Substrate Specificity of an α -Amino Acid Ester Hydrolase Produced by *Acetobacter turbidans* A.T.C.C. 9325

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A partially purified preparation of an α -amino acid ester hydrolase was obtained from *Acetobacter turbidans* A.T.C.C. 9325, which catalyses synthesis of 7-(D - α -amino- α -phenylacetamido)-3-cephem-3-methyl-4-carboxylic acid (cephalexin) from methyl D - α -aminophenylacetate and 7-amino-3-deacetoxycephalosporanic acid. The enzyme preparation catalysed both cephalosporin synthesis from 7-amino-3-deacetoxycephalosporanic acid and suitable amino acid esters (e.g. methyl D - α -aminophenylacetate, L-cysteine methyl ester, glycine ethyl ester, D-alanine methyl ester, methyl DL- α -aminoisobutyrate, L-serine methyl ester, D-leucine methyl ester, L-methionine methyl ester) and the hydrolysis of such esters. The substrate specificity of the enzyme preparation for the hydrolysis closely paralleled the acyl-donor specificity for cephalosporin synthesis, even to the reaction rates. Only α -amino acid derivatives could act as acyl donors. The hydrogen atom on the α -carbon atom was not always required by acyl donors. The hydrolysis rate was markedly diminished by adding 7-amino-3-deacetoxycephalosporanic acid to reaction mixtures, but no effect on the total reaction rate (the hydrolysis rate plus synthesis rate) was observed with various concentrations of 7-amino-3-deacetoxycephalosporanic acid. Both the hydrolytic and the synthetic activities of the enzyme preparation were inhibited by high concentrations of some acyl donors (e.g. methyl D - α -aminophenylacetate, ethyl D - α -aminophenylacetate). The enzyme preparation hydrolysed α -amino acid esters much more easily than α -amino acid derivatives with an acid–amide bond.

Some bacteria belonging to the *Pseudomonadaceae* catalyse *N*-acylation of 7-aminocephem compounds such as 7-amino-3-deacetoxycephalosporanic acid and 7-aminocephalosporanic acid with α -amino acid esters to give the corresponding semi-synthetic cephalosporins (Takahashi *et al.*, 1972). The catalytic properties of a cephalosporin-synthesizing enzyme of *Acetobacter turbidans* A.T.C.C. 9325, one of the bacteria previously studied, is presented here.

The partially purified preparation of the *Acetobacter* enzyme catalysed hydrolysis of α -amino acid esters as well as cephalosporin synthesis from 7-amino-3-deacetoxycephalosporanic acid and such α -amino acid esters. Examination of substrate specificity for both the reactions suggests that the cephalosporin-synthesizing enzyme should be classified as an α -amino acid ester hydrolase. It is well known that some esterases, e.g. ox liver esterase, acetylcholinesterase and acid and alkaline phosphatases, catalyse acyl-transfer reactions, and this is also the case with α -amino acid ester hydrolase. Thus the cephalosporin-synthesizing reaction catalysed by *Acetobacter* α -amino acid ester hydrolase is regarded as an acyl-transfer reaction, the acyl donor and acceptor of which are an α -amino acid ester and a 7-aminocephem compound respectively. In the present paper

the cephalosporin-synthesizing reaction and the substrates used are referred to as an acyl-transfer reaction and as an acyl donor or acceptor, depending on its role in the reaction. Since hydrolysis itself could be regarded as a transfer of the acyl group to water, substrates for the hydrolytic reaction are also referred to as acyl donors.

Materials and Methods

Substrates

Methyl DL- α -hydrazinophenylacetate, methyl DL- α -aminoxiphenylacetate, ethyl α -ethoxycarbonylphenylacetate, methyl α -sulphophenylacetate, D- α -aminophenylacetyl-glycine, ampicillin [6-(D - α -amino- α -phenylacetamido)penicillanic acid], 6-aminopenicillanic acid and 7-amino-3-deacetoxycephalosporanic acid were supplied by the Chemical Research Laboratories, Takeda Chemical Industries Ltd., Osaka, Japan. Ethyl phenylacetate, ethyl phenylglyoxylate, ethyl carbamate, glycine ethyl ester, *N*-acetyl-glycine ethyl ester, L-cysteine methyl ester and L-leucine methyl ester were purchased from Sigma Chemical Company, St. Louis, Mo., U.S.A., and Tokyo Kasei Kogyo Co., Kita-ku, Tokyo, Japan.

