The Absolute Configuration of the Amino Acids in δ-(α-Aminoadipyl)cysteinylvaline from *Penicillium chrysogenum*†

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ABSTRACT: Radioactive carbon-14 L-α-aminoadipic acid, L-cysteine, or L-valine were readily incorporated into the intracellular tripeptide, δ-(α-aminoadipyl)cysteinylvaline (ACV), by washed starved cells of *Penicillium chrysogenum*. The labeled ACV in each case was oxidized with performic acid and isolated as its corresponding sulfonic acid derivative. After acid hydrolysis, the configuration of the component amino acids was determined by L- and D-amino acid oxidases, which showed the tripeptide (ACV) from *P. chrysogenum* to be δ-(L-aminoadipyl)-L-cysteinyl-D-valine.

A noncyclic peptide, commonly known as the Arnstein tripeptide, was first obtained from mycelia of *Penicillium chrysogenum* (Arnstein et al., 1959, 1960) and its structure was established to be δ-(α-aminoadipyl)cysteinylvaline (Arnstein and Morris, 1960) but the configurations of the constituent amino acids remained to be established. These results initiated the tripeptide theory for penicillin biosynthesis and the suggestion was made that the final stage in the biosynthesis of penicillin involved the substitution of the L-α-aminoadipyl side chain of isopenicillin N by acyl groups endogenously derived or exogenously supplied as acids or amides (Demain, 1966). Although the discovery of an enzyme catalyzing the acylation of 6-aminopenicillanic acid (6-APA)[1] by *S*-phenylacetyl-CoA (Brunner et al., 1968; Gatenbeck and Brunsberg, 1968; Spencer, 1968) suggests that various penicillins are formed by acylation of 6-APA rather than by direct substitution of the α-aminoadipyl side chain of isopenicillin N, the question as to whether 6-APA is produced from isopenicillin N by hydrolysis or is synthesized from the Arnstein tripeptide or via cysteinylvaline remains unanswered.

A peptide with the same electrophoretic mobility as the sulfonic acid of δ-(α-aminoadipyl)cysteinylvaline was isolated after oxidation from cells of *Cephalosporium acremonium*. The configurations of the component amino acids were determined, which showed this tripeptide to be δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) (Loder and Abraham, 1971). The α-aminoadipyl side chain of penicillin N from *C. acremonium* was determined to be of the D configuration (Newton and Abraham, 1953) whereas that of isopenicillin N from *P. chrysogenum* possessed the L configuration (Flynn et al., 1962). It was therefore of interest to examine the configuration of the amino acids in the Arnstein tripeptide, for it may render valuable information on the role of the tripeptide in 6-APA biosynthesis.

Experimental Section

Materials. L-Valine-*U*-^{14}C (255 Ci/mol) was a product of New England Nuclear; DL-2-aminoadipic-*1*-^{14}C acid (56 Ci/mol) and L-cysteine-*U*-^{14}C hydrochloride (29.6 Ci/mol) were purchased from Amersham-Searle. L-Amino acid oxidase (0.3 unit/mg, Type 1: crude venom from *Crotalus adamanteus*), D-amino acid oxidase (15 units/mg of protein, hog kidney), catalase (8000–70000 units/mg, beef liver) and flavine adenine dinucleotide disodium salt (FAD) were products of Sigma.

Methods. Paper chromatograms (Whatman No. 1 paper) were developed for 15 hr in the 1-butanol–acetic acid–water (4:1:4) system. Thin-layer chromatography (TLC) of α-ketoisovaleric acid was carried out on silica gel G plates (Brinkmann) using 1-butanol–acetic acid–water (4:1:4) as the developing system (R_f values: valine, 0.33; α-ketoisovaleric acid, 0.43). For α-ketoadipic acid, cellulose plates (EM, 0.1 mm Brinkmann) were used and were developed in the same solvent system (R_f values: α-aminoadipic acid, 0.13; α-ketoadipic acid, 0.46). Paper electrophoresis was performed on a Savant Instruments (Hicksville, N.J.) apparatus using Whatman No. 1 paper at 50 V/cm in 20% (v/v) acetic acid containing 2% formic acid (pH 1.8) (Smith et al., 1967). Radioactivity was assayed in a Packard Model 2002 scintillation spectrometer after addition of 10 ml of Bray's solution. Radiochromatogram was assayed on a Vanguard Model 930 auto-scanner.

