Biosynthesis of D-Alanyl-D-Alanine from L-Alanine by Extracts of a Stabilized L-Form from *Streptococcus pyogenes*

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SUMMARY

A stabilized L-form from *Streptococcus pyogenes* possessed alanine racemase and D-alanyl-D-alanine synthetase, and formed D-alanyl-D-alanine from L-alanine. Comparative rates of synthesis of this dipeptide by coccal and L-form cell-free preparations suggested that this pathway remained unaffected in the L-form, even after prolonged inhibition of wall formation. The terminal nucleotide precursor for wall biosynthesis (UDP-muramic acid-peptide) accumulated in this L-form organism; it probably contained D-alanyl-D-alanine. Lack of D-alanine in glycerol teichoic acid from the L-form only was not caused by a loss of alanine racemase activity.

INTRODUCTION

A stabilized (i.e. non-reverting) L-form from *Streptococcus pyogenes* is completely devoid of a rigid wall comparable to that of the bacterial form from which it was derived (Panos, 1967), but the initial phase of bacterial wall formation (the biosynthesis of uridine nucleotide precursors) continues to function in this osmotically fragile L-form (Edwards & Panos, 1962). One of the nucleotides accumulating in this organism contained a peptide composed of glutamic acid, lysine and excess alanine and was similar to the UDP (uridine diphosphate)-muramic acid-peptide isolated by Park (1952) from *Staphylococcus aureus* treated with penicillin. Although the isomeric configuration of alanine in this accumulated precursor from the derived L-form was not determined, studies by others showed that in certain bacteria the peptide portion of this nucleotide is a pentapeptide which contains D- and L-alanine (Strominger, 1968). A dipeptide of D-alanine comprises the end portion of this pentapeptide and is formed prior to its insertion into this nucleotide used for mucopeptide formation (Ito & Strominger, 1962; Neuhaus, 1962).

Alanine is missing in glycerol teichoic acid from this L-form although present in this polymer from the parent organism, *Streptococcus pyogenes* (Slabyj & Panos, 1971).

Changes may have occurred in this stabilized L-form preventing the use of alanine in reactions leading to wall synthesis after permanent loss of the rigid wall. An inability to isomerize the natural isomer of alanine, for example, could explain the accumulation of UDP-muramic acid-peptide and the lack of D-alanine in its glycerol teichoic acid. Consequently we have determined the ability of preparations from a stabilized L-form, and its parent, to isomerize L-alanine and to synthesize D-alanyl-D-alanine. The results are reported here.

METHODS

Organisms and growth conditions. *Streptococcus pyogenes* (type 12) and its stabilized L-form were grown and harvested at their mid-logarithmic phases of growth (Panos &

Barkulis, 1959) and washed as described by Doolin & Panos (1969). The only difference was that penicillin was omitted from the medium during growth of the L-form.

Bacterial extracts. Washed bacteria (0.5 g wet wt) were suspended in 1 ml of 0.1 M-tris-HCl, pH 7.8, buffer containing 2.5 mM-glutathione and from 15 to 30 ml of this suspension disrupted in a French pressure cell (Milner, Lawrence & French, 1950). Unbroken bacteria and debris were removed by centrifugation at 39 000g for 10 min. The supernatant extract was used as the enzyme source for these studies and was stable for at least 4 months at -15°C .

Assays and synthesis of labelled D-alanyl-D-alanine. D-alanyl-D-alanine synthetase was assayed as described by Ito, Nathenson, Dietzler, Anderson & Strominger (1966). For the combined assay of alanine racemase and D-alanyl-D-alanine synthetase, uniformly labelled [^{14}C]L-alanine (0.5 mCi/mmol) replaced [$\text{U-}^{14}\text{C}$]D-alanine. Radioactive D-alanyl-D-alanine was prepared using the same conditions as for the assay except that carrier free [$\text{U-}^{14}\text{C}$]D-alanine was used and reaction mixtures were scaled-up tenfold. A typical reaction mixture contained 50 μl of 0.5 M-tris-HCl buffer, pH 7.8; 80 μl of 0.05 M- MnCl_2 ; 50 μl of 1.0 M-KCl; 20 μl of 0.1 M-ATP; 5 μCi (0.13 μmol) of [$\text{U-}^{14}\text{C}$]D-alanine and 200 μl (2.4 mg protein) of extract of *Streptococcus pyogenes* in a final volume of 500 μl . Reaction mixtures were incubated for 150 min at 37°C and the reaction terminated by placing all test-tubes in a boiling water bath for 3 min. The precipitate obtained was removed by centrifugation and the radioactive supernatant chromatographed as described by Ikawa & Snell (1958). Biorad AG-50W $\times 8$ (H^+ form), 200 to 400 mesh, was suspended in deionized water and poured into a 1.5 cm column to 57 cm. The radioactive supernatant was quantitatively transferred to the column and eluted successively with deionized water (120 ml), 0.5 N-HCl (120 ml) and 1.0 N-HCl (approx. 500 ml). Fractions (5 ml) were collected and 0.1 ml portions counted from only those eluted with 1.0 N-HCl.

