THE DIRECTION OF PEPTIDE TRIMER SYNTHESIS FROM THE DONOR–ACCEPTOR SUBSTRATE N^{α}-(ACETYL)-N^{ϵ}-(GLYCYL)–L-LYSYL–D-ALANYL–D-ALANINE BY THE EXOCELLULAR DD-CARBOXYPEPTIDASE-TRANSPEPTIDASE OF *STREPTOMYCES* R61

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1. Introduction

In a previous report [1], the exocellular DD-carboxypeptidase-transpeptidase from *Streptomyces* R61 (in short the R61 enzyme) was shown to be able to utilize the tetrapeptide monomer

[^{14}C]Ac-L-Lys–D-Ala–D-Ala (IV)
Gly—┘

as both donor and acceptor substrate. Hydrolysis and transfer reactions occurred concomitantly, yielding free D-Ala, the hydrolyzed tripeptide monomer

[^{14}C]Ac-L-Lys-D-Ala (V)
Gly—┘
the heptapeptide dimer

[^{14}C]Ac-L-Lys–D-Ala–D-Ala (II)
[^{14}C]Ac-L-Lys–D-Ala–Gly—┘
Gly—┘
the hydrolyzed hexapeptide

dimer (i.e. the former dimer lacking one C-terminal D-Alanine) (III) and a mixture of deca- and nona- (i.e. hydrolyzed) peptide trimers (Ia and Ib respectively)

[^{14}C]Ac-L-Lys–D-Ala–(D-A
[^{14}C]Ac-L-Lys–D-Ala–Gly—┘
[^{14}C]Ac-L-Lys–D-Ala–Gly—┘
Gly—┘

The D-Ala–Gly interpeptide bonds in the dimers and trimers formed were identical to those found in the native, completed wall peptidoglycan of *Streptomyces* R61 [2]. In the present report, we show that peptide trimer (I) is preferentially formed by addition of the tripeptide moiety [^{14}C]Ac-L-Lys–D-Ala of the tetra-
Gly—┘
peptide monomer (IV) acting as donor (through its C-terminal D-Ala–D-Ala sequence) to a preformed peptide dimer (II or III) acting as acceptor (through its N-terminal glycine residue) rather than by dimer (II) acting as donor and monomer (IV or V) acting as acceptor. The reactions are illustrated in Scheme 1.

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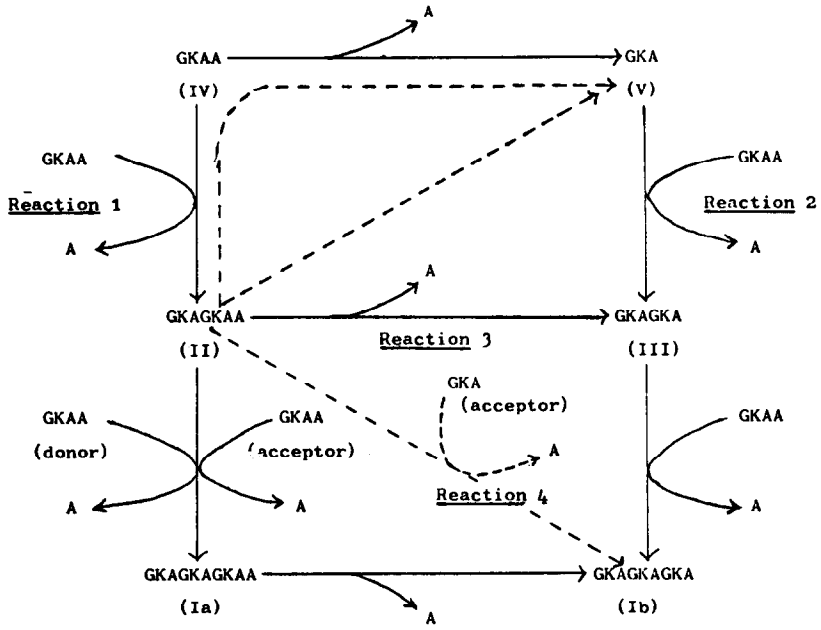

Scheme 1. Reactions leading to synthesis of dimers and trimers. In this scheme G = glycine, K = α-acetyl-L-lysine, A = D-alanine.

2. Materials and methods

The R61 enzyme [3], the tetrapeptide donor–acceptor

[^{14}C]Ac-L-Lys–D-Ala–D-Ala [1] and the tri-Gly—┘
peptide donor Ac_2-L-Lys–D-Ala–D-Ala

[4], were those previously used. In some experiments, *meso*-diaminopimelic acid was used as acceptor (through its amino group located on the D center) [5]. Unless otherwise stated, substrates and enzyme were incubated together in 2.3 mM sodium phosphate buffer pH 7.5, at 37°C, in 17% water, 58% ethylene glycol and 25% glycerol (in order to favor transpeptidation over hydrolysis) [6]. Separation of the reaction products was performed by paper electrophoresis at pH 1.8 [1], except when Ac_2-L-Lys–D-Ala–D-Ala was used as donor substrate in which case separation of the reaction products was performed by paper electrophoresis at pH 6.5 [5]. Radioactive tri-peptide monomer, heptapeptide dimer, hexapeptide dimer and mixed deca- and nona-peptide trimers were eluted from the paper strips and filtered on Sephadex G-15. If necessary, further purification was achieved by repeating the paper electrophoresis and Sephadex filtration.

