The Enzymic Activation of D-Alanine

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D-Alanine is a major component in cell-wall preparations obtained from a variety of micro-organisms (Snell, Radin & Ikawa, 1955; Salton, 1957). In addition, its presence has been demonstrated in intracellular nucleotides (Park, 1952b; Strominger, Scott & Threnn, 1959) which accumulate when *Staphylococcus aureus* and *Escherichia coli* are treated with penicillin. The nucleotides are believed to be precursors of wall material of these bacteria (Park & Strominger, 1957; Strominger & Threnn, 1959). Moreover, D-alanine is required for growth of several lactic acid bacteria in media deficient in vitamin B₆ (Snell, 1945; Holden & Snell, 1949).

Snell *et al.* (1955) have shown that D-alanine is present 'in two distinct forms' in the walls of *Streptococcus faecalis* and various lactic acid bacteria. In *S. faecalis* 40% of the D-alanine of the walls can be extracted with hot trichloroacetic acid as a bound form which is described as 'especially labile to alkaline hydrolysis', whereas the remaining D-alanine, which is insoluble in trichloroacetic acid, appears to be bound as a 'protein-like material'. The similarity in properties and method of extraction of the former fraction suggests that these authors had obtained preparations containing

configuration. Recent investigations by Ikawa & Snell (1958) have demonstrated the presence of D-alanyl-D-alanine as a major product of D-alanine metabolism in *Streptococcus faecalis*.

The wide distribution of D-alanine in a variety of bound forms in bacteria has directed attention to its metabolism in cell-free extracts. This report describes the presence, partial purification and characteristics of an amino acid-activating enzyme for D-alanine from *L. arabinosus* 17-5. A preliminary report of this work has been presented (Baddiley & Neuhaus, 1959). In contrast with other amino acid-activating enzymes, this is the first example of an enzyme for activating a D-amino acid. The process for the activation of D-alanine is analogous to that for the L-amino acids, which was first discovered by Hoagland, Keller & Zamecnik (1956) and demonstrated later with more highly purified enzymes (Davie, Koningsberger & Lipmann, 1956; Berg, 1956; DeMoss, Genuth & Novelli, 1956; Holley & Goldstein, 1959; van de Ven, Koningsberger & Overbeek, 1958; Schweet & Allen, 1958; Webster & Davie, 1959; Bergmann, Berg, Preiss, Ofengand & Dieckmann, 1959). According to currently accepted views, the activation step would be represented by reaction (1):

teichoic acids (Armstrong, Baddiley, Buchanan & Carss, 1958; Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958), which are present in the walls of a variety of micro-organisms including those of *S. faecalis* (Armstrong, Baddiley, Buchanan, Davison, Kelemen & Neuhaus, 1959). These teichoic acids contain alanine ester groups which are labile to neutral hydroxylamine, and in the compounds from *Staphylococcus aureus* H and *Lactobacillus arabinosus* the alanine has the D-

Enzymes of this type catalyse the formation of amino acid hydroxamates from adenosine triphosphate (ATP), hydroxylamine and the amino acid in the presence of Mg²⁺ ions (AMP, adenosine monophosphate). Moreover, with only one exception (Cormier & Novelli, 1958), these enzymes catalyse an amino acid-dependent exchange of inorganic [³²P]pyrophosphate into ATP. The above reactions are represented for the D-alanine-activating enzyme as follows:

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Amino acid-activating enzymes for L-amino acids have been found in micro-organisms (DeMoss & Novelli, 1956; Nismann, Bergmann & Berg, 1957; Novelli, 1958; Schweet, Holley & Allen, 1957),

animal tissues (Schweet *et al.* 1957; Hoagland *et al.* 1956; Davie *et al.* 1956; Lipmann, 1958) and plant material (Davis & Novelli, 1958; Marcus, 1959; Clark, 1958; Webster, 1957).