Optical configuration. The optical configuration of the alanine in this dipeptide was determined after acid hydrolysis and conversion of alanine to pyruvate by D-amino acid oxidase (Lynch & Neuhaus, 1966).

Paper chromatography. All paper chromatography was descending, with Whatman no. 1 paper. Solvent (A) contained *n*-butanol + glacial acetic acid + water (4:1:1, v/v); solvent (B), isobutyric acid + concentrated ammonium hydroxide + water (66:1:33, v/v); solvent (C), pyridine + water (4:1, v/v). Amino acids were detected with ninhydrin.

Measurement of activity. Aqueous samples (0.1 ml) were counted in Bray's scintillation fluid (10 ml) (Bray, 1960). Radioactive spots were cut from chromatograms and deposited into a toluene scintillation fluid (10 ml) of the following composition: 10 g of 2,5-diphenyl-oxazole (PPO), 0.5 g of 1,4-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP), 1 l toluene, before counting. All samples were counted in a Packard Tri-Carb scintillation counter.

Autoradiography. Standard Kodak No-Screen X-ray film was used throughout and exposed at room temperature for from 2 to 10 days depending upon the level of radioactivity expected.

Protein determination. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as the standard.

Chemicals. Uniformly labelled [^{14}C]L-alanine (137 mCi/mmol) and D-alanine (40 mCi/mmol) were purchased from New England Nuclear Co., Boston, Massachusetts, U.S.A. and Amersham/Searle Co., Arlington Heights, Illinois, U.S.A., respectively. Non-radioactive D-alanyl-D-alanine was obtained from Cyclo Chemical Co., Los Angeles, California, U.S.A. and L-alanyl-L-alanyl-L-alanine from Cyclo Chemical Co. and Sigma Chemical Co., St Louis, Missouri, U.S.A. D-Cycloserine, D-amino acid oxidase and glutamic-pyruvic transaminase were purchased from Sigma Chemical Co.

Fig. 1. Ion exchange chromatogram of a reaction mixture with an extract from the streptococcus incubated with $[U-^{14}C]$ D-alanine. Peak 1, alanyl-alanine; peak 2, alanine; peak 3, contaminant or artifact (see text).

Table 1. Paper chromatographic mobility of enzymatically synthesized D-alanyl-D-alanine by streptococcal and L-form extracts

	R_F in solvent		
	A	B	C*
D-Alanine	0.34	0.57	1.00
Synthetic D-alanyl-D-alanine	0.46	0.68	1.09
Enzymatic D-alanyl-D-alanine	0.46	0.68	1.09
L-Alanyl-L-alanyl-L-alanine	0.46	0.76	1.36

$$* R_F = R_{\text{alanine}} = 1.$$

RESULTS

Synthesis of D-alanyl-D-alanine from D-alanine by extracts of Streptococcus pyogenes and its stabilized L-form. Uniformly labelled $[^{14}C]$ D-alanyl-D-alanine was prepared using the assay method for D-alanyl-D-alanine synthetase (see Methods). A typical elution profile obtained with a streptococcus extract is shown in Fig. 1. Peaks 1 and 2 were an alanine dipeptide and alanine, respectively, according to co-chromatography with unlabelled D-alanyl-D-alanine and D-alanine by both paper chromatography and by autoradiography; the R_F of D-alanyl-D-alanine from peak 1 was the same by both techniques. Table 1 tabulates the R_F values obtained for the products in the different solvent systems. The resolution of alanyl-alanine from alanine by column chromatography, though adequate, was not as complete as that obtained by Ikawa & Snell (1958) and Ito & Strominger (1962). Hydrolysis of the material from peak 1 with 6 N-HCl at 110 °C for 16 h always yielded only alanine as identified by paper co-chromatography and autoradiography with unlabelled and labelled alanine (Table 1). The probable origin of peak 3 is discussed below.