Penicillium chrysogenum Wis-54 was grown in corn steep liquor medium for 72 hr (Batchelor et al., 1961). Harvested mycelium was washed well with cold water and pressed between filter papers until superficially dry.

To a suspension of the pressed mycelium (4 g) in 20 ml of oxygen-free distilled water, shaken on a rotary shaker (200 rpm/min) for 30 min at 25°C, was added L-[U-^{14}C]valine (10 μCi). After incubation for 15 min with shaking, the extracellular fluid was removed by centrifugation at 2000*g* for 5 min. The cells were washed twice with water and extracted with three 10-ml aliquots of 70% ethanol followed by three 5-ml aliquots of water. The combined ethanol and aqueous extracts were lyophilized. The residue was dissolved in 4 ml of water and the pH of the suspension was adjusted to 3 with 10% HCl. After removal of the insoluble material by centrifugation, the clear supernatant was applied onto a column (1 × 10 cm) of Dowex 50 (X4, H^+ form, 100–200 mesh) and the column was washed with cold water until the effluent was free of radioactivity. Amino acids and peptides were eluted off the column using 1 *M* aqueous pyridine and 8-ml fractions were collected. Fractions 4–6 were combined and freeze-dried. The dried resi-

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[1] Abbreviations used are: 6-APA, 6-aminopenicillanic acid; ACV, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine.

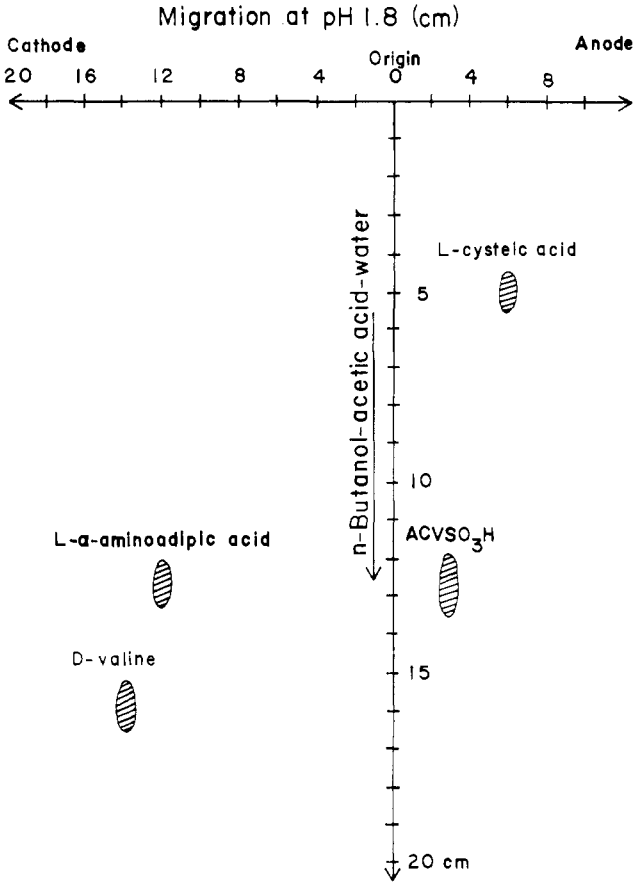

FIGURE 1: Two-dimensional electrophoresis chromatography of ACV-SO_3H, D-valine, L-α-aminoadipic acid, and L-cysteic acid.

due was oxidized with 2 ml of performic acid for 6 hr at −10°C, diluted with 20 ml of water, and again freeze-dried. This residue was dissolved in 2 ml of water and applied onto another Dowex 50 (X4, H^+ form, 100–200 mesh) column (1 × 5 cm). The column was washed with 15 ml of water and 2-ml fractions were collected. Fractions 2–4, containing the peptide sulfonic acids, were pooled, freeze-dried, and further purified by two-dimensional paper electrophoresis (75 min) and chromatography (15 hr) (Loder and Abraham, 1971). δ-(α-Aminoadipyl)cysteinylvalinesulfonic acid (ACV-SO_3H) migrated 3 cm toward the anode from the origin and 12 cm from the origin on the paper chromatogram. The ACV-SO_3H was eluted from the paper by three 5-ml extractions with water. After lyophilization, the residue was hydrolyzed with 2 ml of 6 *N* HCl for 14 hr at 105°C. The radioactive component amino acids (either valine, cysteic acid, or α-aminoadipic acid) were purified by two-dimensional paper electrophoresis (45 min) and paper chromatography (Figure 1). In two other separate similar incubation experiments, L-[^{14}C]cysteine (20 μCi) or DL-[1-^{14}C]-α-aminoadipic (40 μCi) acid were substituted for the L-[^{14}C]valine.

