FORMATION OF D-ALANYL–D-ALANINE AND D-ALANINE FROM UDPMurNAc–L-ALA–D-isoGLU–L-LYS–D-ALA–D-ALA BY EXTRACTS OF *STAPHYLOCOCCUS AUREUS* AND *STREPTOCOCCUS FAECALIS*

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1. Introduction

Formation of UDPMurNAc-pentapeptide in bacteria is catalyzed by enzymatic addition of D-alanyl–D-alanine to UDPMurNAc-tripeptide in the presence of ATP, with concomitant formation of ADP and inorganic phosphate [1–3].

Removal of the terminal D-alanine residue from UDPMurNAc-pentapeptide results from the action of D-alanine carboxypeptidases that are inhibited by penicillins [4–7].

In the present study we describe a different mode of cleavage of the lysine containing UDPMurNAc-pentapeptide that occurs in cell-free preparations of several strains of *Staphylococcus aureus* and of *Streptococcus faecalis*. This cleavage is ADP-dependent and leads to formation of D-alanyl–D-alanine and free D-alanine. Both enzymatic activities are penicillin- insensitive, but the formation of D-alanine is inhibited by D-cycloserine (DCS). We suggest that formation of D-Ala–D-Ala is a consequence of reversal of the UDP–*N*-acetylmuramoyl–L-alanyl–D-glutamyl–L-lysyl: D-alanyl–D-alanine ligase (ADP-forming) (EC 6.3.2.10) [8], and that free D-alanine is formed by subsequent cleavage of the initially released dipeptide by the reverse action of D-alanine: D-alanine ligase (ADP-forming) (EC 6.3.2.4). The possible physiological significance of these cleavages is briefly discussed.

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2. Materials and methods

Uniformly labeled [^{14}C] D-alanine was obtained from Radiochemical Center (Amersham), adenine nucleotides from P-L Biochemicals, Inc., pyruvate kinase from Boehringer (GmBH Manheim); phosphoenol pyruvate and D-cycloserine from Sigma Chemical Company.

2.1. *Preparation of UDPMurNAc–L-Ala–D-isoGlu–L-Lys–D-Ala–[^{14}C] D-Ala and UDPMurNAc–L-Ala–D-isoGlu–L-Lys–[^{14}C] D-Ala–D-Ala.*

Preparation of UDPMurNAc-pentapeptide labeled in either the ultimate D-alanine or the penultimate D-alanine residue was carried out by enzymatic addition of chemically synthesized D-Ala–[^{14}C] D-Ala or [^{14}C] D-Ala–D-Ala, respectively, to UDPMurNAc–L-Ala–D-isoGlu–L-Lys islated from *S. aureus* [7,8]. The preparation of adding enzyme used was obtained either from *S. faecalis* or from *S. aureus* 52A5 by slight modifications of known procedures [1–3].

2.2. *Bacterial strains and preparation of enzymic extract*

The bacterial strains used in these experiments were *S. faecalis* [7], *S. aureus H.*, *S. aureus* 52A5 [9] and *S. aureus* Mp29 – a laboratory derived strain, that is methicillin resistant and penicillinase negative. This latter mutant was stored and grown in the presence of 500 μg/ml of methicillin.

Cells from the early logarithmic phase of growth were ground in the cold with levigated alumina as previously described [8]. The supernatant fraction

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Table 1
The effects of ATP, ATP-regenerating system, fluoride, penicillin G and D-cycloserine on formation of D-Ala–D-Ala and free alanine from UDPMurNAc-pentapeptide by extracts of *S. aureus* 52A5.

Compound formed	Amount (nmoles)					
	-ATP	complete system*	+ATP regenerating system**	+fluoride 20 mM	+DCS 250 μg/ml	+penicillin G 7 mg/ml
D-Ala–[^{14}C]D-Ala	0.3	4.5	0.01	0.1	3.2	4.3
[^{14}C]alanine	0.05	0.3	0	0.05	0	0.4

* The reaction mixture (130 μl) contained in μmoles: UDPMurNAc–L-Ala–D-isoGlu–L–Lys–D–Ala–[^{14}C]D-Ala. (16 × 10^6 cpm/μmole), 0.03; $MgCl_2$, 1.0; ATP, 0.5; 2-mercaptoethanol, 0.5; Tris–HCl (pH 7.6), 10; and enzyme, 0.66 mg protein. The reaction was carried out at 37°C for one hr.

** The ATP regenerating system consisted of 1 μmole of phosphoenol pyruvate and 10 units of pyruvate kinase.

(6–12 mg protein per ml) obtained by centrifugation at 105 000 *g* served as the enzyme source and was immediately tested for enzymatic activity.