Identical column (Fig. 1) and paper (Table 1) chromatographic results were obtained

Fig. 2. Ion exchange chromatograms of reaction mixtures with extracts from the L-form incubated with (a) $[U-^{14}C]$ L-alanine and (b) $[U-^{14}C]$ L-alanine plus D-cycloserine. Peak 1, alanyl-alanine; peak 2, alanine; peak 3, contaminant or artifact (see text).

Table 2. *Determination of optical configuration of alanine residues in alanyl-alanine synthesized from $[U-^{14}C]$ L-alanine by an extract from the L-form*

	Untreated	Counts per minute after treatment with	
		D-amino acid oxidase	transaminase
$[U-^{14}C]$ D-Alanine	62 853	56 701	—
$[U-^{14}C]$ L-Alanine	68 106	209	65 391
Dipeptide hydrolysate	27 680	27 794	760

when extracts of the L-form (8 mg protein/500 μ l reaction mixture) replaced those of the parent *Streptococcus pyogenes*.

Synthesis of D-alanyl-D-alanine from L-alanine by extracts of the L-form. Since the previous experiments indicated that the L-form was capable of alanine dipeptide formation, they were repeated with labelled L-alanine to detect any racemase activity. A typical elution profile (Fig. 2a) illustrates the ability of the L-form to continue to synthesize alanyl-alanine from L-alanine. D-Cycloserine is a potent inhibitor of alanyl-alanine synthetase and alanine racemase (Strominger, Ito & Threnn, 1960). When $[^{14}C]$ L-alanine was incubated with extracts of the L-form in the presence of 4 mM-D-cycloserine and the reaction mixture resolved by column chromatography, only alanine (peak 2) and peak 3 were observed (Fig. 2b). Clearly synthesis of alanine dipeptide was inhibited by this antibiotic; indicating that the dipeptide formed in the absence of D-cycloserine was probably D-alanyl-D-alanine.

Fig. 3. Combined rate of racemase and D-alanyl-D-alanine synthetase activities in extracts. Specific activity of enzymes = 1.0 and 1.3 nmol/min/mg protein for streptococcal and L-form extracts, respectively. Radioactivity from dipeptide spot from segmented paper chromatograms.

The DNP-derivative of this peptide was prepared as described by Grebner, Hall & Neufeld (1966) except that DNP-peptide was extracted into ethyl ether instead of ethyl acetate after acidification of the reaction mixture. It was hydrolysed in 6 N-HCl, 105 °C for 6 h, and DNP-alanine and alanine separated and counted (Ikawa & Snell, 1958). Of the original counts in the sample (70 700 c.p.m. in a typical experiment), 16% (11 524 c.p.m.) appeared in DNP-alanine and 16% (11 892 c.p.m.) in alanine. This equal distribution of radioactivity in the two halves of this dipeptide confirms the evidence that the dipeptide was D-alanyl-D-alanine. Low recoveries of radioactivity in such experiments have also been obtained by others (Ikawa & Snell, 1958) and probably reflect incomplete formation of derivatives.

The optical configuration of the alanine residues was confirmed enzymatically. The radioactive dipeptide (peak 1, Fig. 2a) was hydrolysed with 6 N-HCl as above and the acid removed *in vacuo*. Portions of this hydrolysate were treated with D-amino acid oxidase and glutamic-pyruvic transaminase and the pyruvate formed, if any, separated as described by Lynch & Neuhaus (1966) and counted. Adequate controls included use of labelled L- and D-alanine. The quantitative conversion of D-alanine to pyruvate by D-amino acid oxidase, but not by the transaminase, is apparent in Table 2 and indicates that the alanine residues in newly synthesized alanyl-alanine are of the D configuration.