EXPERIMENTAL

Materials

D-Alanine, D- α -amino-*n*-butyric acid and D-serine were purchased from the California Corp. for Biochemical Research. Crystalline ATP and adenosine diphosphate (ADP) were the products of Pabst Laboratories and the triphosphates of cytidine, uridine and guanosine were purchased from Sigma Chemical Co. [32 P]Pyrophosphate containing less than 0.5% of inorganic phosphate was obtained from The Radiochemical Centre, Amersham, Bucks.

Calcium phosphate (hydroxylapatite modification) was prepared according to the method of Tiselius, Hjertén & Levin (1956). DL-Alanine hydroxamic acid was synthesized by the method of Safir & Williams (1952) and used as a colorimetric standard in the hydroxamate assay. Salt-free hydroxylamine was prepared by the method of Beinert *et al.* (1953), and its concentration was determined colorimetrically (Frear & Burrell, 1955). Yeast inorganic pyrophosphatase was prepared by the 'Alternative purification procedure' of Heppel (1955) through the ethanol fractionation. The specific activity of this preparation was 11 200 μ -moles/hr./mg. of protein.

Analytical procedures

In enzyme preparations that had not been treated with protamine sulphate, the protein concentration was determined with the Folin reagent (Lowry, Rosebrough, Farr & Randall, 1951) with bovine serum albumin as a standard. In preparations containing less than 8% of nucleic acid, protein was determined by the spectrophotometric method (Warburg & Christian, 1941). Good agreement was observed between the two methods. Orthophosphate was determined by the method of Fiske & Subbarow (1925). Radioactivity measurements were made with a thin-window Geiger-Müller counter on polyethylene planchets (1.77 cm.²) to which 0.05 ml. of 0.01 M-cetyltrimethylammonium bromide had been added. All radioactivity measurements were made with a counting error of less than 5%.

Assay procedures

Hydroxamate assay. The complete system contained: 10 μ moles of $MgCl_2$; 100 μ moles of 2-amino-2-hydroxy-methylpropane-1:3-diol (tris)-HCl buffer, pH 7.4; 800 μ moles of salt-free hydroxylamine, pH 7.4; enzyme preparation; 9 μ moles of neutralized ATP; 100 μ moles of D-alanine; water to a final volume of 1 ml. Unless otherwise stated the assay solutions contained 40 μ g. of purified inorganic pyrophosphatase/ml. The tubes were incubated for the indicated period at 37°. The reaction was stopped and the colour developed by the method of Cormier & Novelli (1958). In every case control experiments were performed without added D-alanine to eliminate the effects of chromogenic impurities and endogenous activity due to the presence of other amino acids.

A unit of enzyme is defined according to various authors (van de Ven *et al.* 1958; Davie *et al.* 1956; Schweet & Allen, 1958) as the amount of enzyme which catalyses the forma-

tion of 1 μ mole of hydroxamate/hr. Specific activity is expressed as the number of units/mg. of protein.

The proportionality of the hydroxamate assay is shown in Fig. 1. Fig. 2 (curve B) demonstrates that the rate of hydroxamate formation is linear for 1 hr. All assays for hydroxamate formation were measured under conditions in which linearity was observed.

Exchange assay. The complete system for measuring the [32 P]pyrophosphate-ATP-exchange reaction contained: 10 μ moles of $MgCl_2$; 50 μ moles of tris-HCl buffer, pH 7.8;

Fig. 1. Effect of enzyme concentration on hydroxamate formation. The purified preparation through step 3 was used in the hydroxamate assay.

Fig. 2. Exchange of [32 P]pyrophosphate (curve A) and hydroxamate formation (curve B) as a function of time. The exchange assay was performed with 20 μ g. of purified enzyme preparation and the hydroxamate assay was carried out with 23 μ g. of protein.

enzyme preparation; 9 μ moles of dipotassium ATP neutralized with NaOH; 7 μ moles of [32 P]pyrophosphate (average specific activity 50 000 counts/min./ μ mole); 100 μ moles of D-alanine; water to a final volume of 1 ml. Unless otherwise stated the tubes were incubated at 37° for 30 min., and the reaction was terminated by the addition of 1 ml. of 10% trichloroacetic acid. ATP and pyrophosphate were separated by the method of Crane & Lipmann (1953). After acid hydrolysis of the ATP, radioactivity and orthophosphate were determined in measured portions. The amount of [32 P]pyrophosphate exchanged was calculated by the method of Duffield & Calvin (1946). Unless otherwise specified all values are corrected for a small amount of apparent non-enzymic [32 P]pyrophosphate exchange. Exchange results are reported as μ moles of [32 P]pyrophosphate exchanged/hr. or, in some cases, as % exchanged/30 min.