2.3. *Determination of radioactive D-Ala–D-Ala and free alanine*

Reactions were terminated by boiling for 2 min in a water bath. The reactants and products were separated by paper chromotography in isobutyric acid–1 M NH_4OH (5:3 v/v) and located by autoradiography [8]. [^{14}C]alanine and radioactive D-Ala–D-Ala were extracted from the paper and further identified and determined quantitatively using a Beckman amino acid analyser equipped with a Tri-Carb flow analyzer [8].

3. Results and discussion

Table 1 shows that on incubation of the 105 000 *g* supernatant fraction of *S. aureus* 52A5 with UDPMurNAc-pentapeptide in the presence of ATP, D-Ala–D-Ala is formed as the main product and a small amount of free alanine is also detected. Without exogeneously added ATP or with ATP in the presence of fluoride (added to inhibit ATPase activity), low but significant activity is observed presumably due to the endogenous pool of adenine dinucleotide. In the precence of ATP supplemented with an ATP-regenerating system and thus precluding the accumulation of ADP from ATP, neither D-Ala–D-Ala nor D-alanine are formed. This indicates that not ATP per se but ADP is the active component in the reaction system. This is also confirmed by the fact that ADP+P_i can substitute for ATP and the rate of formation of D-Ala–D-Ala in the presence of ADP+P_i is indeed higher than in the presence of ATP alone (table 2). AMP cannot substitute for ATP and neither can inorganic phosphate.

Table 2
Formation of D-alanyl–[^{14}C]D-alanine by extracts of *S. aureus* 52A5 in the presence of adenine nucleotides.

	Addition	D-Ala–[^{14}C]D-Ala (cpm)
1	none	212
2	ATP	12 550
3	ADP + P_i	71 453
4	As for 3 + DCS (100 μg/ml)	70 161
5	AMP	443
6	P_i	354

The reaction mixture (150 μl) contained in μmoles: UDPMurNAc-pentapeptide (11 × 10^6 cpm/μmole), 0.03; 2–mercaptoethanol, 0.5; Tris–HCl (pH 7.6), 10; $MgCl_2$, 5; enzyme fraction, 0.42 mg protein. The reaction was carried out at 37°C for 20 min. In experiments 2–6 the concentration of each nucleotide and of the inorganic phosphate (pH 7.0) was 10 mM.

Table 3
Formation of D-Ala–D-Ala and free D-alanine from UDPMurNAc-pentapeptide by supernatant fractions of *S. aureus* H, *S. aureus* Mp 29 and *S. faecalis**.

	Substrate	Products	amount (nmoles)								
			No ATP		+ATP**			+ADP + P_i**			
			–	+pen G	–	+pen G	+DCS	–	+pen G		+DCS
Exp. I *S. aureus* Mp 29	UDPMurNAc–L-Ala–D-isoGlu–L-Lys–D-Ala–[^{14}C]D-Ala 25 × 10^6 cpm/μmole, 12 nmoles	D-Ala–[^{14}C]D-Ala	0.29	0.34	5.45	5.42	5.51	5.39			5.65
		[^{14}C]alanine	0.05	0.03	0.67	0.57	0	0.94			0
Exp. II *S. aureus* H	UDPMurNAc–L-Ala–D-isoGlu–L-Lys–D-Ala–[^{14}C]D-Ala 11 × 10^6 cpm/μmole, 42 nmoles	D-Ala–[^{14}C]D-Ala			10.30		9.92				
		[^{14}C]alanine			1.04		0				
Exp. III *S. faecalis*	UDPMurNAc–L-Ala–D-isoGlu–L-Lys–D-Ala–[^{14}C]D-Ala 25 × 10^6 cpm/μmole, 12 nmoles	D-Ala–[^{14}C]D-Ala	0	0	5.62			6.71			7.43
		[^{14}C]alanine	2.0	0	1.34			3.6			0.90
Exp. IV *S. faecalis*	UDPMurNAc–L-Ala–D-isoGlu–L-Lys–[^{14}C]D-Ala–D-Ala 2 × 10^6 cpm/μmole, 65 nmoles	[^{14}C]D-Ala-D-Ala	1.7		19.3			15.5	14.9		10.5
		[^{14}C]alanine	0		2.2			6.0	6.0		0

* The reaction mixture (150 μl) contained in μmoles, Tris–HCl (pH 7.6), 10; $MgCl_2$, 5; mercaptoethanol, 2; and substrate as indicated. The amount of enzyme protein in Exp. I was 2 mg; Exp. II, 0.46 mg; Exp. III, 1.4 mg and Exp. IV, 0.7 mg. The incubations were carried out at 37°C for one hr.

** Where indicated, 0.4 μmole of ATP was added or 0.4 μmole of ADP + 0.4 μmole of phosphate buffer (pH 7.0). The final concentration of penicillin G was 100 μg/ml; DCS, 250 μg/ml in Exp. I and 100 μg/ml in Exp. II – IV. A zero time control using heat denatured enzyme preparation was used in each experiment and neither free alanine nor alanine dipeptide were formed.