3. Results

3.1. *Time course experiment (fig. 1)*

Tetrapeptide monomer (IV) (1 μmol) and 12 μg of enzyme were incubated at 37°C in a final volume of 150 μl. The substrate was utilized very rapidly, all of it disappearing after 40 min of incubation. At this time, 30% of the substrate was converted into tri-peptide monomer (V), 35% into heptapeptide dimer (II), 20% into hexapeptide dimer (III) and 10% into deca- and nonapeptide trimers (I). Tetramers were not detected.

The heptapeptide dimer (II) was produced rapidly (Reaction 1. Maximum yield: 40% of the total radioactivity after 10 min) and then it disappeared very slowly from the reaction mixture. The hexa-peptide dimer (III) was produced somewhat more slowly but it continued to appear even when the tetrapeptide monomer (IV) had completely dis-appeared, evidently because of hydrolysis of dimer

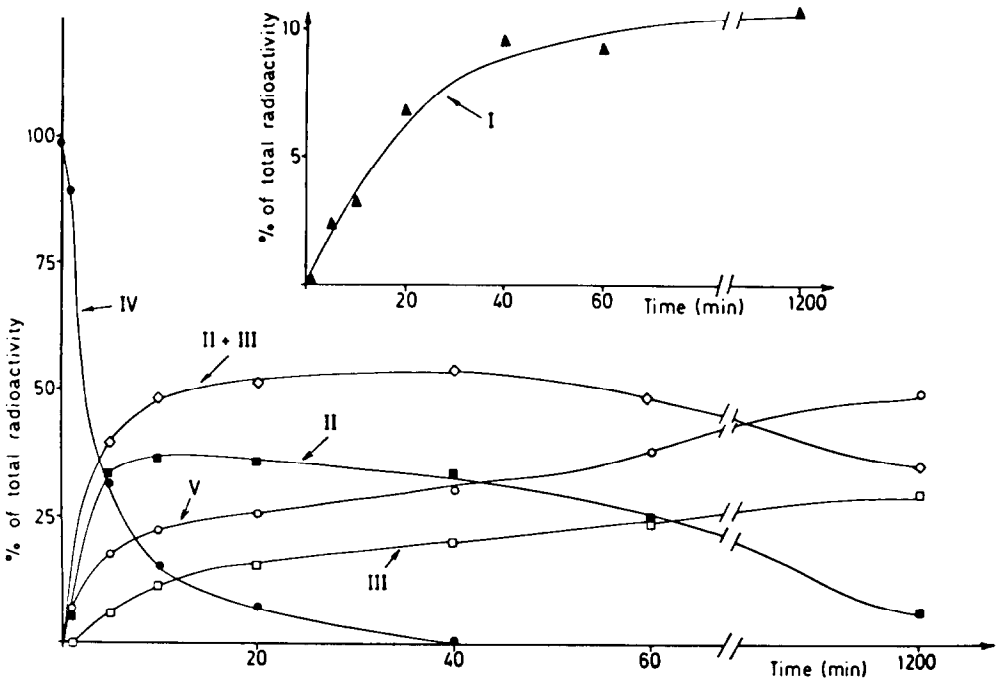

Fig.1. Time-course of the reaction. Tetrapeptide monomer (1 μmol) was incubated with 12 μg of enzyme in 3.3 mM sodium phosphate buffer pH 7.5, at 37°C and in 17% water, 58% ethylene glycol and 25% glycerol. After increasing times, samples (20 μl) were removed and analyzed by paper electrophoresis at pH 1.8. Numbering of the compounds refers to increasing electrophoretic mobilities (see 1): I = trimer (decapeptide + nonapeptide); II = heptapeptide dimer; III = hexapeptide dimer; IV = tetrapeptide monomer (substrate); V = tripeptide monomer.

(II) (Reaction 3). Since this latter reaction was so slow even after dimer II had attained its maximum concentration, the majority of dimer III present at 40 min must have been formed by transpeptidation between tetrapeptide monomer (IV) and tripeptide monomer (V) (Reaction 2). Tripeptide monomer (V) was synthesized at such a rate that it must have been present in sufficient concentration to act as an acceptor for synthesis of dimer III by Reaction 2. With time, the amount of tripeptide monomer (V) continued to increase slowly even after the disappearance of the tetrapeptide monomer (IV). The only explanation was that the enzyme preparation had a weak endopeptidase activity through which the peptide dimers formed were re-hydrolyzed into monomers. In fact, by incubating the isolated heptapeptide dimer (II) (1 mM) with 1 μg of enzyme in 20 μl (final volume) for 5 h at 37°C, tripeptide monomer (V) was formed (yield: 12% of the total radioactivity). At present, it is not known whether this endopeptidase activity is a genuine property of the R61 enzyme or is due to a contaminating enzyme.

