THE ENZYMATIC SYNTHESIS OF D-ALANYL-D-ALANINE[1]

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D-alanine is a major component of the mucopeptide (Snell et al., 1955; Salton, 1957; Strominger and Threnn, 1959; Ikawa and Snell, 1960) and teichoic acid fractions (Armstrong et al., 1958) of the cell walls from a number of gram-positive bacteria. The mucopeptide precursor, UDP-GNAc-lactyl-L-ala-D-glu-L-lys-D-ala-D-ala, contains a terminal D-alanyl-D-alanine moiety (Strominger, 1959) in peptide linkage; teichoic acid contains a single D-alanine in a labile ester linkage (Armstrong et al., 1959). Formation of the mucopeptide precursor occurs by addition of D-alanyl-D-alanine to UDP-GNAc-lactyl-L-ala-D-glu-L-lys (Ito and Strominger, 1960). D-alanyl-D-alanine has been shown to accumulate as a major product in the cold trichloroacetic acid extracts of Streptococcus faecalis, strain R, grown on a vitamin B_6-deficient medium supplemented with D-alanine-1-C^{14} (Ikawa and Snell, 1958).

The occurrence of the D-alanine activating enzyme in a number of bacteria (Baddiley and Neuhaus, 1960) suggested the possibility of its action as catalyzing the first step of D-alanine incorporation into wall components. The present studies with S. faecalis, strain R (A.T.C.C. 8043), demonstrate the presence of an enzyme, independent of the D-alanine activating enzyme, which catalyzes the formation of D-alanyl-D-alanine. This enzyme is here

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termed D-alanyl-D-alanine synthetase. During the course of this work Ito and Strominger (1960) made similar observations with extracts of Staphylococcus aureus.

The separation of the D-alanine activating and D-alanyl-D-alanine synthesizing activities can be readily effected by purification of the latter. By the steps shown in Table I, the D-alanyl-D-alanine synthetase activity has been increased 200-fold and the D-alanine activating enzyme removed. The DEAE eluate, fraction 5, is free of ATPase and PPase, but it still contains a small amount of adenylate kinase.

TABLE I

Separation of D-alanyl-D-alanine synthetase[1] and D-alanine activating enzyme

Purification step	D-alanyl-D-alanine synthetase[2]	D-alanine activating enzyme[3]
	μmoles dipeptide/hr. per mg. protein	μmoles hydroxamate/hr. per mg. protein
1. Sonic extract	1.3	0.46
2. 0-50% ammonium sulfate; Protamine sulfate treatment	4.6	0.47
3. Heat treatment; 63°-5' pH 5.5, 1mM ATP, 0.1M Mg acetate	22	0
4. 29-37% acetone precipitate	125	0
5. DEAE eluate	250	0

[1]A complete description of this procedure will be published elsewhere.

[2]Assay contained 0.01M $MgCl_2$; 0.04M Tris, pH 7.8; 0.01M ATP; 2.5mM glutathione; 0.05M D-alanine-1-C^{14} (4,380 c.p.m./μmole); and enzyme. Incubated at 37° for 30'; reaction terminated by placing the tubes in a boiling water bath for two minutes. Analyzed by descending chromatography on Whatman 3MM in butanol-acetic acid-water (4:1:5), organic phase. Representative sections of the chromatogram containing either the D-alanyl-D-alanine-C^{14} or D-alanine-1-C^{14} were counted in the Packard Tri-Carb liquid scintillation counter according to Wang and Jones (1959). The μmoles of dipeptide synthesized have been calculated from the per cent conversion.

[3]Assayed as described by Baddiley and Neuhaus (1960).

Addition of the purified D-alanine activating enzyme to the D-alanyl-D-alanine synthetase preparations does not stimulate dipeptide synthesis.

The requirements for D-alanyl-D-alanine synthesis are ATP, D-alanine, a divalent metal cation (Mn^{++} or Mg^{++}) and enzyme. At low concentrations (6mM) Mn^{++} is more active than Mg^{++} whereas at saturation concentrations (10mM) Mg^{++} shows a higher activity than Mn^{++}. If the D-alanine activating enzyme were a component of the D-alanyl-D-alanine synthetase, formation of one mole of dipeptide should result with the elimination of one mole of pyrophosphate and one mole of AMP. As shown in Table II, the synthesis of one mole of dipeptide results in the formation of one mole of P_i and one mole of ADP.

TABLE II

Stoichiometry of Product Formation

Products[1]	D-alanine concentration (moles/liter)	
	0.100	0.010
	μmoles formed/30'-37°/ml.	
D-alanyl-D-alanine	1.23	0.27
P_i	1.21	0.29
ADP	1.06	0.26
AMP	0.14[2]	---[3]

[1]Protocol as for Table I; D-alanine-1-C^{14} (4,380 c.p.m./μmole) and enzyme, Fraction 5, 15 μgms./ml. Reaction terminated and dipeptide analysis as in Table I. P_i determined by Marsch (1959). ATP, ADP and AMP separated by descending chromatography on Whatman 3MM in isobutyric acid: NH_4OH: water (66:1:33); concentrations determined spectrophotometrically after elution with 0.01M HCl.

[2]AMP formation is the result of a small amount of adenylate kinase.

[3]No significant AMP detected.

As a result of these data, the synthesis of D-alanyl-D-alanine can be formulated as follows:

$$\text{ATP} + 2\ \text{D-alanine} \xrightarrow{Mg^{++}} \text{D-alanyl-D-alanine} + \text{ADP} + P_i$$

The purified synthetase catalyzes the liberation of P_i from ATP in the presence of D-α-amino-n-butyric acid at 10% the rate for D-alanine; all other amino acids tested were inactive. The K_m for D-alanine is 0.05M and the pH optimum is 8.0-9.5. In contrast to the D-alanine activating enzyme (Baddiley and Neuhaus, 1960) and as expected from the stoichiometry, the synthetase is insensitive to addition of pyrophosphate or PPase and does not catalyze a D-alanine dependent PP-ATP exchange. These observations eliminate the participation of D-alanyl adenylate as an intermediate in the biosynthesis of D-alanyl-D-alanine.

These data suggest that the incorporation of D-alanine into cell wall mucopeptide is independent of the D-alanine activating enzyme. It is possible that the latter functions in the introduction of D-alanine into teichoic acid polymers. The synthetase described here, on the other hand, catalyzes the biosynthesis of the dipeptide, which has been shown by Ito and Strominger (1960) to be subsequently incorporated into the cell wall mucopeptide precursor, UDP-GNAc-lactyl-L-ala-D-glu-L-lys-D-ala-D-ala.

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