Other esters were prepared in this laboratory from the corresponding commercially available acids by the conventional method (Kupryszewski & Sokolowska, 1957). All the amino acid esters used were hydrochloride salts. D- α -Aminophenylacetic acid hydroxamate was prepared from methyl D- α -aminophenylacetate by the method of Lewis (1926). D- α -Aminophenylacetamide was prepared by the conventional method (Smith & Slonim, 1948). Cephalixin [7-(D- α -amino- α -phenylacetamido)-3-deacetoxycephalosporanic acid] was prepared as described by Takahashi *et al.* (1972).

Cell preparation

A medium (100 litres) containing meat extract (Wako Pure Chemical Industries Ltd., Higashi-ku, Osaka, Japan; 1% w/v), peptone (Takeda Chemical Industries Ltd., Higashi-ku, Osaka, Japan; 1% w/v), glucose (2% w/v), glycerol (3% w/v), NaCl (0.5%) and antifoam (Antiflos; Daiichi Seiyaku Kogyo, Shimogyo-ku, Kyoto, Japan; 0.005%) was sterilized in a 200-litre fermentor, inoculated with 2.5 litres of a 16h shaken-flask culture of *Acetobacter turbidans* A.T.C.C. 9325 prepared as described previously (Takahashi *et al.*, 1972) and stirred at 170 rev./min with an air flow of 50 litres/min at 28°C. During cultivation, the pH of the medium was controlled at 6.0 by the addition of 5M-NaOH. After 40h of growth the cells (about 1.4kg wet wt.) were collected by passing the culture through a Sharples centrifuge.

Enzyme preparation

All subsequent operations were carried out at 5°C. The cells of *A. turbidans* A.T.C.C. 9325 were suspended in 0.1M-Tris-HCl buffer (pH 8.0) to give a final volume of 5 litres and disrupted by passage through a Sorvall Ribi Cell Fractionator model RF-1 at 1.02×10^8 Pa. To the viscous fluid obtained were added 3mg of crystalline deoxyribonuclease I (Sigma) and 6.25g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and the mixture was stirred for 2h. The resulting fluid was centrifuged at 11600g for 20min and the supernatant (fraction 1) retained was the crude extract. Solid calcium acetate (106g) was slowly dissolved in the crude extract, to which 87g of K_2HPO_4 was added as an aqueous solution (500ml). The mixture was stirred for 2h and centrifuged at 11600g for 15min, giving 4.4 litres of supernatant (fraction 2). Solid $(\text{NH}_4)_2\text{SO}_4$ (2075g) was added to the supernatant to give a 70% saturated solution and the mixture was stirred for 2h. The precipitate was collected by centrifuging at 11600g for 20min and dissolved in 500ml of cold water. The solution was dialysed against deionized water (100 litres) overnight and the precipitate that formed was removed by centrifuging at 11600g for 20min and discarded. The supernatant (fraction