Fig. 2 (curve A) illustrates the effect of time on the [32 P]pyrophosphate-ATP-exchange reaction. Under the assay conditions employed the velocity of the [32 P]pyrophosphate-ATP exchange to hydroxamate formation is 4.5.

Growth of bacteria and preparation of extracts

Lactobacillus arabinosus 17-5. This was maintained on litmus-milk culture and was grown at 28° in the following medium: glucose, 2% (autoclaved separately); tryptone, 2%; sodium acetate, 1%; yeast extract (Oxoid), 1%; potassium dihydrogen phosphate, 0.45%; N-sodium hydroxide, 26 ml./l.; inorganic salts 'B' (Barton-Wright, 1946), 5 ml./l. The cells were grown in the medium for 18 hr. and then harvested in a Sharples centrifuge. The wet cells were resuspended in 0.01 M-phosphate buffer, pH 6.8, and centrifuged again. Acetone-dried cells were prepared by suspending 40 g. of wet cells in 400 ml. of acetone (previously chilled to -20°) and homogenizing in a blender for 1 min. The mixture was filtered on a Büchner funnel through Whatman no. 1 filter paper, and the filter cake was suspended in 200 ml. of acetone at -20°. The suspension was filtered and approximately 200 ml. of peroxide-free ether at 20° was used to wash the cells on the funnel. The cells were then dried at room temperature, and last traces of ether were removed *in vacuo*. The dried cells were stored at -20°. The product from 220 g. of wet cells weighed 41 g.

To prepare the crude extract dried cells (9 g.) suspended in 75 ml. of 0.01 M-phosphate buffer, pH 6.8, were disrupted at 2° in three lots for 1 hr. with the aid of the 20 kcyc./sec., 60 w MSE-Mullard Ultrasonic Disintegrator (Measuring and Scientific Equipment Ltd.) with the 1:1 vibrator probe. Unbroken cells and debris were removed by centrifuging at 5° for 30 min. at 21 500 g.

Lactobacillus casei, *Bacillus subtilis* and *Staphylococcus aureus* H. *L. casei* ATCC 7469 (kindly provided by Dr E. F. Gale) was grown under the same conditions as *L. arabinosus*. A cell-free extract was prepared by disrupting 2 g. of acetone-dried cells in 20 ml. of 0.02 M-phosphate buffer, pH 6.8, for 30 min. Unbroken cells and debris were removed by centrifuging at 21 500 g at 2° for 30 min. *S. aureus* H (kindly provided by Dr J. T. Park) was grown overnight at 37° with aeration on a medium similar to that described by Park (1952a). A cell-free extract was prepared by disrupting 0.35 g. of acetone-dried cells in 5 ml. of 0.01 M-phosphate buffer, pH 6.8, for 25 min., and then centrifuging as described for *L. casei*. *B. subtilis*

(kindly provided by Dr M. R. J. Salton) was grown overnight at 28° with aeration on the following medium: peptone, 2%; NaCl, 0.5%; DL-alanine, 0.4%. The DL-alanine was included in the medium in an effort to inhibit spore formation (Foster & Heiligman, 1949). Wet cells (4.5 g.) in 20 ml. of 0.01 M-phosphate buffer, pH 6.8, were disrupted for 25 min. and centrifuged as described before.

