S. & Wolfe, S. (1984) Biochem. Biophys. Res. Commun. **120**, 607–613
Bradford, M. M. (1976) Anal. Biochem. **12**, 248–254
Darnell, J., Lodish, H. & Baltimore, D. (1986) Molecular Cell Biology, pp. 31–34, Scientific American Books, New York
Enriquez, L. A. & Pisano, M. A. (1979) Antimicrob. Ag. Chemother. **16**, 392–397
Fromm. H. J. (1975) Initial Rate Enzyme Kinetics, pp. 51–56, Springer-Verlag, Berlin
Good, N. E., Winget, G. D., Winter, W., Connolly, T. N., Izawa, S. & Singh, R. M. M. (1966) Biochemistry **5**, 467–477
Jensen, S. E., Westlake, D. W. S. & Wolfe, S. (1988) FEMS Microbiol. Lett. **49**, 213–218
Kupka, J., Shen, Y.-Q., Wolfe, S. & Demain, A. L. (1983) Can. J. Microbiol. **29**, 488–496
Langer, R. S. (1974) Sc.D. Massachusetts Institute of Technology, Cambridge
Lara, F., Mateos, R. C., Vazquez, G. & Sanchez, S. (1982) Biochem. Biophys. Res. Commun. **105**, 172–178
Lipmann, F. (1980) Adv. Microbiol. Physiol. **21**, 227–266
Piret, J., Resendiz, B., Mahro, B., Zhang, J., Serpe, E., Romero, J., Connors, N. & Demain, A. L. (1990) Appl. Microbiol. Biotechnol. **32**, 560–567
Scopes, R. K. (1987) Protein Purification: Principles and Practice, pp. 253–259, Springer-Verlag, New York
Van Liempt, H., von Dohren, H. & Kleinkauf, H. (1989) J. Biol. Chem. **264**, 3680–3684

Wolfe, S., Hollander, I. J. & Demain, A. L. (1984) Bio/Technology **2**, 635–636
Wolfe, S., Lubbe, C., Jensen, S. E., Hernandez, H. & Demain, A. L. (1985) J. Antibiot. **38**, 1550–1554
Zhang, J. & Demain, A. L. (1990*a*) Biochem. Biophys. Res. Commun. **137**, 528–535
Zhang, J. & Demain, A. L. (1990*b*) Biotechnol. Lett. **12**, 649–654
Zhang, J. & Demain, A. L. (1992) Biotechnol. Adv., in the press

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