Penicillin G has no effect on these reactions (table 1) but D-cycloserine strongly inhibits formation of D-alanine (tables 1 and 3) while formation of D-Ala–D-Ala appears to be relatively unaffected (tables 1 and 2).

The two enzymatic activities described above, were also observed in *S. aureus* H, *S. aureus* Mp29 and *S. feacalis* (table 3).

Unlike the strains of *S. aureus* presently described, *S. faecalis* also contains a D-alanine carboxypeptidase which is inhibited by penicillin G [7]. Accordingly, the enzyme preparation obtained from *S. faecalis*, also catalyzes a penicillin G sensitive release of the terminal D-alanine residue in the absence of exogenous ATP; this activity, however, is not abolished by DCS (table 3, Exp. III, see also [7]). UDPMurNAc-pentapeptide labeled exclusively in the penultimate D-alanine residue was therefore used as a specific substrate to asses the nucleotide-dependent activities in the presence of contaminating D-alanine carboxypeptidase. As can be seen from table 3 Exp. IV, release of the labeled penultimate alanine residue is dependent on the presence of ADP (or ATP) and that this activity is insensitive to penicillin G., and is completely inhibited by DCS.

From the data given we suggest that D-Ala–D-Ala is released from UDPMurNAc-pentapeptide by UDPMurNAc–L-Ala–D-isoGlu–L-Lys: D-alanyl–D-alanine ligase (ADP-forming) operating in the reverse direction. Although the present results were obtained using a 105 000 *g* supernate fraction, formation of D-Ala–D-Ala from UDPMurNAc-pentapeptide was also demonstrated in particulate fractions of *S. aureus* 52A5 [8], suggesting that this activity is only loosely associated with cell membranes.

Since release of D-alanine occurs only in the

presence of ADP and is DCS sensitive (tables 1 and 3), this activity presumably also reflects the reverse action of D-alanine:D-alanine ligase (ADP-forming) [10–12]. This conclusion is in accordance with results obtained from experiments in which D-Ala–[^{14}C] D-Ala was used as a substrate in the absence of adenine nucleotides. Under these conditions free alanine was not detected but its formation was observed upon addition of ATP or ADP+P_i, and this activity was completely inhibited by DCS.

In view of the results reported here, it is important to point out that in studies in which UDPMurNAc-pentapeptide is used as a substrate for peptidoglycan synthesis and where ATP is added for amidation reactions of the isoglutamyl residue of the peptide moiety (cf. [13]), release of the terminal D-alanine residue obviously does not represent exclusively transpeptidase activity.

With the finding that both ligases can readily operate in a reversible manner, one may consider a functional regulatory mechanism which determines the level of the lysine-containing UDPMurNAc-pentapeptide depending on the intracellular ATP/ADP ratio.

It might also be of interest to ascertain whether nucleotide-dependent cleavages of UDPMurNAc-pentapeptide occur in bacteria containing diamino-pimelic acid in their cell walls.

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References

[1] Ito, E. and Strominger, J. L. (1960) J. Biol. Chem. 235, PC5–6.
[2] Comb, D. G. (1962) J. Biol. Chem. 237, 1601–1604.
[3] Neuhaus, F. C. and Struve, W. G. (1965) Biochemistry 4, 120–131.
[4] Izaki, K. and Strominger, J. L. (1968) J. Biol. Chem. 243, 3193–3201.
[5] Lawrence, P. J. and Strominger, J. L. (1970) J. Biol. Chem. 245, 3660–3666.
[6] Blumberg, P. M. and Strominger, J. L. (1971) Proc. Natl. Acad. Sci. U.S. 68, 2814–2817.
[7] Oppenheim, B., Koren, R. and Patchornik, A. (1974) Biochem. Biophys. Res. Commun. 57, 562–571.
[8] Oppenheim, B., Burstein, Y., Patchornik, A. (1972) Isr. J. Chem. 10, 43–49.
[9] Chatterjee, A. N., Mirelman, D., Singer, H. J. and Park, J. T. (1969) J. Bacteriol. 100, 846–853.
[10] Neuhaus, F. C. (1962) J. Biol. Chem. 237, 778–786; 3128–3135.
[11] Neuhaus, F. C., Carpenter, C. V., Lynch Miller, J., Lee, N. M., Gragg, M. and Stickgold, R. A. (1969) Biochemistry 8, 5119–5124.
[12] Ito, E. and Strominger, J. L. (1962) J. Biol. Chem. 237, 2696–2703.
[13] Strominger, J. L., Izaki, K., Matsuhashi, M. and Tipper, D. J. (1967) Federation Proc. 26, 9–21.