Purification of enzyme from extracts of Lactobacillus arabinosus

Ammonium sulphate precipitation and protamine sulphate treatment. All subsequent steps were carried out at 0-4°. The extract (50 ml.) was brought to 0.80 saturation by the slow addition of 26 g. of ammonium sulphate. When the solution had been stirred for 1 hr. the precipitate was removed by centrifuging for 20 min. at 21 500 g and dissolved in 0.01 M-phosphate buffer, pH 6.8. This fraction was dialysed in Visking cellophan tubing for 4 hr. against 0.01 M-phosphate buffer, pH 6.8. Protamine sulphate (1%), which had been adjusted to pH 5, was added dropwise to this fraction until the supernatant obtained by centrifuging had an absorption ratio at 280-260 m μ of 0.75. The precipitate was removed by centrifuging.

Ammonium sulphate fractionation. The supernatant (53 ml.) from the above step was brought to 0.55 saturation by the slow addition of 17.3 g. of ammonium sulphate. The precipitate was removed by centrifuging at 21 500 g and discarded. The supernatant was brought to 0.70 saturation by the addition of 4.9 g. of ammonium sulphate. After the mixture had been stirred for 15 min., the precipitate was collected by centrifuging at 21 500 g and dissolved in 0.01 M-phosphate buffer, pH 6.8. This fraction was dialysed against 4 l. of the same buffer for 4 hr.

Chromatography on calcium phosphate (hydroxylapatite modification). A column (13 cm. $\times$ 1 cm.) was packed with calcium phosphate under slight positive pressure and washed for 12 hr. with 0.01 M-phosphate buffer, pH 6.8. The enzyme solution (1 ml., 125 units) was applied to the column, which was then developed successively with 0.01, 0.03, 0.05, 0.10 and 0.20 M-phosphate buffer, pH 6.8. A flow rate of 0.6 ml./min. was maintained by a pressure system. The fractions (3 ml.) which contained the bulk of the D-alanine-activating enzyme were combined and stored at -10°. Fig. 3 illustrates a typical separation.

The original extract, the supernatant from the treatment with protamine sulphate and the ammonium sulphate (0.55-0.70) fraction were stable for 6 months at -10°. However, the fraction obtained from the column lost approximately 25% of its activity at -20° during 1 week. The specific activities reported for the fractions from the column refer to freshly prepared material. A summary of the purification procedure is presented in Table 2.

RESULTS

Distribution of activating enzyme in various bacteria

The results in Table 1 demonstrate that *L. casei* was the most abundant source of the D-alanine-activating enzyme. The presence of the enzyme has now been demonstrated in extracts of *Streptococcus faecalis* R (ATCC 8043). In every case activity towards L-alanine was either absent or very low.

Schweet *et al.* (1957) noted that histidine, methionine, serine and valine are activated in extracts of *Lactobacillus plantarum*. In addition to D- and L-alanine, these amino acids were also activated in the hydroxamate assay by unpurified enzyme preparations from *L. arabinosus*. The D-alanine-activating enzyme from *L. arabinosus* was chosen for purification because of its possible participation in the system which incorporates D-alanine into the teichoic acid of that micro-organism.

Properties of the purified enzyme

The requirements for the hydroxamate system and the [32 P]pyrophosphate-ATP-exchange reaction are given in Table 3. Both reactions show an absolute requirement for Mg^{2+} ions and D-alanine. The hydroxamic acid was identified as alanine hydroxamate by cochromatography with authentic material by the following procedure. A system containing 200 μ moles of D-alanine, 1600 μ moles of

hydroxylamine (pH 7.4), 200 μ moles of tris-HCl buffer (pH 7.4), 20 μ moles of $MgCl_2$, 4.5 mg. of enzyme preparation purified through step 3, 18 μ moles of neutralized ATP and water to 2 ml. was incubated for 1 hr. at 37°. The reaction was terminated by placing the incubation mixture in a boiling-water bath for 3 min. After removal of the protein by centrifuging, the supernatant was evaporated to dryness in a desiccator over H_2SO_4 to decompose the hydroxylamine. The sample was partially deionized on Dowex-1 (OH^- form) according to the procedure of Stein (1953). The residue was chromatographed in the following ascending solvent systems: (a) butan-2-ol-formic acid-water (75:15:10, by vol.) (Hoagland *et al.* 1956); (b) butan-1-ol-acetic acid-water (4:1:5, by vol.) (organic layer); (c) ethanol-aq. NH_3 (sp.gr. 0.88)-water (10:1:2, by vol.) (Schweet *et al.* 1957). The hydroxamic acids were detected with acidic ferric chloride (Schweet *et al.* 1957). The R_f values for the hydroxamic acid cochromatographed with authentic material were: (a) 0.30, (b) 0.35 and (c) 0.60.