3) was mixed with 250g of DEAE-cellulose (Whatman DE-11; W. and R. Balston Ltd., Maidstone, Kent, U.K.), previously equilibrated with 0.01M- K_2HPO_4 - KH_2PO_4 buffer (pH 6.8). The suspension was stirred for 30min, filtered and the residual pellet washed with 1 litre of 0.1M- K_2HPO_4 - KH_2PO_4 buffer (pH 6.8). The filtrate and washings were combined and adjusted to pH 6.0 with 5% (v/v) acetic acid to give fraction 4. This fraction was applied to a CM-cellulose (Whatman CM-11) column (5cm $\times$ 100cm; bed vol. 1.8 litres) previously equilibrated with 0.01M- K_2HPO_4 - KH_2PO_4 buffer (pH 6.0), and washed with 4 litres of the same buffer. The enzyme was then eluted with 4 litres of 0.01M- K_2HPO_4 - KH_2PO_4 buffer (pH 6.0) containing 0.05M-NaCl and the fractions with a high specific activity of the enzyme were pooled. The active fraction was dialysed against deionized water (45 litres) overnight (fraction 5), centrifuged at 11600g for 5min to remove insoluble materials and freeze-dried to give 1.4g of powder. The powder was dissolved in 1.4 litres of 0.05M- K_2HPO_4 - KH_2PO_4 buffer (pH 6.0) and centrifuged at 11600g for 5min to remove undissolved materials, giving the final enzyme preparation used in this paper (fraction 6).

Enzyme assay

The following assay method was used during the partial purification of the enzyme. The reaction mixture containing methyl D- α -aminophenylacetate (20mM) and enzyme (0.2–1.0 unit/ml) was incubated at 27°C and acid release followed at pH 6.0 (the optimum pH of the enzyme) with a Radiometer TTT1C titrator. A gentle stream of N_2 was passed over the surface. The titrant was 5mM-NaOH. The observed rate of acid liberation was multiplied by 1.22 to correct for the difference in ionization constant of the amino groups of methyl D- α -aminophenylacetate and D- α -aminophenylacetic acid. One unit of activity was defined as the amount of enzyme required to hydrolyse 1 μmol of methyl D- α -aminophenylacetate/min under the above standard conditions.

Determination of reaction rates

Hydrolysis of carboxylic acid esters was carried out at pH 6.0 and 27°C in a 5ml reaction mixture containing 0.1–50mM-acyl donor and 0.3–2.5 units of enzyme/ml with a Radiometer TTT1C titrator, a stream of N_2 being passed over the surface. The titrant was 2–5mM-NaOH. For amino acid esters, the observed rate of acid liberation was corrected for the difference in ionization constant of an amino group between an ester and the corresponding acid. Hydrolysis of compounds other than the esters was carried out at 27°C in a reaction mixture containing 0.2M- K_2HPO_4 - KH_2PO_4 (pH 6.0), 0.1–40mM-acyl

donor and 0.3–2.5 units of enzyme/ml and was terminated by adding the colorimetric reagents. A reaction time of 5 or 10 min was usually used. The enzyme concentration was chosen to ensure that the product formed in this time was 10% or less of the theoretical yield. These reaction conditions were also applied to ester hydrolysis carried out in a buffer solution. Determination of 7-amino-3-deacetoxycephalosporanic acid and 6-aminopenicillanic acid, products of hydrolysis of cephalixin and ampicillin respectively, was done by a colorimetric method (Marrelli, 1968). Amino acids produced by hydrolysis of α -amino acid derivatives were determined by the ninhydrin method (Yemm & Cocking, 1955). For hydrolysis of D- α -aminophenylacetic acid hydroxamate, the remaining substrate was measured by the colorimetric method of Lipmann & Tuttle (1945).

The transfer reaction of acyl groups from acyl donors to 7-aminocephalosporanic acid was carried out at 27°C in a 2 ml reaction mixture containing 0.2 M-K₂HPO₄-KH₂PO₄ (pH 6.0), 20–60 mM-acyl donor, 20 mM-7-amino-3-deacetoxycephalosporanic acid and 0.3–2.5 units of enzyme/ml. After 5 min incubation, a sample (0.1 ml) of the reaction mixture was removed and diluted 100-fold with cold 0.05 M-Tris-HCl buffer (pH 8.0). A portion (2.5 ml) of the diluted solution was taken out and the E_{260} was measured with a Hitachi 181 spectrophotometer. β -Lactamase solution (20 μ l) was then added to this solution and the mixture incubated at 25°C for 2 min, resulting in decrease of the E_{260} . From the difference in E_{260} before and after the β -lactamase treatment, the concentration of the product was calculated with cephalixin as a standard. The preparation of β -lactamase was supplied by Mr. H. Shirafuji who has selected a potent, β -lactamase producing strain of *Aerobacter cloacae* I.F.O. 12937 and has obtained a partially purified preparation of the enzyme capable of hydrolysing the β -lactam ring of cephalosporins, by means of ultrasonic irradiation, followed by CM-cellulose column chromatography (personal communication from H. Shirafuji of the Microbiological Research Laboratories, Takeda Chemical Industries

