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Purification and Properties of Acyltransferases from *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa, unlike other pseudomonads, possesses the ability to hydrolyse acetamide (Bühlmann, Vischer & Bruhin, 1961) and to utilize a variety of aliphatic amides as sole carbon and nitrogen source for growth; both these properties are associated with the presence of inducible amidases (Kelly & Clarke, 1962). The rate of appearance of amidase activity in succinate-grown *P. aeruginosa* 8602/A adapting to growth on acetamide has been shown to be discontinuous and to proceed in two distinct and separable phases (Kelly & Kornberg, 1962*a*). In phase I, amidase was produced before the onset of growth on acetamide: this phase was completed when, as a consequence of amidase action, the acetamide content of the medium fell to a low level. In phase II, amidase was produced, rapidly but gratuitously (cf. Monod & Cohn, 1952), during the subsequent growth on the acetate that was formed through hydrolysis of acetamide. Adaptation of succinate-grown cells to growth on acetate was accompanied by phase II but not phase I of amidase formation: this production of amidase was abolished or arrested by the addition of 5 mM-cyanoacetamide to the medium. In contrast, cyanoacetamide at this concentration did not affect the formation of amidase necessary for growth on acetamide (phase I).

These observations suggested that the nature of the carbon source influenced either the nature of the amidase(s) formed or the mode of their formation. Since preliminary purification of the enzyme (Kelly & Kornberg, 1962*b*) produced by acetate-

grown cells (phase II) had shown it to catalyse not only the hydrolysis of aliphatic amides (reaction 1) but also the transfer of the acyl moiety of such amides (reaction 2) or of the corresponding acids (reaction 3) to hydroxylamine, it was feasible to test the possible identity of the enzyme produced in phase I by comparing its ability to effect the following three reactions:

The results obtained indicate that the enzyme produced in phase I is identical with that produced in phase II; since the amidase activity (reaction 1) is but one example of the ability to transfer the acyl moieties to suitable acceptors, it is suggested that these enzymes be designated 'aliphatic acyl-transferases' rather than 'amidases'.

Similar findings have been obtained by W. B. Jakoby & Joan D. Fredericks (personal communication).

MATERIALS AND METHODS

Maintenance and growth of the organisms. Cultures of *P. aeruginosa* 8602/A were maintained on slopes containing minimal salts medium (Kelly & Clarke, 1962) plus acetamide (50 mM), solidified with 2% (w/v) Ionagar no. 2 (Oxo Ltd., London). The organisms were subcultured at weekly intervals, grown at 37° and stored at 5°.

For growth in liquid media, loopfuls of bacteria from a freshly grown slope were inoculated into 2 l. conical flasks, each containing 1 l. of minimal salts medium with either a mixture of 80 mM-sodium acetate and 80 mM-ammonium

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chloride, or 80 mM-acetamide, as carbon and nitrogen sources. Cyanoacetamide (5 mM) was included in all media containing acetamide as growth substrate. The cultures were shaken at 37° in a Gyrotory incubator shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.). The bacteria were harvested at times empirically determined to yield maximal acyltransferase activities; thus acetate-grown cells were harvested shortly before the end of growth (at cell densities of approx. 1 mg. dry wt./ml.), whereas acetamide-grown cells were harvested in an earlier stage of growth (at cell densities of approx. 0.35 mg. dry wt./ml.). The cells were collected by centrifugation at 0° and 12000g for 10 min. and were stored as a thick paste at -15°. There was no significant loss of acyltransferase activity in cells thus stored for at least 6 months.

Estimation of protein. Soluble protein was generally measured by the biuret method, as modified by Gornall, Bardawill & David (1949). The protein content of dilute solutions, such as those obtained during chromatography on columns of DEAE-cellulose, was estimated by the procedure of Warburg & Christian (1941).

Purification of acyltransferase from acetate-grown cells (phase II enzyme). Step 1: preparation of ultrasonic extracts. Frozen bacteria (approx. 15 g. wet wt.) were suspended at 0° in 200 ml. of 0.5M-potassium phosphate buffer, pH 7.2, and, in 20 ml. portions, were exposed for 4 min. to the output of a M.S.E. ultrasonic disintegrator, operating at 1.5 A. The suspensions of broken cells were combined and centrifuged for 15 min. at 15000g and 0°. The supernatant solution was decanted and retained; the sediment was suspended in 200 ml. of 0.2M-potassium phosphate buffer, pH 7.2, and the ultrasonic treatment and centrifugation procedures were repeated. The supernatant solution from this treatment was combined with that obtained previously, one or two crystals of deoxyribonuclease (L. Light and Co., Colnbrook, Bucks.) were added and the suspension was left at room temperature for 15 min.