Substrate specificity. Table 4 demonstrates the absolute specificity of the purified enzyme preparation for amino acids with the D-configuration. Preliminary results (Baddiley & Neuhaus, 1959) with the relatively insensitive hydroxamate assay had indicated that this enzyme preparation was specific for D-alanine; however, specificity studies with the more sensitive exchange reaction demon-

Fig. 3. Fractionation of D-alanine-activating enzyme on calcium phosphate. The shaded area represents the enzymic activity, expressed as units/ml., and the open area represents the protein concentration ($E_{280 m\mu}$). The hydroxamate assay was performed on one-half scale.

Table 1. Distribution of D-alanine-activating enzyme in various bacteria

The hydroxamate assay was used without addition of inorganic pyrophosphatase, and the exchange assay was performed as described in the Experimental section. All extracts were dialysed for 18 hr. against 0.01 M-phosphate buffer, pH 6.8.

	Hydroxamate formation (μ moles/hr./mg. of protein)		[32 P]Pyrophos- phate-ATP exchange (μ moles/hr./mg. of protein). D-Alanine
	D-Alanine	L-Alanine	
<i>L. arabinosus</i>	0.94	0.09	4.63
<i>L. casei</i>	3.90	0	13.63
<i>B. subtilis</i>	0.28	0.10	1.79
<i>S. aureus</i> H	0.53	0	4.72

Table 2. Purification of D-alanine-activating enzyme

Purification step	Volume (ml.)	Activity (units)	Specific activity (units/mg. of protein)	Yield (%)
1. Extraction	50	1200	1.37	100
2. Protamine sulphate treatment	54	1050	1.95	88
3. Ammonium sulphate fractionation	5.4	680	3.95	57
4. Column eluate	5.2	34.8	29.8	15*

* The results for step 4 represent a typical experiment on a 1 ml. sample from step 3 (125 units). The yield is calculated on the assumption that the whole ammonium sulphate fraction was chromatographed.

strated significant activity towards D- α -amino-*n*-butyric acid (14.6 %) and D-serine (7.5 %). In contrast with the absolute specificity for amino acids with the D-configuration, it appears that slight modifications introduced into the β -carbon atom of D-alanine are possible. Although it is not established that the D-alanine-activating enzyme is responsible for the activation of these two amino acids, it seems likely that they are activated by this enzyme by virtue of their similarity in structure.

Of the nucleotide triphosphates tested only ATP could catalyse the pyrophosphate-ATP exchange effectively; triphosphates of uridine, cytidine and guanosine showed less than 5 % of the activity of ATP. ADP had 10 % of the activity observed for ATP; a slight contamination of the ADP with ATP or the presence of myokinase could account for this activity. According to Pabst Laboratories (1959), ADP contains a trace of ATP.

pH *optima*. As illustrated in Fig. 4, the pH optimum for the hydroxamate system is 7.0–8.0,

whereas the pH optimum for the [32 P]pyrophosphate-ATP-exchange reaction is 7.5–8.0.

Effect of pyrophosphatase and pyrophosphate. Purified preparations of this enzyme were markedly stimulated by adding yeast inorganic pyrophosphatase. As illustrated in Table 5, only 41 % of the maximum activity was observed in incubation for 1 hr. if the pyrophosphatase was omitted. Addition of 0.1 μ mole of pyrophosphate in the absence of pyrophosphatase resulted in a reduction to 29 % of the maximum activity. In order to observe maximum activity with all preparations, including the crude extract, it was necessary to add pyrophosphatase.