Ltd.). The β -lactamase solution used in the present work showed a specific activity of 90 units/mg of protein. One unit of activity was defined as the amount of enzyme required to hydrolyse 1 μ mol of sodium cephalosporin C/min at pH 8.0 and 25°C. The preparation of β -lactamase hardly hydrolysed 7-amino-3-deacetoxycephalosporanic acid as well as other 7-aminocephem compounds under similar conditions and was utilized for selective hydrolysis of the product (a cephalosporin) in the presence of 7-amino-3-deacetoxycephalosporanic acid (Takahashi *et al.*, 1972).

Determination of protein

The method of Lowry *et al.* (1951) was used with bovine serum albumin (Sigma) as a standard.

Results

Preparation and activity of the partially purified component of the enzyme

Table 1 summarizes the partial purification of α -amino acid ester hydrolase of *Acetobacter turbidans* A.T.C.C. 9325. Incubation of the enzyme (the final preparation) with methyl D- α -aminophenylacetate and 7-amino-3-deacetoxycephalosporanic acid led to synthesis of cephalixin in good yield. A typical example is shown in Fig. 1. Besides cephalixin, D- α -aminophenylacetic acid invariably appeared in the reaction mixtures (Fig. 1) and it soon became apparent that the enzyme preparation readily hydrolysed methyl D- α -aminophenylacetate itself, though cephalixin was also slowly hydrolysed by the enzyme to give D- α -aminophenylacetic acid and 7-amino-3-deacetoxycephalosporanic acid. In the absence of 7-amino-3-deacetoxycephalosporanic acid, the hydrolysis rate of the ester was even faster than that of cephalixin synthesis (a transfer of D- α -aminophenylacetyl group from methyl D- α -aminophenylacetate to 7-amino-3-deacetoxycephalosporanic acid) under similar conditions. The optimum pH value (6.0) for the hydrolytic reactions (of the ester and

Table 1. Partial purification of α -amino acid ester hydrolase of *Acetobacter turbidans* A.T.C.C. 9325

Experimental details are given in the Materials and Methods section.

Fraction no.	Stage of purification	Volume (litres)	Total protein (g)	$10^{-3} \times$ Total activity (units)	Specific activity (units/mg of protein)	Recovery (%)	Purification
1	Crude extract	4.80	147	288	1.96	100	1
2	Calcium phosphate gel treatment	4.40	80.1	222	2.77	77	1.4
3	70%-satd. (NH ₄) ₂ SO ₄ fractionation	0.96	68.5	220	3.21	76	1.6
4	DEAE-cellulose treatment	1.90	22.0	223	10.1	78	5.2
5	CM-cellulose column	0.80	2.0	126	63.2	44	32.2
6	Final enzyme solution	0.20	1.31	122	93.5	43	47.6

Fig. 1. Formation of cephalixin and D- α -aminophenylacetic acid from methyl D- α -aminophenylacetate and 7-amino-3-deacetoxycephalosporanic acid by α -amino acid ester hydrolase of *Acetobacter turbidans* A.T.C.C. 9325

A reaction mixture containing 50mM-methyl D- α -aminophenylacetate, 20mM-7-amino-3-deacetoxycephalosporanic acid and 0.3 unit of enzyme/ml was incubated at 27°C and pH 6.0 with a Radiometer TTT1C titrator, a stream of N₂ being passed over the surface. A portion (100 μ l) of the reaction mixture was removed for determination of the cephalixin concentration at a given reaction time. Cephalixin and D- α -aminophenylacetic acid were determined as described in the text. ○, Cephalixin concentration; ●, D- α -aminophenylacetic acid concentration.

cephalexin) was the same as that for the transfer reaction.