The absolute configuration of the amino acids derived from ACV-SO_3H was established by the stereospecific oxidation to their corresponding keto acids with L- and D-amino acids oxidases (Smith et al., 1967). (a) L-Amino acid oxidase. The reaction mixture contained either radioactive valine or α-aminoadipic acid, derived from ACV-SO_3H in 0.4 ml of water, 0.6 ml of 1 *M* KCl, 10 ng of L-amino acid oxidases, and 800 units of catalase and the total volume was adjusted to 2 ml with 0.1 *M* Tris buffer, (pH 8.3). After incubation for 5 hr at 37°C, the reaction mixture was acidified to pH 3.0 and extracted three times with 3-ml portions of ethyl acetate. The radioactivity of the ethyl acetate layer was assayed as a quantitative measure of the corresponding keto acids produced, whose purity is further confirmed chromatographically. The oxidation of cysteic acid (Meister et al., 1954) was carried out similarly except oxygen gas was continuously bubbled into the incubation mixture for 12 hr at 37°C and pH 7.4. The following procedure was used for the isolation of β-sulfopyruvic acid. The L-cysteic acid incubation mixture was placed onto a Dowex 50 column (1 × 5 cm) (X4, H^+ form, 100–200 mesh). The oxidation product was eluted from the column with three 5-ml portions of water, and the aqueous eluate was then lyophilized. The residue was dissolved in 40 μl of water and placed on Whatman No. 1 paper and analyzed by paper electrophoresis at pH 1.8 (1 hr). L-Cysteic acid migrated 6 cm whereas its radioactive oxidation product migrated 18 cm toward the anode, corresponding to the mobility of known β-sulfopyruvic acid. The radioactive β-sulfopyruvic acid and the residual cysteic acid were eluted from the paper using water and the radioactivities of the eluates were determined in the scintillation counter. (b) D-Amino acid oxidase. To the radioactive valine or α-aminoadipic acid in 0.2 ml of water was added 1 μmol of FAD, 800 units of catalase, and 30 μg of D-amino acid oxidase in a total volume of 2 ml of 0.1 *M* sodium pyrophosphate buffer (pH 8.3). After incubation for 5 hr at 37°C, the reaction mixture was worked up and assayed as described for L-amino acid oxidase incubations. For cysteic acid, oxygen gas was continuously bubbled into the reaction mixture for 12 hr.

Table I: The Incorporation of ^{14}C-Labeled Amino Acids into ACV.

Substrate	ACV-SO_3H (μCi)	Amino Acid from ACV-SO_3H Hydrolysis (μCi)
L-[^{14}C] Valine (10 μCi)	0.11	0.05
DL-α-Aminoadipic acid (40 μCi)	0.06	0.03
L-Cysteine (20 μCi)	0.17	0.08

Results

Washed cells of *P. chrysogenum* readily incorporated the radioactive amino acids, L-valine, L-cysteine, and DL-α-aminoadipic acid, into the intracellular sulfur-containing tripeptide, ACV (Table I). To facilitate its purification, ACV was first transformed into its corresponding sulfonic acid derivative (ACV-SO_3H) by oxidation with performic acid. Figure 1 shows that ACV-SO_3H is clearly separated from the component amino acids in the two- dimensional electrophoresis–chromatography system. In each incubation, ACV-SO_3H was the predominant labeled component when either one of the three component amino acids was exposed to *P. chrysogenum*. After elution of ACV-SO_3H from the paper, it was hydrolyzed into its component amino acids with 6 *N* HCl, which was again purified using two-dimensional electrophoresis–chromatography. Approximately 50% of the radioactivity of ACV-SO_3H was recovered in the desired amino acid (Table I).