Step 2: heat treatment. The combined supernatant solutions were divided between two thin-walled glass flasks and each batch (approx. 210 ml.) was held in a water bath at $60 \pm 1^\circ$ for 10 min. The flasks were then cooled rapidly by swirling in ice-water for 5 min.; all subsequent operations were performed at 2°. The two batches of material were combined and centrifuged at 25000g for 60 min., the supernatant solution was decanted and the sediment, resuspended in 50 ml. of 0.1M-potassium phosphate buffer, pH 7.2, was centrifuged once more at 25000g for 60 min.

The supernatant solutions were combined and the bulk of their protein content was precipitated by the addition of ammonium sulphate (300 g. to 450 ml. of supernatant solution) to give 90% saturation. After standing for 30 min., the precipitated material was collected by centrifugation at 25000g for 15 min., and was dissolved in the minimum volume of potassium phosphate buffer, pH 7.2 (5 mM), containing EDTA (disodium salt) (1 mM) and cysteine (1 mM); this solution was dialysed against four changes of 2 l. of this buffer overnight.

Step 3: column chromatography. A batch of 14 g. of DEAE-cellulose was washed as prescribed by Peterson & Sober (1962), adjusted to pH 7.2 with phosphoric acid and, with the aid of a pressure of 5 lb./in.² (0.068 atm.) applied from a nitrogen cylinder, was packed into a chromatographic column to form a cylinder of dimensions 2 cm. ×

35 cm. The packed DEAE-cellulose was washed with 2 l. of the phosphate-EDTA-cysteine buffer at a flow rate of about 40 ml./hr., after which the dialysed protein was applied to the column at 15 ml./hr. The loaded column was developed initially with the phosphate-EDTA-cysteine buffer and subsequently with a linear gradient of phosphate; this gradient was obtained by allowing 300 ml. of potassium phosphate buffer, pH 7.2 (400 mM), containing EDTA (disodium salt) (1 mM) and cysteine (1 mM), to flow, with constant stirring, into a vessel containing 300 ml. of the phosphate (5 mM)-EDTA (1 mM)-cysteine (1 mM) buffer, and allowing the resultant mixtures to pass through the column at 20 ml./hr. Fractions (10 ml.) of the effluent were collected throughout; their protein content was estimated as the extinction at 280 mμ.

Every fifth fraction was tested for amidase (reaction 1) and amide-transferase (reaction 2) activities until these activities were located, when more frequent assays were performed in order to define the peak of enzymic activity. Both the amidase and amide-transferase activities were eluted between 40 mM- and 120 mM-phosphate; fractions with a specific activity greater than 300 μmoles of amide hydrolysed/min./mg. of protein (Fig. 1: fractions 29-39) were combined. The protein content of this pooled material was precipitated by the addition of ammonium sulphate (80 g. to 110 ml. of solution) to give 90% saturation, and was collected by centrifugation at 25000g for 15 min.

Step 4: absorption and elution from alumina C_γ gel. The precipitate from step 3 was dissolved in approx. 20 ml. of 10 mM-potassium phosphate buffer, pH 6.5, containing EDTA (disodium salt) (1 mM) and cysteine (1 mM), and was dialysed overnight against two changes of 2 l. of the same buffer. The dialysed material was then stirred for 10 min. with 10 ml. of alumina C_γ gel (13 mg. dry wt./ml.); the mixture was centrifuged at 5000g for 5 min. and the supernatant solution was discarded. The gel was washed with 5 ml. of 10 mM-potassium phosphate buffer,

Fig. 1. Elution of acyltransferase of *P. aeruginosa* 8602/A from DEAE-cellulose. Experimental conditions are given in the text. The protein concentration (○) was measured as $E_{280\text{ m}\mu}$; the amidase (▲) and amide-transferase (●) activities were measured with propionamide as substrate. The phosphate concentration of the gradient is indicated as △.

pH 7.2, which was removed by centrifuging as above: the supernatant solution was discarded. The protein was then eluted from the gel by stirring with 3 ml. of 10% (w/v) ammonium sulphate in 100 mM-potassium phosphate buffer, pH 7.2, containing EDTA (disodium salt) (1 mM) and cysteine (1 mM). The eluate was collected by centrifugation and the elution procedure was repeated on the gel twice more. The protein in the combined eluates was precipitated by the addition of ammonium sulphate (5.3 g. to 8.3 ml. of eluate) to give 90% saturation and was centrifuged at 25000*g* for 15 min. This material contained amidase with a specific activity of 801 μ moles of propionamide hydrolysed/mg. of protein/min.; it was stored as a paste at -15° . For subsequent experiments small amounts were dissolved in 10 mM-potassium phosphate buffer, pH 7.2, containing EDTA (disodium salt) (1 mM) and cysteine (1 mM).

A summary of this purification procedure is given in Table 1 (preparation A).