Substrate specificity of the enzyme

The hydrolytic and transfer activities of the enzyme were examined with various carboxylic acid derivatives as acyl donor and 7-amino-3-deacetoxycephalosporanic acid as acyl acceptor. The results are given in Table 2, which shows that the substrate specificity of the enzyme for the hydrolysis of acyl donors closely paralleled, even to the reaction rates, that for the transfer of the acyl group from the acyl donors to 7-amino-3-deacetoxycephalosporanic acid to give the corresponding cephalosporins. To clarify the catalytic properties of the enzyme, the compounds tested for acyl-donor activity were divided into three groups: I, compounds closely related to α -aminophenylacetate; II, various amino acids; and III, derivatives of α -aminophenylacetate. From comparison of the sensitivity of group I compounds to the enzyme, it is clear that the substrate must have a free amino group. Further evidence was obtained that the essential amino group of the substrate must be located on the α -carbon atom of the acyl groups from the experiments with group II compounds. In spite of the strict requirement for a free α -amino group,

the enzyme showed rather broad specificity for the configuration of the α -amino group. Methyl α -aminophenylacetate, however, exceptionally displayed a pronounced difference in reaction rate between D and L enantiomorphs. Thus of the α -amino acid esters examined, methyl D- α -aminophenylacetate gave the highest V value (88.8 μ mol/min per mg of protein) and its L isomer the lowest (1.2 μ mol/min per mg of protein) in the hydrolytic reaction. L-Cysteine methyl ester was the second best substrate. Methyl DL- α -aminoisobutyrate was also a good substrate for the enzyme, indicating that a hydrogen atom on the α -carbon atom is not necessarily essential for substrate activity. Esters of various carboxylic acids having no amino group, such as ethyl acetate, ethyl propionate, ethyl *n*-butyrate, ethyl benzoate and *p*-nitrophenyl acetate were also examined and found not to be substrates. The results with group III compounds clearly show that the enzyme hydrolysed ester bonds much more easily than the acid-amide linkages. Of the compounds having an acid-amide bond to be split, cephalixin was the best substrate, the hydrolysis proceeding at a rate only one-sixth that for methyl D- α -aminophenylacetate. Ampicillin was also hydrolysed, but at one-seventh the rate for cephalixin.

Substrate inhibition

Hydrolysis of acyl donors such as methyl D- α -aminophenylacetate, ethyl D- α -aminophenylacetate and ampicillin was inhibited by high substrate concentrations, whereas activation of hydrolysis by excess of substrate was observed with ethyl DL- α -aminobutyrate and L-leucine methyl ester as shown in Table 2.

Substrate inhibition of the transferring activity of the enzyme was also observed with each acyl donor that exhibited either substrate inhibition or substrate activation against the hydrolytic activity. In addition, L-valine methyl ester, L-S-benzylcysteine ethyl ester and *n*-butyl D- α -aminophenylacetate inhibited the respective transfer reactions at 60mM. These esters did not exert any influence on the hydrolytic reactions at a concentration of 20mM but may affect the rate at a higher concentration.

Nomenclature of the enzyme

In view of these catalytic properties, it appears appropriate to designate the enzyme as α -amino acid ester hydrolase. The alternative name, α -amino acid ester-7-aminocephem α -aminoacyltransferase, even though it has the advantage of referring to both the substrates, seems to be improper, since enzymes that can catalyse both hydrolysis and transfer are classified not as transferases but as hydrolases according to the enzyme nomenclature (Rule 22)