Because only small quantities of ACV-SO_3H were obtained, it would be difficult to establish the absolute configuration of its component amino acids using conventional spectroscopic methods. We therefore elected to resolve this problem by taking advantage of the stereospecificity of L-

Table II: Stereospecific Oxidation of Valine and α-Aminoadipic Acid by L- and D-Amino Acid Oxidases.

	L-[^{14}C] Valine (Control)		D-[^{14}C] Valine (Control)		Valine (from ACV-SO_3H)		DL-α-Aminoadipic Acid (Control)		α-Aminoadipic (from ACV-SO_3H)	
	Amino Acid Oxidase (cpm)									
	L	D	L	D	L	D	L	D	L	D
EtOAc layer	30700	32	100	15600	320	15200	20200	200	20100	50
Aqueous layer	2100	32200	15900	900	16200	410	24000	45700	200	21000

Table III: Stereospecific Conversion of Cysteic Acid into β-Sulfopyruvic Acid.

	L-[^{14}C] Cysteic Acid (47000 cpm) (Control)		Cysteic Acid (46884 cpm) (from ACV-SO_3H)	
	Amino Acid Oxidase (cpm)			
	L	D	L	D
β-Sulfopyruvic acid	36710		35120	
Cysteic Acid		39210		38410

and D-amino acid oxidases. It is well documented that L- and D-amino acid oxidases readily oxidize L- and D-valine into α-ketoisovaleric acid (Smith et al., 1967); L- and D-cysteic acid into β-sulfopyruvic acid (Meister et al., 1954). While L-α-aminoadipic acid is transformed into α-ketoadipic acid by L-amino acid oxidases, D-α-aminoadipic acid is resistant to attack by D-α-amino acid oxidase (Smith et al., 1967). The resulting α-ketoisovaleric and α-ketoadipic acids may be conveniently separated from their parent amino acids since the keto acids are readily extractable into organic solvents such as ethyl acetate, whereas the hydrophilic amino acids remain in the aqueous phase. Thus, the amount of radioactivity residing in the organic layer may be taken as a quantitative measure of the concentration of α-keto acids.

A series of preliminary experiments were conducted to confirm the stereospecificity of the amino acid oxidases as well as the validity of this extraction procedure (Table II). After incubation of L-[^{14}C]valine with L-amino acid oxidase, it was found that major portions of the radioactivity were extracted into the organic phase, consistent with the formation of α-ketoisovaleric acid. This supposition was corroborated by thin-layer chromatographic analysis of the ethyl acetate layer, which showed only one radioactive peak corresponding in mobility to α-ketoisovaleric acid. As expected, D-amino acid oxidase did not oxidize L-[^{14}C]valine as the radioactivity remained in the aqueous phase. Complementary results were obtained when D-[^{14}C]valine was employed as the substrate. When DL-[^{14}C]-α-aminoadipic acid was exposed to L-amino acid oxidase, 50% of the radioactivity appeared in the organic phase upon extraction. Thin-layer chromatographic analysis of the ethyl acetate layer revealed a radioactive band with mobility corresponding to α-ketoadipic acid. No oxidation was observed with D-amino acid oxidase.

Having verified the analytical procedures, we then proceeded to establish the stereochemistry of the valine and α-aminoadipic acid samples, derived from acid hydrolysis of ACV-SO_3H. It was found (Table II) that only the D-amino oxidase oxidized the valine sample to α-ketoisovaleric acid and only the L-amino acid oxidase converted the α-aminoadipic acid into α-ketoadipic acid. These results clearly establish that the absolute configuration of the valine and α-aminoadipic acid in ACV is that of D and L, respectively.

The rate of oxidation of L-cysteic acid to β-sulfopyruvic acid, catalyzed by L-amino acid oxidase, may be enhanced by bubbling molecular oxygen into the reaction mixture continuously (Meister et al., 1954). As β-sulfopyruvic acid is highly polar and not separable from cysteic acid by extraction methods, these two compounds were isolated by means of paper electrophoresis. The results in Table III showed that the cysteic acid derived from ACV-SO_3H possessed the L configuration as it was converted into β-sulfopyruvic acid by L-amino acid oxidase but remained unchanged when exposed to D-amino acid oxidase.