Effect of D-alanine, ATP, pyrophosphate, hydroxylamine, and Mg $^{2+}$ concentration on the hydroxamate system and on the exchange reaction. The concentration of D-alanine required to give one-half maximal activity in the hydroxamate system is approximately 0.4M. Fig. 5 (curve A) shows the effect of D-alanine on the rate of the [32 P]pyrophosphate-ATP-exchange reaction. From a Line-

Table 3. Requirements for the enzymic activation of D-alanine

The hydroxamate assay with 200 μ g. of enzyme preparation was used without added inorganic pyrophosphatase. The exchange assay was carried out with 50 μ g. of enzyme preparation.

Modification to system	Hydroxamate formation (μ moles/hr./mg. of protein)	[32 P]Pyrophosphate-ATP exchange	
		(%)	(μ moles/hr./mg. of protein)
Complete	3.5	15.7	28.0
Without Mg $^{2+}$ ions	0	0.30	—
Without ATP	0	—	—
Without D-alanine	0	0.17	—
Without D-alanine; with L-alanine (100 μ moles)	0	0.26	—
Complete (boiled)	0	0.33	—

Table 4. Specificity of D-alanine-activating enzyme

The hydroxamate assay was carried out with 23 μ g. of purified enzyme on one-half scale. In the exchange assay, series A contained 20 μ g. of protein, whereas series B contained 23 μ g. of protein.

Addition	Concn. of substrate (mole/l.)	Hydroxamate formation (μ moles/hr./mg. of protein)	[32 P]Pyrophosphate-ATP exchange (%)	
			A	B
None	—	0	0.22	0.18
D-Alanine	0.100	25.0	20.80	35.40
L-Alanine	0.100	0	0.22	—
D- α -Amino- <i>n</i> -butyric acid	0.100	1.3	3.26	5.27
L- α -Amino- <i>n</i> -butyric acid	0.100	0	—	0.17
D-Serine	0.100	0	1.71	2.90
L-Serine	0.100	0	—	0.40
D-Phenylalanine	0.100	—	0.50	—
L-Phenylalanine	0.100	—	0.34	—
D-Glutamic acid	0.104	—	0.19	—
meso- α - ϵ -Diaminopimelic acid	0.012	—	0.19	—
DL-Alanyl-DL-alanine	0.100	0	0.28	—
β -Alanine	0.100	0	—	—
Glycine	0.094	0	—	—
Acetate (sodium)	0.100	0	—	—
DL-Valine	0.108	0	—	—

Table 5. *Effect of pyrophosphatase and pyrophosphate on the formation of D-alanine hydroxamate*

The complete system contained: 50 μ moles of tris-HCl buffer, pH 7.4; 5 μ moles of $MgCl_2$; 5 μ moles of neutralized ATP; 50 μ moles of D-alanine; 20 μ g. of purified enzyme; 400 μ moles of hydroxylamine, pH 7.4; water to a total volume of 0.50 ml. The tubes were incubated for 60 min. at 37°. The reaction was stopped and the colour developed by the method of Cormier & Novelli (1958).

Modification to system	Activity	
	(μ mole/hr.)	(% of max. rate)
Complete	0.590	[100]
Without pyrophosphatase	0.250	41
Without pyrophosphatase; with 0.1 μ mole of pyrophosphate	0.175	29
Without pyrophosphatase; with 1 μ mole of pyrophosphate	0.075	12

Fig. 4. Effect of pH on the rate of D-alanine activation. In the hydroxamate assay (O) the incubations contained: 10 μ moles of $MgCl_2$; 1.2 mg. of enzyme purified through step 3; 9.5 μ moles of neutralized ATP; 100 μ moles of D-alanine; 100 μ moles of tris-HCl buffer and 800 μ moles of hydroxylamine adjusted to the specified pH; water to a total volume of 1 ml. Controls incubated without D-alanine at the specified pH were used to correct these results. In the exchange assay (●) the following three components were adjusted to the specified pH: 25 μ moles of tris-maleate buffer, 9 μ moles of ATP and 5.3 μ moles of [^{32}P]-pyrophosphate. In addition, each tube contained: 18 μ g. of purified enzyme, which had been preincubated with 1 μ mole of glutathione for 15 min.; 100 μ moles of D-alanine; 10 μ moles of $MgCl_2$; water to a total volume of 1 ml. The tubes were incubated for 30 min. at 37°.

weaver & Burk (1934) plot of these results (Fig. 5, curve B) a K_m for D-alanine was determined (70 mM).