Table 2. *Substrate specificity of α -amino acid ester hydrolase*

Hydrolysis of esters was carried out at pH 6.0 and 27°C in a 5ml reaction mixture containing 0.1–50mM-acyl donor and 0.3–2.5 units of enzyme/ml with a Radiometer TTT1C titrator, a N₂ stream being passed over the surface. The titrant was 2–5mM-NaOH. For amino acid esters the observed rate of acid liberation was corrected for the difference in ionization constant of an amino group between an ester and the corresponding acid. Hydrolysis of compounds other than esters was carried out at 27°C in a reaction mixture containing 0.2M-K₂HPO₄-KH₂PO₄ (pH 6.0), 0.1–40mM-acyl donor and 0.3–2.5 units of enzyme/ml. Transfer reaction was carried out at 27°C in a 2ml reaction mixture containing 0.2M-K₂HPO₄-KH₂PO₄ (pH 6.0), 20 or 60mM-acyl donor, 20mM-7-amino-3-deacetoxycephalosporanic acid and 0.3–2.5 units of enzyme/ml. The rates of these reactions were determined as described in the Materials and Methods section. The maximum velocity (*V*) and Michaelis constant (*K_m*) for hydrolysis of acyl donors were obtained from double-reciprocal plots (Lineweaver & Burk, 1934) by using the method of least squares. Determination of *V* and *K_m* values in the transfer reaction was difficult because of substrate inhibition displayed by some acyl donors and low solubility of 7-amino-3-deacetoxycephalosporanic acid. Blank spaces in the columns indicate that no determinations were done.

Group	Compound (used as acyl donor)	Hydrolysis of acyl donor			Transfer of acyl group from acyl donor to 7-amino-3-deacetoxycephalosporanic acid: initial rate (μ mol/min per mg of protein) at the concn. of acyl donor	
		<i>V</i> (μ mol/min per mg of protein)	<i>K_m</i> (mM)	Substrate inhibition or activation	20 mM	60 mM
I	Ethyl phenylacetate	0.0			0.0	0.0
	Ethyl phenylglyoxylate	0.0			0.0	0.0
	Methyl DL- α -hydroxyphenylacetate	0.0			0.0	0.0
	Methyl DL- α -aminooxyphenylacetate	0.0			0.0	0.0
	Methyl DL- α -hydrazinophenylacetate	0.0			0.0	0.0
	Ethyl α -ethoxycarbonylphenylacetate	0.0			0.0	0.0
	Methyl DL- α -sulphophenylacetate	0.0			0.0	0.0
	Methyl D- α -aminophenylacetate	88.8	4.3	Inh.* (>40mM)	60.6	53.8
	Methyl L- α -aminophenylacetate	1.2	24.0	—† (<20mM)	0.2	0.3
	Methyl N-acetyl-D- α -aminophenylacetate	0.0			0.0	0.0
II	Ethyl carbamate	0.0			0.0	0.0
	Glycine ethyl ester	21.8	5.0	— (<20mM)	3.8	7.2
	N-Acetylglycine ethyl ester	0.0			0.0	0.0
	D-Alanine methyl ester	18.8	40.9	— (<50mM)	1.8	3.7
	β -Alanine methyl ester	0.0			0.0	0.0
	Methyl DL- α -aminoisobutyrate	18.6	32.2	— (<20mM)	3.4	4.7
	Ethyl DL- α -amino- <i>n</i> -butyrate	17.2	2.6	Act.‡ (>5mM)	3.6	3.1
	Methyl γ -amino- <i>n</i> -butyrate	0.0			0.0	0.0
	L-Valine methyl ester	3.8	0.8	— (<20mM)	2.3	2.1
	D-Leucine methyl ester	8.4	3.3	— (<20mM)	7.3	9.8
	L-Leucine methyl ester	2.6	3.6	Act. (>10mM)	3.4	3.1
	L-Serine methyl ester	14.2	12.2	— (<20mM)	3.4	5.3
	L-Cysteine methyl ester	47.3	1.2	— (<20mM)	35.3	36.5
	L-S-Benzylcysteine ethyl ester	8.2	0.5	— (<1mM)	1.7	1.1
	D-Methionine methyl ester	5.6	9.8	— (<20mM)	0.7	0.7
	L-Methionine methyl ester	10.0	1.0	— (<20mM)	3.8	5.2
III	Ethyl D- α -aminophenylacetate	53.3	5.6	Inh. (>20mM)	37.0	31.0
	<i>n</i> -Propyl D- α -aminophenylacetate	42.4	6.1	— (<20mM)	27.6	25.6
	Isopropyl D- α -aminophenylacetate	14.9	5.9	— (<20mM)		
	<i>n</i> -Butyl D- α -aminophenylacetate	78.9	3.2	— (<20mM)	54.6	44.2
	D- α -Aminophenylacetamide	<0.1			<0.1	<0.1
	D- α -Aminophenylacetic acid hydroxamate	6.0				0.5
	D- α -Aminophenylacetyl glycine	<0.1			<0.1	<0.1
	Ampicillin	2.1	0.6	Inh. (>2mM)	<0.1	<0.1
	Cephalexin	14.7	3.7	— (<20mM)		