Most of the trimers (I) were formed during the first 40 min of incubation (yield: 8–9% of the total radioactivity), while tetrapeptide monomer (IV) was still available. After that only a very small further increase occurred. This small increase was attributed to a transfer reaction between a heptapeptide dimer (II) acting as donor and a tripeptide monomer (V) acting as acceptor. Alternatively, the small amount of endopeptidase activity referred to above could have produced some trimer by transpeptidation of tripeptide from one hexapeptide dimer (III) to another, but at most this could only be a minor process. Hence, the great majority of the trimers formed during the first 40 min of incubation, must have resulted from transfer reactions between a tetrapeptide monomer (IV) acting as donor and hexa- and heptapeptide dimers (III and II) acting as acceptor.

3.2. *Direction of peptide trimer synthesis*

The results of the time-course experiment suggested that addition of tripeptide units at the N-terminus by transpeptidation from tetrapeptide monomer (IV) accounted for most of dimer and trimer formed. To confirm this idea the following experiments were carried out:

(1) Ac_2-L-Lys–D-Ala–D-Ala (which can act only as donor) (2 mM) was used in transpeptidation tests with 2 mM of either the tripeptide monomer (V), or the tetrapeptide monomer (IV) or the heptapeptide dimer (II) as acceptor (enzyme: 1 μg; final volume: 20 μl). The peptides were 100%, 70% and 20% respectively as efficient acceptors as *meso*-diaminopimelic acid (which is one of the best acceptors for the R61 enzyme). Hence, although dimers are formed most readily, a further tripeptide unit is also easily added at the N-terminal glycine of a dimer to form a trimer.

(2) *meso*-Diaminopimelic acid (which can act only as acceptor) (7.4 mM) was used in transpeptidation tests with the heptapeptide dimer (II) (0.55 mM). The incubation was carried out for 60 min at 37°C in fully aqueous medium (2.3 mM phosphate buffer pH 7.5), in the presence of 1.6 μg of enzyme. Of the heptapeptide dimer (II) used, 50% remained unchanged, 25% was converted to hexapeptide dimer (III) and 25% to the transpeptidation product hexapeptide-diaminopimelic acid. The ratio of transpeptidation to hydrolysis was thus 1.0, a value which was very close to the 1.7 value obtained by incubating the same acceptor *meso*-diaminopimelic acid and 1.7 mM of Ac_2-L-Lys–D-Ala–D-Ala (which is one of the best donor peptides) with the R61 enzyme [6]. The velocity of the reaction, however, was only 2% of that obtained with the donor Ac_2-L-Lys–D-Ala–D-Ala. Thus whereas tetrapeptide monomer (IV) is as good an acceptor for the R61 enzyme as the best known, the dimer (II) is relatively a very poor donor. Hence polymerization of monomer units by accretion at the N-terminus is strongly favoured.

(3) Tripeptide monomer (V) (which can act only as acceptor) (1.25 mM) and heptapeptide dimer (II) (1 mM) were incubated for 90 min with 1 μg of enzyme (final volume: 20 μl). Some dimer (0.9% of the total radioactivity) was converted into trimer (I). However, when tripeptide (V) monomer was replaced by the tetrapeptide monomer (IV), which can also act as a donor, the rate of trimer formation was at least ten times higher.

3.3. *Peptide tetramer formation*

Under the conditions devised for the time course experiment, tetramer formation was not detected. However, by incubating a large amount of tetrapeptide monomer (IV) (400 nmol) with 3 μg of enzyme for 80 min at 37°C, a radioactive compound representing about 1% of the total radioactivity was formed which had an electrophoretic mobility (8 cm/h, i.e. 75% of that of the heptapeptide dimer; see [1]) lower than that of the trimers. After extraction, this compound eluted from Sephadex G-15 with a K_D value lower than 0.1. Both values (see fig.2 in [1]) suggested that this high molecular weight compound was a peptide tetramer. Attempts to synthesize a peptide tetramer by transpeptidation between two units of dimer (II) failed.

4. Conclusions

Wall peptidoglycan growth in Gram-positive bacteria proceeds by attachment through transpeptidation, of the newly synthesized, nascent peptidoglycan to the 'old', pre-existing wall peptidoglycan. At least in *Bacillus licheniformis,* it has been shown that the peptide monomer units of the nascent peptidoglycan serve as donors in the reaction [7]. On the basis of the proportions of peptide monomers, dimers and trimers found in the wall peptidoglycan of *Streptococcus faecalis,* Oldmixon et al. [8] have reached the conclusion that peptide crosslinking is not a random polymerization process but that it proceeds by a monomer addition mechanism. Similarly, the in vitro synthesis of a peptide trimer by the exocellular DD-carboxypeptidase-transpeptidase of *Streptomyces* R61 is not a random process. It preferentially occurs by transpeptidation between a peptide monomer acting as donor and a preformed peptide dimer acting as acceptor. Moreover, peptide tetramer formation, if it occurs, does not occur by addition of dimers, but by serial addition of monomer units.

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