Much lower concentrations of ATP and pyrophosphate are required for maximal activity. Fig. 6 illustrates the effect of [^{32}P]pyrophosphate concentration on the exchange reaction. The K_m for pyrophosphate calculated from a Lineweaver-Burk plot is 0.13 mM. In the hydroxamate assay the maximum activity is obtained when the concentrations of ATP and Mg^{2+} ions are approximately equimolar. In Fig. 7 the effect of ATP concentration with equimolar Mg^{2+} ion concentration on the exchange reaction is shown. The concentration of ATP required for one-half maximal velocity is 2.5 mM. This agrees with the value

Fig. 5. Effect of D-alanine concentration on the [^{32}P]-pyrophosphate-ATP-exchange reaction (curve A) and the Lineweaver-Burk plot of these results (curve B); 20 μ g. of protein was used.

Fig. 6. Effect of [^{32}P]pyrophosphate concentration on the exchange reaction; 23 μ g. of purified enzyme was used.

Fig. 7. Effect of ATP concentration on the [³²P]pyrophosphate-ATP-exchange reaction; 23 μg. of protein and 3.5 μmoles of [³²P]pyrophosphate were used. The concentration of MgCl₂ was equimolar with ATP.

Fig. 9. Effect of enzyme concentration on the [³²P]pyrophosphate-ATP-exchange reaction in the presence of glutathione. The purified enzyme had been previously incubated with mM-glutathione for 15 min. at 37° (pH 6.8) before addition to the assay mixture.

Fig. 8. Effect of enzyme concentration on the [³²P]pyrophosphate-ATP-exchange reaction in the absence of glutathione. Varying amounts of purified enzyme were used. In curve A the rate of pyrophosphate incorporation is plotted against the enzyme concentration, and in B against the square of the enzyme concentration.

obtained when the ATP concentration was varied in the hydroxamate assay. The maximum velocity in the hydroxamate system was observed when the hydroxylamine concentration was approximately 1M; however, the hydroxamate assay was always carried out with 0.8M-hydroxylamine.

Effect of glutathione on the enzyme. The effect of enzyme concentration on the [³²P]pyrophosphate-ATP-exchange reaction (Fig. 8, curve A), shows a non-linear relationship with the 25-fold-purified preparation. However, if the purified enzyme preparation is first incubated with mM-glutathione proportionality between activity and enzyme concentration is observed (Fig. 9). The amount of reactivation with glutathione appeared to depend on the age of the ammonium sulphate fraction (step 3) before chromatography on calcium phosphate. The maximum amount of activation observed in one preparation was fourfold; this was obtained by previous incubation for 15 min. with 10 mM-glutathione, pH 7.8. The effect was not observed with preparations that had been purified through step 3. From the data presented in Fig. 8 (curve A) the velocity of the exchange reaction is proportional to the square of the enzyme concentration.

Effect of possible inhibitors on the enzyme. The purified preparations contained approximately 2–3% of nucleotide material, as determined by the method of Warburg & Christian (1941). Treatment of the purified preparation with ribonuclease

produced no significant inhibition in either the hydroxamate system or the exchange reaction. The possibility was considered that penicillin or chloramphenicol might affect this enzyme. However, mM-chloramphenicol and 10 mM-benzylpenicillin had no effect on the activation of D-alanine in the exchange assay.

DISCUSSION

The available evidence on the specificity of amino acid-activating enzymes indicates the occurrence of separate enzymes for each amino acid. The demonstration of an enzyme which activates D-alanine lends new support to this proposal.

Snell *et al.* (1955) concluded that the amount of D-alanine in intracellular protein of *L. casei* and *S. faecalis* is less than one-fifteenth of that present in the cell wall. Thus since the major fraction of the D-alanine is localized in the wall it seems likely that the D-alanine-activating enzyme is primarily concerned with the biosynthesis of cell-wall material rather than with the synthesis of intracellular protein.