* Inhibition by acyl donor.

† No effect was observed.

‡ Activation by acyl donor.

Table 3. Comparison of specific activities for hydrolytic and transfer reactions of α -amino acid ester hydrolase preparations at different purification stages

All reactions were carried out at 27°C and pH 6.0. Hydrolysis of methyl D- α -aminophenylacetate was performed in a 10ml reaction mixture containing 20mM-methyl D- α -aminophenylacetate and about 0.2 unit of enzyme/ml with a Radiometer TTT1C titrator. Hydrolysis of cephalixin was carried out in a 1.0ml reaction mixture containing 0.1 M-K₂HPO₄-KH₂PO₄ (pH 6.0), 20mM-cephalexin and about 0.3 unit of enzyme/ml and terminated by addition of 1 ml of 10% (v/v) formic acid after 20min incubation. Cephalixin synthesis, which was chosen as a typical acyl-transferring reaction, was carried out in a 8ml reaction mixture containing 0.1M-K₂HPO₄-KH₂PO₄ (pH 6.0), 20mM-7-amino-3-deacetoxycephalosporanic acid, 40mM-methyl D- α -aminophenylacetate and about 0.5 unit of enzyme/ml. The reaction rates were determined as described in the text and specific activities for each reaction with relative activities in parentheses are shown in the table.

Enzyme preparation	Specific activity (μ mol of product/min per mg of protein) for		
	Hydrolysis of		Transfer (synthesis of cephalixin)
	Methyl D- α -aminophenylacetate	Cephalixin	
Crude extract (fraction 1)	1.93 (100)	0.37 (19)	1.10 (57)
Partially purified preparation (fraction 6)	90.2 (100)	16.4 (18)	45.6 (51)

Fig. 2. Inhibitory effect of 7-amino-3-deacetoxycephalosporanic acid on hydrolysis of methyl D- α -aminophenylacetate catalysed by α -amino acid ester hydrolase of *Acetobacter turbidans* A.T.C.C. 9325

Reaction mixtures containing 40mM-methyl D- α -aminophenylacetate, various concentrations of 7-amino-3-deacetoxycephalosporanic acid as indicated and 0.7 unit of enzyme/ml were incubated at 27°C and pH 6.0 in a Radiometer TTT1C titrator with 5mM-NaOH as a titrant. Initial rates of acid liberation (V_H , ●) and cephalixin formation (V_S , ○) were determined as described in the text. The total reaction rate (□) calculated from V_H and V_S is also shown.

recommended by the Enzyme Commission of the International Union of Biochemistry (1965).