Purification of acyltransferase from acetamide-grown cells (phase I enzyme). The procedure for purification of the acyltransferase produced in the early stages of growth on acetamide (and in the presence of cyanoacetamide) was essentially the same as that used for the purification of the enzyme from acetate-grown cells, except that an additional step was introduced between the heat treatment and the column chromatography. The protein, precipitated by ammonium sulphate from the supernatant solution obtained after the heat treatment, was dissolved in 50 mM-potassium phosphate buffer, pH 7.2, containing EDTA (disodium salt) (1 mM) and cysteine (1 mM), and was dialysed against three changes of 2 l. of this buffer for 18 hr. Ammonium sulphate was added to make a 45% saturated solution (125 g. to 490 ml. of dialysed solution), the mixture was stirred for 30 min. at 2° and the protein precipitate was removed by centrifuging at 25000*g* for 15 min. Solid ammonium sulphate (58 g.) was added to the 500 ml. of supernatant solution, to bring it to 60% saturation, the mixture was centrifuged, and the precipitated protein was dialysed and subjected to chromatography on DEAE-cellulose as described for the enzyme purified from acetate-grown cells. The acyltransferase finally obtained, after elution from alumina C_{γ} gel, had a specific activity of 698 μ moles of propionamide hydrolysed/min./mg. of protein.

A summary of the purification procedure is given in Table 1 (preparation B).

Assay procedures. (a) Amidase activity (reaction 1). The rates of enzymic hydrolysis of aliphatic amides were determined by measuring the quantities of ammonia released at various times. All assays were performed at 37° . The incubation mixture contained 1 m-mole of the amide tested, 1 m-mole of potassium phosphate buffer, pH 7.2, 20 μ moles of cysteine hydrochloride, enzyme, and water to 5 ml. The reaction was initiated by the addition of amide, and samples (1 ml.) were removed at 2–5 min. intervals for estimation of their ammonia content by the micro-diffusion method (Conway, 1957). The results of such estimations were corrected for any ammonia contained in the enzyme samples and for the low non-enzymic hydrolysis of the amides that occurred in a similar incubation mixture but from which the enzyme had been omitted. The specific activity of amidase is defined as the quantity (μ moles) of amide hydrolysed/min./mg. of soluble protein.

(b) Transferase activities (reactions 2 and 3). The rates of enzymic hydroxamate formation, with either amides (reaction 2) or acids (reaction 3) as substrates, were determined as described by Kelly & Kornberg (1962*b*). Again, all results were corrected for possible non-enzymic acyl group transfer by subtracting the quantities of hydroxamates formed in an incubation system from which the enzyme had been omitted. The specific activities of the transferases are expressed as the quantities (μ moles) of hydroxamate formed/min./mg. of soluble protein under the particular conditions.

Estimation of phosphate. The phosphate content of fractions eluted from DEAE-cellulose columns was estimated by the method of Fiske & Subbarow (1925).

Chemicals. Lactamide, β -hydroxypropionamide and glycollamide were synthesized by the methods described by Kelly & Clarke (1962). Acetamide, butyramide, formamide and propionamide were purchased from British Drug Houses Ltd., Poole, Dorset, and acrylamide was obtained from L. Light and Co. Ltd., Colnbrook, Bucks. All these amides, except formamide, were recrystallized from butan-2-one before use. Glycinamide was purchased from Mann Research Laboratories Inc., New York, U.S.A. Propionohydroxamate was synthesized by the procedure of Inoue & Yukawa (1940). Other reagents used were of the highest purity commercially available.

Table 1. *Purification of amidase(s) from Pseudomonas aeruginosa 8602/A*

Experimental details are given in the text. Preparation A was obtained with acetate-grown cells, preparation B with acetamide-grown cells. One unit is defined as that quantity of enzyme catalysing the hydrolysis of 1 μ mole of propionamide/min. at 37° .

Step	Total vol. (ml.)		$10^{-3} \times$ total units		Concn. of protein (mg./ml.)		Total protein (mg.)		Specific activity (units/mg. of protein)	
	Prep. A	Prep. B	Prep. A	Prep. B	Prep. A	Prep. B	Prep. A	Prep. B	Prep. A	Prep. B
1 Ultrasonic extract	420	1080	198	102	21.0	23.0	8800	24800	22.6	4.1
2 Dialysed protein from heat treatment	105	490	190	98	24.2	14.0	2520	6850	75.0	14.3
2a Ammonium sulphate fraction	—	36	—	76.5	—	42.5	—	1530	—	50.0
3 Pooled protein from DEAE-cellulose chromatography	—	10	84.8	36.7	—	11.8	197	118	432	310
4 Alumina C_{γ} -gel eluate	—	—	63.2	24.4	—	—	79	35	801	698