Moreover, Snell *et al.* (1955) observed that the D-alanine required by *L. arabinosus* and *S. faecalis* that were grown in media deficient in vitamin B₆ could be replaced in part by D- α -amino-n-butyric acid. L- α -Amino-n-butyric acid and all other related amino acids were inactive. The D-alanine-activating-enzyme preparation had a slight action on both D- α -amino-n-butyric acid and D-serine. This suggests an intimate relationship between the growth experiments and the specificity of this enzyme. The response of the deficient micro-organism to D-serine has not been examined.

The D-alanine-activating enzyme is similar in many respects to other amino acid-activating enzymes that have been characterized (Davie *et al.* 1956; van de Ven *et al.* 1958; Schweet & Allen, 1958); however, it differs in requiring a relatively high concentration of amino acid for maximal activity. No explanation is offered for this observation, but alanine racemase from *S. faecalis* also has a rather high K_m for alanine (8.5 mM; Wood & Gunsalus, 1951).

Novelli (1958) has observed a wide variation in the ability of bacterial extracts to catalyse amino acid-dependent [³²P]pyrophosphate-ATP-exchange reactions and the formation of hydroxamic acids. Webster (1959) proposed that this difference in activity towards specific amino acids might be the result of either a preferential inactivation of some of the amino acid-activating enzymes or a variation in their pH optima. The characteristics of the D-alanine-activating enzyme suggest another possible explanation for the low activities observed in some cases. If the K_m values of some of the enzymes are

high then routine activation tests at a fixed amino acid concentration might be inadequate for demonstrating such enzymes.

In contrast with the high K_m for D-alanine, this enzyme exhibits a low K_m for pyrophosphate and ATP. The stimulation of the purified preparation by glutathione suggests the presence of an essential sulphhydryl group on the enzyme. The purified enzyme preparations contain 2–3 % of a nucleotide material. With the L-alanine-activating system from rabbit liver, Ogata & Nohara (1957) found a reduction in activity after treatment with ribonuclease. In contrast with these results the purified tryptophan-activating enzyme (Davie *et al.* 1956) is not dependent on the 3–4 % of nucleotide material which it contains. In addition, the tyrosine-activating enzyme (Schweet & Allen, 1958) does not contain any detectable nucleotide. When the D-alanine-activating enzyme was treated with ribonuclease there was no significant decrease in its activity. This suggests that ribonucleic acid is not an essential factor in the activation of D-alanine.

The data which are presented in this paper are consistent with the formation during the activation of D-alanine of an enzyme-AMP-amino acid complex as shown in reaction 1. It is possible that this system may constitute the first step in the introduction of D-alanine into either teichoic acid or the muramic acid-peptide fraction of cell walls.

SUMMARY

1. An amino acid-activating enzyme for D-alanine has been detected in *Lactobacillus arabinosus*, *L. casei*, *Bacillus subtilis* and *Staphylococcus aureus* H. It has been purified 25-fold from ultrasonic extracts of acetone-dried cells of *L. arabinosus*.

2. The enzyme catalyses a [³²P]pyrophosphate-adenosine triphosphate (ATP) exchange in the presence of D-alanine and Mg²⁺ ions and the formation of D-alanine hydroxamate in the presence of ATP, Mg²⁺ ions, hydroxylamine and D-alanine. These reactions are consistent with the formation of an adenosine monophosphate-D-alanine anhydride in combination with the enzyme.

3. In the [³²P]pyrophosphate-ATP exchange the K_m for D-alanine is 70 mM, and for inorganic pyrophosphate it is 0.13 mM. The concentration of ATP required for one-half maximal velocity is 2.5 mM. The purified enzyme preparation is specific for D-amino acids. In addition to D-alanine this preparation catalyses a small but significant [³²P]-pyrophosphate-ATP exchange in the presence of D- α -amino-n-butyric acid (14.6 %) and D-serine (7.5 %).

4. The properties of this enzyme have been

compared with those of other amino acid-activating enzymes. In addition, its possible significance in the biosynthesis of bacterial cell-wall components is discussed.

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