Some evidence for a single enzyme capable of catalysing both hydrolysis and transfer

It is the primary purpose of the present paper to show that the *Acetobacter* enzyme capable of cata-

lysing cephalosporin synthesis is a kind of esterase. However, there is a possibility that the ester-hydrolysing and acyl-transferring activities are due to two separate enzymes. This appears unlikely because both the activities showed the same strict specificity for the acyl donors as well as the same optimum pH value and were influenced by some acyl donors at high concentrations. The activity ratio between the two also did not change during the partial purification procedures (Table 3). Further support was given to this hypothesis by an experiment in which the effect of 7-amino-3-deacetoxycephalosporanic acid on the hydrolysis rate of methyl D- α -aminophenylacetate by the enzyme was determined. As is shown in Fig. 2, addition of various amounts of 7-amino-3-deacetoxycephalosporanic acid to a reaction mixture containing the enzyme and a large excess of the ester resulted in a profound diminution in the rate of hydrolysis of the ester. There was a concomitant formation of cephalixin but no change in the total rate of ester consumption (the hydrolysis rate of the ester plus the formation rate of cephalixin). This observation appears to be difficult to explain on the hypothesis of two separate enzymes. It can be most satisfactorily explained by the following acyl-enzyme hypothesis, which was originally proposed by Wilson *et al.* (1950) for acetylcholinesterase and was supported by numerous experiments with carboxylesterases (Adler & Kistiakowsky, 1962; Stoops *et al.*, 1969; Goldberg & Fruton, 1970; Greenzaid & Jencks, 1971) and with α -chymotrypsin (Bender & Kezdy, 1964, and the reference cited therein): (a) a single enzyme catalysed both the reactions, which proceeded through a common acyl-enzyme intermediate; (b) the intermediate was then subjected to a nucleophilic attack by either a water or a 7-amino-3-deacetoxycephalosporanic acid molecule, resulting in formation of D- α -aminophenylacetic acid or cephalixin; and (c) the rate-

determining step of both the reactions was acylation of the enzyme. According to the hypothesis, a ratio of hydrolysis/transfer rate should depend on the molar ratio of water to 7-amino-3-deacetoxycephalosporanic acid and should not vary with the ester moiety of a given acyl donor under the same reaction conditions. As shown in Table 2, this appears to be the case with D- α -aminophenylacetic acid esters. The ratio hardly changed from methyl ester to *n*-butyl ester. The additive rule shown in Fig. 2 was also observed with *n*-butyl D- α -aminophenylacetate.

In addition, it is noteworthy that another α -amino acid ester hydrolase having both the ester-hydrolysing and acyl-transferring activities with a similar activity ratio to that of *Acetobacter* enzyme was recently obtained from *Xanthomonas citri* I.F.O. 3835 (T. Takahashi, K. Kato & Y. Yamazaki, unpublished work).

Discussion

Hydrolysis of α -amino acid esters is effected by some hydrolases with wide specificities, such as ox liver esterase (Goldberg & Fruton, 1969), leucine aminopeptidase (Shippey & Binkley, 1958), trypsin (Dixon & Webb, 1964) and *Escherichia coli* penicillin amidase (Kaufmann & Bauer, 1964), from which the *Acetobacter* enzyme is very different in substrate specificity.

Kaufmann *et al.* (1960) reported the transfer of the phenylacetyl group from various phenylacetic acid derivatives to 6-aminopenicillanic acid by penicillin amidase bound to *E. coli* cells. Besides 6-aminopenicillanic acid, glycine and hydroxylamine have also been found to be active as acyl acceptors for the cell-bound enzyme (Cole, 1969). On the other hand, penicillin acyltransferase, which possessed no penicillin amidase activity, has been obtained from *Penicillium chrysogenum* (Pruess & Johnson, 1967; Spencer, 1968). It is particularly notable that the *Penicillium* enzyme catalyses the transfer of phenylacetyl group from benzylpenicillin or *S*-phenylacetyl-CoA to 6-aminopenicillanic acid, penicic acid amide or amino acid esters, but not to 7-aminocephalosporanic acid, 7-aminodeacetylcephalosporanic acid, amino acids or ethanol. Our preliminary experiments indicate that 7-amino-3-deacetoxycephalosporanic acid esters, 6-aminopenicillanic acid and methanol are also active as acyl acceptors for *Acetobacter* α -amino acid ester hydrolase.

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