RESULTS

Rates of enzymic reactions. Both the acyltransferase purified from acetate-grown cells and that purified from acetamide-grown cells catalysed the hydrolysis of propionamide (reaction 1) more rapidly than they catalysed the formation of propionhydroxamate from hydroxylamine and either propionamide (reaction 2) or propionate (reaction 3). Under the assay conditions used, an amount of acyltransferase sufficient to catalyse the hydrolysis of 2.1 μ moles of propionamide/min. also catalysed the formation of propionhydroxamate at 0.65 μ mole/min. when propionamide was the substrate, and at 0.25 μ mole/min. when propionate was used. Since these 17:5:2 proportions in the rates of the three reactions were observed with both acyltransferases, and did not change significantly throughout their purification, it is probable that the two enzymes were closely similar and that all three acyl group transfer reactions were catalysed by one protein in each preparation. The rates of the individual reactions, which were linear with time over at least 15 min., were also proportional to the quantities of acyltransferase added over at least the range 1–10 μ moles of propionamide hydrolysed/min.

Optimum pH. The effects of pH on the amidase and amide-transferase activities of the purified enzymes, as studied over the pH range 5.5–8.5 with phosphate buffers, were similar for both acyltransferases (Fig. 2). Transferase activity appeared to be less influenced by alteration in the pH than was amidase. The rate of propionamide hydrolysis proceeded maximally at pH 7.2, whereas the rate of amide-transferase activity increased beyond this pH; however, no sharp pH optimum could be distinguished, and, since the quantities of propionhydroxamate formed non-enzymically increased with increasing pH, assays for both amidase and amide-transferase activities were performed at pH 7.2.

Position of equilibrium. In the presence of propionamide and hydroxylamine, the acyltransferase could presumably catalyse not only (1) the hydrolysis of propionamide to propionate and ammonia, (2) the transfer of the propionyl moiety of propionamide to hydroxylamine, with the formation of propionhydroxamate and ammonia, and (3) the formation of propionhydroxamate from the propionate produced through reaction (1), but also the reverse of these reactions. This was tested by determination of the changes, with time, in the concentration of the products of the various reactions: the position of equilibrium was taken to be that point at which no further significant change occurred in 10–20 min. (Fig. 3).

The hydrolysis of propionamide (reaction 1), measured as the rate of ammonia production in the absence of hydroxylamine, proceeded to completion (Fig. 3): no significant reversal of this reaction was detected. In the presence of hydroxylamine, about 55 % of the added propionamide was converted

Fig. 2. Effect of variation in pH on activities of acyltransferases purified from *P. aeruginosa* 8602/A. With propionamide as substrate the amidase (Δ) and amide-transferase ($\circ$) activities of the enzyme purified from acetate-grown cells are compared with the amidase (Δ) and amide-transferase ($\bullet$) activities of that purified from acetamide-grown cells.

Fig. 3. Equilibria of reactions catalysed by the acyltransferase purified from acetate-grown *P. aeruginosa* 8602/A. The variations with time in the extent (% of quantity added) of hydrolysis of propionamide ($\circ$), of propionhydroxamate formation from propionamide (Δ) and from propionate ($\bullet$), and of hydrolysis of synthetic propionhydroxamate ($\bullet$), were determined as described in the text.

into propionohydroxamate; a similar proportion of propionohydroxamate formation was observed when propionate was the substrate (Fig. 3). These observations indicate that the formation of propionohydroxamate is reversible.

Support for this conclusion was derived from an experiment in which synthetic propionohydroxamate was incubated with the purified acyltransferase in the absence of hydroxylamine: propionohydroxamate was hydrolysed at a rate considerably less than was propionamide, but the amount of propionohydroxamate remaining at equilibrium approximated to the quantities formed from hydroxylamine and either propionamide or propionate (Fig. 3).

When acetamide was used as substrate in the presence of acyltransferase and hydroxylamine, the rate of acetohydroxamate formation was considerably more rapid than had been observed with propionamide and nearly all the added acetamide initially gave rise to acetohydroxamate (Fig. 4). However, the concentration of acetohydroxamate in the incubation mixture fell with time until, at equilibrium, only 50% of that initially formed remained. This quantity of acetohydroxamate was also formed when acetate was used as substrate instead of acetamide (Fig. 4). These observations, which indicate that the amide-transferase reaction (2) with acetamide as substrate is considerably more rapid than the hydrolysis of acetamide (1) or the formation of acetohydroxamate from acetate (3), are in agreement with the direct measurements reported by Kelly & Kornberg (1962b).

Effect of substrate concentration. The effect of varying the concentration of substrates on the rates of amidase (1) and amide-transferase (2) activities of the acyltransferases purified from cells grown respectively on acetate and acetamide was determined by the method of Lineweaver & Burk (1934). The K_m values thus measured were not significantly different for the two enzymes (Table 2), which further indicated their close similarity.