Discussion

Our results clearly demonstrate that ACV of *P. chrysogenum* is δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine, identical with the ACV derived from *C. acremonium*. Initial support for the tripeptide theory was based on the observation that lysine inhibition of penicillin synthesis could be reversed by addition of α-aminoadipic acid to the *Penicillium* fermentation (Somerson et al., 1961). Also, there is convincing genetic evidence that α-aminoadipic acid is an obligatory intermediate for all penicillins by both *Penicillium* (Goulden and Chattaway, 1968) and *Cephalosporium* (Lemke and Nash, 1972). Recently, it was reported that ACV is a precursor to penicillin N as manifested by its incorporation into the latter β-lactam antibiotic by protoplast lysates of *C. acremonium* (Fawcett et al., 1975). Since the configuration of the α-aminoadipyl moiety of penicillin N is epimeric to that found in ACV, one could surmise that the α-aminoadipyl unit may participate in the cyclization step(s) leading to the penam nucleus; in the process there is an overall inversion of configuration from L to D. However, our finding that the α-aminoadipyl moiety of ACV in *P. chrysogenum* possesses the same L configuration as isopenicillin N tends to favor the view that no obligatory inversion of the α-aminoadipyl moiety is needed during the construction of the penam nucleus.

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Chromatin-Bound Protease: Degradation of Chromosomal Proteins under Chromatin Dissociation Conditions†

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ABSTRACT: A chromatin-bound protease, active in 2 *M* NaCl–5 *M* urea or 5 *M* urea alone, was demonstrated in rat liver, thymus, kidney, testes, brain, rabbit bone marrow, chicken reticulocyte, and Ehrlich ascites chromatin. Chicken erythrocyte chromatin did not possess any detectable proteolytic activity in salt and urea. The proteolytic activity of rat liver chromatin in salt and urea was found to be independent of the methods of chromatin preparation. The protease can be inhibited by the serine specific reagents phenylmethanesulfonyl fluoride and diisopropyl fluorophosphate and the alkylating reagent, carbobenzoxyphenylalanine chloromethyl ketone, in the presence of organic solvents at 1 m*M* concentration. The inhibitions of chromatin-bound protease in rat liver by these compounds are irreversible. On the other hand, carbobenzoxyphenylalanine and *p*-nitrophenyl acetate were shown to be reversible inhibitors of rat liver chromatin-bound protease. The application of these inhibitors during the dissociation of chromatin by salt and urea may be useful to researchers interested in purifying various chromosomal proteins or to those researchers doing reconstitution studies with labile chromatins.

It has been shown previously that chromatin from calf thymus and rat liver tissue contains a proteolytic activity which degrades histones as well as non-histone proteins (Furlan and Jericijo, 1967a,b; Furlan et al., 1968; Bartley and Chalkley, 1970; Garrels et al., 1972; Kurecki and Toczko, 1972, 1974; Chae and Carter, 1974). Furlan and coinvestigators and Kurecki and Toczko have established that the calf thymus chromatin-bound proteolytic activity resides in a protein which has a molecular weight of between 16000 and 24000, a pH optimum of 7.8–8.5, and which is insensitive to thiol-blocking and chelating reagents. Furlan and Jericijo (1967b) report that the calf thymus protease is active in the presence of high salt concentrations (>1 *M* NaCl). Chong et al. (1974) have isolated a high molecular weight protease from rat liver chromatin prepared from whole tissue which is sensitive to sulfhydryl and chelating reagents and is inactive in salt concentrations greater than 1 *M* NaCl. These data suggest that a chromatin-bound protease may be a normal constituent of animal chromatin.

That a protease does exist in rat liver chromatin which is active in the presence of 2 *M* NaCl–5 *M* urea (pH 6–8) was reported by Chae and Carter (1974). These observations are significant for those investigators interested in studying the structure and reconstitution of chromatin, since chromatin generally has been dissociated in high salt and urea (Bekhor et al., 1969; Huang and Huang, 1969; Gilmour and Paul, 1969; Stein et al., 1972). Other investigators have dissociated chromatin in 2 *M* NaCl–5 *M* urea or 3 *M* NaCl in order to fractionate non-histone proteins (Gilmour and Paul, 1970; MacGillivray et al., 1972; Richter and Sekeris, 1972; van den Broek et al., 1973).

This investigation characterizes the chromatin-bound proteolytic activity of rat liver in the presence of denaturing solvents commonly used for dissociation of chromatin and establishes techniques for inhibition of the protease. Proteolytic activities of chromatin from tissues other than rat liver are also reported.

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