Combined rate of alanine racemase and alanyl-alanine synthetase in extracts. The combined rate of the racemase and synthetase enzymes in each preparation was assayed as detailed by Ito *et al.* (1966) with [U-¹⁴C]L-alanine as substrate. Fig. 3 illustrates the formation of D-alanyl-D-alanine from labelled L-alanine by these two preparations. The rate of formation remained linear over 40 min for each preparation and all reactions were terminated after

60 min. The specific activity for these two enzymatic reactions in preparations of the parent coccus was 1.3 nmol/min/mg protein as compared with 1.0 nmol/min/mg protein in those from the L-form.

Origin of the third peak eluted during column chromatography. The material comprising peak 3 (Fig. 1 to 3) had the same mobility as alanine in solvent system A. However, it continued to appear in elution profiles of reaction mixtures incubated with D-cycloserine (Fig. 2*b*). It was detected in labelled D- and L-alanine as received from two different vendors so it was either an impurity present initially or formed by radiolysis (Oldham, 1970).

DISCUSSION

This L-form has been unable to synthesize a rigid wall for the past 15 years (Panos, 1969). The importance of the D and L isomers of alanine and D-alanyl-D-alanine in bacterial wall mucopeptide formation is well known and will not be pursued here. However, the detection of these two reactions still operating in a streptococcal L-form completely devoid of a wall is significant. It demonstrates that the accumulation of the terminal wall precursor (uridine nucleotide-muramic acid-peptide) in this L-form (Edwards & Panos, 1962) is not due to the inability of this organism to isomerize the natural isomer of alanine or to synthesize the dipeptide end of the peptide portion of this precursor. Therefore, these findings, together with our earlier results (Edwards & Panos, 1962), indicate that this nucleotide which accumulated in the L-form is not an alternative nucleotide with an altered peptide side chain. Finally, no significant difference was noted in the rate of synthesis of D-alanyl-D-alanine from L-alanine by coccal or L-form extracts. This suggests that prolonged inhibition of wall formation has not diminished the rate at which this metabolic route can operate.

Plapp & Kandler (1964) established that the L-phase variants (stable and unstable L-forms) of *Proteus mirabilis* contain alanine racemase. They also stated that this enzyme '... is not lost during the penicillin induced transition of the bacterial form of *P. mirabilis* to the L-phase, although the enzyme has no further function in the mucopolymer-free L-phase'. As now shown, however, there is a definite need for this enzyme in this mucopeptide-free streptococcal L-form. It lies in the continued formation of the terminal nucleotide precursor for wall synthesis still found accumulating in this L-form (Edwards & Panos, 1962). The inhibition of D-alanyl-D-alanine, the dipeptide end of this peptide containing nucleotide, by D-cycloserine (see below) substantiates this belief.

As already indicated, glycerol teichoic acid isolated from this L-form, but not that from its parental *Streptococcus pyogenes*, is devoid of D-alanine (Slabyj & Panos, 1971). Yet an actively functioning alanine racemase is present and D-alanyl-D-alanine is formed by the L-form. The inability to insert D-alanine into glycerol teichoic acid in the L-form may be due, instead, to a lack or inhibition of D-alanine:membrane acceptor ligase (Reusch & Neuhaus, 1971).

Alanine racemase seems to be absent from the few mycoplasmas examined thus far (Plapp & Kandler, 1964; Smith, 1971). This has led investigators to suggest that this enzyme may serve as a criterion for differentiating between a mycoplasma and an L-form of a bacterium (Plapp & Kandler, 1964). However, the finding of this enzyme in this coccus and its stabilized L-form precludes its usefulness for differentiating between stabilized and unstabilized bacterial L-forms.

D-Cycloserine inhibits bacterial wall formation by inhibiting reactions catalysed by alanine racemase and D-alanyl-D-alanine synthetase which leads to formation of this dipeptide; it has been successfully employed in the transformation of a bacterium to an L-form (Ward &

Martin, 1962). This antibiotic is without affect upon growth of this streptococcal L-form (Panos, Cohen & Fagan, 1967); it affected growth of '... the mucopolymer containing L-phase only' (Plapp & Kandler, 1964). These results now suggest that, while not affecting growth, D-cycloserine probably causes accumulation of UDP-muramic acid-tripeptide in this streptococcal L-form instead of UDP-muramic acid-pentapeptide (with its dipeptide of D-alanine).

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