Specificity of amidase and amide-transferase activities. It has been shown (Kelly & Clarke, 1962) that the amidase activity of crude extracts of acetamide-grown *P. aeruginosa* is maximal with propionamide as substrate, but that a number of other aliphatic amides are also hydrolysed. This applies to an equal degree to both types of acyltransferases (Table 3): both the enzyme purified from acetate-grown cells and that from acetamide-grown cells hydrolysed propionamide, acrylamide, glycollamide and acetamide rapidly though in decreasing order of velocity, and formamide, lactamide, glycineamide and β -hydroxypropionamide at a much smaller rate. Cyanoacetamide, which inhibits phase II of acyltransferase pro-

Fig. 4. Effect of hydroxylamine on enzymic acyl group transfer from acetamide. The variations with time in the amounts (% of quantity of substrate added) of acetohydroxamate formed from hydroxylamine and either acetamide (O) or acetate (Δ) when incubated with the acyltransferase purified from acetate-grown *P. aeruginosa* 8602/A were determined as described in the text.

Table 2. Effect of substrate concentration on acyltransferases of *Pseudomonas aeruginosa* 8602/A

The effect of substrate concentration on the amidase and amide-transferase activities of the enzymes purified from *P. aeruginosa* 8602/A grown on acetate or acetamide was tested by the method of Lineweaver & Burk (1934). The results given are the K_m values determined from the plot of the reciprocal rate against the reciprocal of the substrate concentration.

Enzymic activity tested	Substrate concentration varied	K_m (mm) for acyl-transferase from cells grown on:	
		Acetate	Acetamide
Amidase	Propionamide	20	20
Amide transferase	Propionamide (excess of hydroxylamine)	20	22
Amide transferase	Hydroxylamine (excess of propionamide)	167	168
Acid transferase	Propionate (excess of hydroxylamine)	200	200

duction, was, however, not hydrolysed by the purified enzyme. Although the quantities of enzymes added in these tests differed, the proportions of their activities against the different substrates remained constant, which again supported the view that the enzymes were not significantly different.

A similar conclusion may be drawn from comparison of their amide-transferase and acid-transferase activities in the presence of hydroxylamine and a number of different substrates. Both enzymes catalysed the formation of hydroxamates most rapidly with acetamide as substrate, though the acyl moieties of acrylamide, glycollamide, propionamide, acetate, propionate and formamide

were also transferred to hydroxylamine in decreasing order of reaction velocity. The rates of transfer noted with glycylamide, β -hydroxypropionamide and acrylate were less than 0.25 % of that observed with acetamide (Table 3).

The ability of the purified enzymes to catalyse the hydrolysis of esters was tested by incubating them with either methyl propionate or ethyl propionate under the conditions of the amidase assay procedure, but with the buffer omitted. The possible formation of propionic acid in this system would have been readily detected as a pH change, which would have been automatically measured with 20 mM-sodium hydroxide added from an automatic titrator. However, no hydrolysis of these esters was detected, and the acid-transferase action of the acyltransferases is therefore not akin to the non-specific formation of hydroxamates from acids, in the absence of ATP, catalysed by esterases (Lipmann & Tuttle, 1945).

Effect of inhibitors. (a) Cyanoacetamide. Although the formation of acyltransferase during growth on acetate was abolished by cyanoacetamide, whereas the formation of the enzyme in response to acetamide was not, neither the purified enzyme from acetate-grown cells nor that from acetamide-grown cells was inhibited in either its amidase or amide-transferase activity when incubated with cyanoacetamide at up to 100 mM concentration.

(b) Thiol reagents. Both amidase and amide-transferase activities of the purified enzymes were abolished when they were incubated with 0.1 mM-*p*-hydroxymercuribenzoate in the absence of cysteine; this inhibition was prevented by the inclusion of cysteine (5 mM). When *p*-hydroxymercuribenzoate was added in the course of enzymic reaction (but in the absence of cysteine), this inhibition could be followed as the gradual decrease in enzymic activity; both amidase and amide-transferase activities were affected (Fig. 5). The addition of cysteine to the inhibited system completely restored the rates of hydrolysis, and of amide transfer, observed before the addition of *p*-hydroxymercuribenzoate.

Iodoacetate also inhibited both amidase and amide-transferase activities of the purified enzymes to a similar extent (Table 4). However, the concentrations of this inhibitor required for complete inhibition were more than 3000 times greater than those of *p*-hydroxymercuribenzoate, and the activities of the enzymes inhibited by iodoacetate were not restored by the addition of cysteine.

(c) Urea. The amidase activity of both acyltransferases was powerfully inhibited by urea. The inhibition was non-competitive (K_i 1.1 mM) with propionamide as substrate (Fig. 6). Amidase activity was not affected by thiourea even at 100 mM concentration, but was totally inhibited by 5 mM-*N*-methyleurea.

Table 3. *Specificity of acyltransferase purified from Pseudomonas aeruginosa 8602/A grown on acetate plus ammonium chloride, or acetamide, as carbon and nitrogen sources*

Preparation A was obtained with acetate-grown cells, preparation B with acetamide-grown cells. The rates of reaction with the substrates listed are given as the specific activities (units/mg. of protein).

Substrate	Specific amidase activity			Specific amide-transferase activity			Specific acid-transferase activity		
	Prep. A	Prep. B	A/B ratio	Prep. A	Prep. B	A/B ratio	Prep. A	Prep. B	A/B ratio
Acetate	—	—	—	—	—	—	104	100	1.0
Acetamide	292	260	1.1	1590	1280	1.2	—	—	—
Acrylate	—	—	—	—	—	—	1.7	1.6	1.1
Acrylamide	553	446	1.2	740	565	1.3	—	—	—
Butyrate	—	—	—	—	—	—	0	0	—
Butyramide	0	0	—	< 1	< 1	—	—	—	—
Cyanoacetate	—	—	—	—	—	—	0	0	—
Cyanoacetamide	0	0	—	0	0	—	—	—	—
Formate	—	—	—	—	—	—	0	0	—
Formamide	47	36	1.3	18.3	14.4	1.3	—	—	—
Glycine	—	—	—	—	—	—	0	0	—
Glycinamide	15.3	12.8	1.2	3.0	2.8	1.1	—	—	—
Glycollate	—	—	—	—	—	—	< 1	< 1	—
Glycollamide	490	410	1.2	285	221	1.3	—	—	—
β -Hydroxypropionate	—	—	—	—	—	—	0	0	—
β -Hydroxypropionamide	3.0	2.7	1.1	3.8	3.0	1.3	—	—	—
Lactate	—	—	—	—	—	—	0	0	—
Lactamide	29.4	23.8	1.2	< 1	< 1	—	—	—	—
Propionate	—	—	—	—	—	—	95	91	1.1
Propionamide	800	698	1.1	249	212	1.2	—	—	—

Table 4. *Effect of iodoacetate on amidase and amide-transferase activities of Pseudomonas aeruginosa 8602/A*

The enzyme purified from acetate-grown *P. aeruginosa* 8602/A was incubated with propionamide (Expt. 1), or propionamide plus hydroxylamine (Expt. 2), and iodoacetate. Specific activities are units/mg. of protein.

	Expt. 1			Expt. 2	
	Concn. of iodoacetate added (mm)	Specific amidase activity	Inhibition (%)	Specific amide-transferase activity	Inhibition (%)
	0	800	—	250	—
	1.25	760	5	235	6
	6.25	690	14	220	12
	70.0	560	30	170	32
	125	280	65	78	69
	300	80	90	12.5	95

Fig. 5. Effect of *p*-hydroxymercuribenzoate and cysteine on the amidase (▲) and amide-transferase (●) activities of the enzyme purified from acetate-grown *P. aeruginosa* 8602/A. The experimental conditions used, with propionamide as substrate, were those described in the text, except that cysteine was omitted initially, 0.1 mM *p*-hydroxymercuribenzoate was added at the arrows marked H, and 5 mM-cysteine hydrochloride at those marked C.

In contrast with the effects observed on amidase activity, urea inhibited the amide-transferase activity of the purified enzymes competitively, both in the presence of limiting propionamide and excess (twice K_m value) of hydroxylamine (K_i 10 mM) and in the presence of limiting hydroxylamine and excess (ten times K_m value) of propionamide (K_i 167 mM; Fig. 7). These observations suggest an explanation for the apparent dissociation of amidase and amide-transferase activities that differs from that advanced by Kelly &

Fig. 6. Effect of urea on the amidase activity of the purified acyltransferases. The rates of hydrolysis of various concentrations of propionamide by the acyltransferase purified from *P. aeruginosa* 8602/A grown on acetamide (▲) or acetate (●), and the effect of 1 mM-urea (○) and 2 mM-urea (Δ) on the latter enzyme, were determined as described in the text.

Kornberg (1962*b*). Since assays of amide-transferase activity are necessarily conducted in the presence of hydroxylamine, whereas assays of amidase are performed in its absence, the differential effect of urea on the two enzymic activities might be a consequence of the presence of hydroxylamine in one case and not the other. This is supported by the results of measurements of the effects of urea, *N*-methylurea and thiourea on the quantities of ammonia formed in the absence of hydroxylamine, and of ammonia and hydroxamate formed in its presence, when the acyltransferase purified from acetate-grown cells was incubated with these materials and propionamide (Table 5). Concentrations of urea and of *N*-methylurea that totally abolished hydrolysis of propionamide in the absence of hydroxylamine had little or no effect in

Fig. 7. Effect of urea on the amidase activity of the purified acyltransferases. The rates of propionhydroxamate formation from excess of propionamide ($10 \times K_m$ value) and various concentrations of hydroxylamine by the acyltransferase purified from acetate-grown *P. aeruginosa* 8602/A (●), and the effect of 100 mM-urea (▲) and 200 mM-urea (△) on these rates, were determined as described in the text.

its presence. Thiourea did not affect amidase activity in the presence or absence of hydroxylamine.

(d) Fluoride. Whereas fluoride exerted only a slight inhibitory effect on the amidase activity of the purified acyltransferases, the amide-transferase activity was inhibited more strongly (Kelly & Kornberg, 1962b). Thus, in the presence of 100 mM-potassium fluoride, the amidase activity was decreased by only 4 %, whereas the amide-transferase activity was inhibited by 82 %. The kinetics of fluoride inhibition of amide-transferase activity were complex: in the presence of excess of hydroxylamine and limiting propionamide, the inhibition appeared to be competitive, but, in the presence of excess of propionamide and limiting hydroxylamine, a type of inhibition similar to that observed by Dodgson, Spencer & Williams (1956) for the effect of hydrazine on arylsulphatase was obtained; this manifested itself as an increase in the extent of inhibition caused by a constant concentration of fluoride when the reactions were performed with increasing concentrations of hydroxylamine. These results imply that it was not fluoride itself but a complex of fluoride and hydroxylamine that was the inhibitor. Since hydroxylamine was not present in tests for the effects of fluoride on amidase activity, the formation of such a complex would not occur: this difference in conditions would thus provide an explanation for the differential effects of fluoride on amidase and amide-transferase activities, alternative to the postulate of a

Table 5. Effect of hydroxylamine on inhibitors of amidase of *Pseudomonas aeruginosa* 8602/A

The enzyme purified from acetate-grown *P. aeruginosa* 8602/A was incubated for 2 min. with propionamide and the inhibitors listed, under the conditions usually employed for assay of amidase activity (*C*) and of amide-transferase activity (*A* and *B*). The quantities of ammonia formed as a consequence of amide transfer to hydroxylamine under these latter conditions were taken to be equivalent to the quantities of hydroxamate formed (*A*); the difference between these values and those experimentally measured (*B*) was the result of amidase action in the presence of hydroxylamine (*B* - *A*).

Inhibitor used	Concn. of inhibitor (mM)	Hydroxamate formed (μmoles) (<i>A</i>)	Total ammonia formed (μmoles) (<i>B</i>)	Ammonia formed by amidase	
				In the presence of hydroxylamine (μmoles) (<i>B</i> - <i>A</i>)	In the absence of hydroxylamine (μmoles) (<i>C</i>)
Urea	0	4.8	19.8	15.0	15.3
	10	4.8	19.6	14.8	0
	20	4.7	19.1	14.4	0
	100	3.7	14.1	10.4	0
	200	2.0	8.1	6.1	0
<i>N</i> -Methylurea	10	4.9	19.8	14.9	0
	40	3.9	15.8	11.9	0
	100	3.2	10.1	7.0	0
	200	1.7	5.7	4.0	0
Thiourea	10	4.8	19.7	14.9	15.4
	100	4.9	19.7	14.8	15.3

Table 6. *Effect of hydroxylamine on the inhibition by fluoride of amidase of Pseudomonas aeruginosa 8602/A*

The conditions were similar to those described in Table 5.

Concn. of fluoride (mM)	Hydroxamate formed (μ moles) (A)	Total ammonia formed (μ moles) (B)	Ammonia formed by amidase	
			In the presence of hydroxylamine (μ moles) (B - A)	In the absence of hydroxylamine (μ moles) (C)
0	5.4	23.0	17.6	17.8
10	2.8	17.0	14.2	17.2
40	1.2	10.0	8.8	16.0
100	0.6	6.6	6.0	15.4
200	0.3	1.6	1.3	14.0

'multi-headed enzyme' advanced by Kelly & Kornberg (1962b). Support for this view is derived from measurements of the quantities of hydroxamate and ammonia formed when the purified acyltransferase was incubated with excess of propionamide and hydroxylamine in the presence of various amounts of fluoride (Table 6): the results demonstrate that quantities of fluoride that exert a negligible action on amidase activity in the absence of hydroxylamine sharply decrease the rate of propionamide hydrolysis in its presence.

DISCUSSION

The formation by *P. aeruginosa* 8602/A of enzymes that catalyse the hydrolysis of a variety of aliphatic amides may be elicited in at least two ways (Kelly & Kornberg, 1962a). One mode of enzyme induction is observed when the organisms become adapted to growth on amides, e.g. acetamide or propionamide, as sole carbon and nitrogen source. Under these conditions, amidase formation precedes growth and is not affected by the addition of cyanoacetamide to the medium. A second type of amidase formation is observed during the later stages of growth on acetate: this appears to be gratuitous, as it can be abolished by the inclusion of cyanoacetamide in the growth medium without impairing the growth of cells. This second type of amidase formation is also elicited by the addition of substituted (non-substrate) amides to cultures growing on succinate (Kelly & Clarke, 1962), but does not accompany the growth of the organism on acetate precursors, e.g. butyrate.

These observations suggested either that *P. aeruginosa* produced two different amidases in response to these different substrates, or that the same enzyme was formed, but that cyanoacetamide did not effectively compete with the inducer-like action exerted by amides when these were the sole growth substrates though it prevented enzyme induction when this enzyme was not required for growth.

The results reported above argue against the view that *P. aeruginosa* forms two distinct amidases. The enzyme produced by cells grown on acetamide in the presence of cyanoacetamide and that of cells grown on acetate and ammonium chloride were purified to roughly similar final specific activities, and a variety of different properties of these enzymes were compared. Both enzymes exhibited the same specificity towards substrates and inhibitors and the same kinetic behaviour. Moreover, both enzymes catalysed not only the transfer of the acyl moiety of amides to water (amidase activity), but also its transfer to hydroxylamine (amide-transferase activity); to a smaller extent, both enzymes also catalysed the transfer to hydroxylamine of the acyl moiety of aliphatic acids. Although the ratio of these three enzymic activities varied with different substrates, these variations were identical for both enzymes.

It is therefore probable that only one form of amidase is produced by *P. aeruginosa* 8602/A. Since the properties of this enzyme indicate that its amidase activity is but one aspect of the cleavage of an intermediate acyl-protein linkage by reagents of the type $R \cdot OH$ (where R may be H or NH_2), it would be preferable to refer to this inducible enzyme as an 'aliphatic acyltransferase'.

SUMMARY

1. Two preparations of amidase, of similar final specific activities, were obtained from *Pseudomonas aeruginosa* 8602/A grown on acetate plus ammonia, and on acetamide, as carbon and nitrogen sources.

2. The purified enzymes catalysed the transfer of the acyl moieties of a number of aliphatic amides to water (amidase activity) and to hydroxylamine (amide-transferase activity); to a smaller extent they also catalysed the transfer to hydroxylamine of the acyl moieties of several aliphatic acids (acid-transferase activity). Both enzymes exhibited the same substrate specificity.

3. The ratios of the amidase, amide-transferase and acid-transferase activities exhibited by each enzyme varied in an identical manner with different substrates. Both enzymes exhibited identical kinetic properties.

4. The amidase activity of both enzymes was non-competitively inhibited by urea (K_i 1.1 mM) and by *N*-methylurea; these inhibitions were reversed by hydroxylamine. Thiourea did not inhibit.

5. The amide-transferase and acid-transferase activities of both enzymes were inhibited by iodoacetate and by *p*-hydroxymercuribenzoate: the inhibition caused by the latter compound was reversed by cysteine. Both activities were also inhibited by low concentrations of fluoride in the presence but not in the absence of hydroxylamine.

6. These results support the view that the enzyme from cells grown on acetamide was identical with that from cells grown on acetate. The properties of the enzyme were those of an acyl-transferase, in which acyl-enzyme complexes were split by hydroxylamine or water.

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Antigenicity of Some New Synthetic Polypeptides and Polypeptidyl Gelatins

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Synthetic polypeptides may be immunogenic and such synthetic immunogens possess well-defined antigenic specificities (Sela, Fuchs & Arnon, 1962; Fuchs & Sela, 1963; Gill & Doty, 1961; Gill & Matthews, 1963; Maurer, 1963; Maurer, Gerulat & Pinchuck, 1962). The availability of synthetic antigens allows systematic variation of different structural parameters of the molecule so that these can be correlated with antigenic properties (Sela, 1962). For this purpose it is desirable to deal with as simple a system as possible that would possess well-defined immunogenic regions. In two previous papers (Sela *et al.* 1962;

Fuchs & Sela, 1963) we discussed the immunogenicity and antigenic specificity of various linear and multichain synthetic polypeptides in which tyrosine was the immunogenic factor. The copolypeptides investigated contained all or some of the amino acids tyrosine, glutamic acid, alanine and lysine.

Conclusions about the role of shape and size in the antigenic features of a molecule, as well as of the accessibility of the antigenically important area of the molecule to the site of the biosynthesis of the antibody, were presented by Sela *et al.* (1962). Tyrosine was present in all of the polymers found to be immunogenic. Thus, multichain poly-DL-alanine (pAla-pLys*) is not immuno-

* For nomenclature, see Sela *et al.* (1962).