Formation of adenosine 5′-tetraphosphate from the acyl phosphate intermediate: a difference between the MurC and MurD synthetases of *Escherichia coli*

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Abstract The mechanism of the Mur synthetases of peptidoglycan biosynthesis is thought to involve in each case the successive formation of an acyl phosphate and a tetrahedral intermediate. The existence of the acyl phosphates for the MurC and MurD enzymes from *Escherichia coli* was firmly established by their in situ reduction by sodium borohydride followed by acid hydrolysis, yielding the corresponding amino alcohols. Furthermore, it was found that MurD, but not MurC, catalyses the synthesis of adenosine 5′-tetraphosphate from the acyl phosphate, thereby substantiating its existence and pointing out a difference between the two enzymes.

Key words: Acyl phosphate; Adenosine 5′-tetraphosphate; MurC; MurD; Peptidoglycan

1. Introduction

The biosynthesis of bacterial peptidoglycan is a complex two-stage process. The first stage consists in the formation of the disaccharide-peptide monomer unit, whereas the second stage concerns polymerisation and maturation reactions. The peptide moiety of the monomer unit is assembled by a series of cytoplasmic synthetases responsible for the successive additions of L-alanine, D-glutamic acid, *meso*-diaminopimelic acid (or L-lysine) and a dipeptide (generally D-alanyl-D-alanine) to UDP-*N*-acetylmuramoyl (MurNAc); these enzymes are referred to as MurC, MurD, MurE and MurF, respectively [1]. They catalyse the formation of an amide or peptide bond with concomitant cleavage of ATP into ADP and inorganic phosphate:

$$R - COOH + H_2N - R' + ATP \leftrightarrow R - CO - NH - R' + ADP + P_i$$

By analogy with enzymes catalysing the same type of reaction (e.g. glutamine synthetase [2]), it has been thought that their reaction mechanism consists in the activation of substrate R-COOH by ATP into an acyl phosphate; this species then undergoes the nucleophilic attack by substrate R′-NH_2 to yield a tetrahedral intermediate, which breaks down into amide (or peptide) and inorganic phosphate (Scheme 1). Work aimed at verifying this mechanism for the Mur synthetases has recently been performed, in particular to demonstrate the occurrence of acyl phosphate intermediates in their reaction pathway [3–8]. In the present paper, we firmly establish, by in situ sodium borohydride reduction, the formation of acyl phosphates by MurC and MurD; furthermore, we also show that MurD, but not MurC, can synthesise adenosine 5′-tetraphosphate (p_4A) from the acyl phosphate intermediate, thereby substantiating its existence and pointing out a difference between the two enzymes.

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP (110 TBq mmol^{-1}) and [8-^{14}C]ATP (1.92 Gbq mmol^{-1}) were purchased from Amersham France (Les Ulis, France), and p_4A and L-alaninol from Sigma (St. Louis, MO, USA). UDP-MurNAc [9], UDP-MurNAc-L-Ala [10], UDP-MurNAc-L-[^{14}C]Ala (5.44 Gbq mmol^{-1}) [11] and 2-amino-2-deoxy-3-*O*-[(*S*)-1-(hydroxymethyl)ethyl]-D-glucose [12] were prepared according to the published procedures. UDP-[^{14}C]MurNAc (9.27 Gbq mmol^{-1}) was synthesised from UDP-*N*-acetyl-D-[^{14}C]glucosamine (9.27 Gbq mmol^{-1}; Amersham France) by the actions of MurA and MurB ([13]; A. Bouhss and C. Parquet, unpublished results). MurC was purified from *Escherichia coli* JM83(pAM1005) by the procedure already described [14], except that phosphate was replaced by HEPES throughout the purification (G. Auger and D. Blanot, unpublished results); the enzyme was stored in 20 mM potassium HEPES, 1 mM EDTA, 2 mM dithiothreitol, 15% (v/v) glycerol, pH 7.2. MurD was purified from JM83(pMLD58) [10]; it was dialysed against 20 mM sodium HEPES, 1 mM dithiothreitol, pH 7.5.

2.2. Analytical procedures

HPLC was performed with the system of Hill and Arrio [15], slightly modified. A Supelcosil LC-18-DB column (3 μm, 33×4.6 mm; Supelco, L'Isle d'Abeau, France) and a mixture of 5% methanol and 95% 0.1 M potassium phosphate, 25 mM tetrabutylammonium hydrogen sulphate, pH 7, at 0.6 ml min^{-1} were used. The column was protected by a guard column of Hypersil ODS (5 μm, 10×4.6 mm; Hypersil France, Les Ulis, France). In some experiments, a UV2000 spectrophotometer (Thermo Separation Products, Les Ulis, France) allowing the on-line recording of the absorption spectra of the eluted compounds was included in the device. Detection was performed with a radioactive flow detector (model LB506-C1, EG&G Wallac/Berthold, Evry, France) using the Quicksafe Flow 2 scintillator (Zinsser Analytic, Maidenhead, UK) at 0.6 ml min^{-1}. Quantitation was carried out with a computer connected to the detector and equipped with the Winflow software (EG&G Wallac/Berthold). TLC was performed either on plates of silica gel 60 (Merck, Darmstadt, Germany) developed in dioxane/ammonium hydroxide/water 6:1:6 (v/v), or on PEI-cellulose plates (Macherey-Nagel, Düren, Germany) developed in 1.2 M guanidinium chloride. High-voltage electrophoresis was performed on 3469 filter paper (Schleicher and Schuell, Dassel, Germany) in water/pyridine/acetic acid 971:6:23 (v/v; pH 4.0) for 1 h at 40 V cm^{-1}, using an LT36 apparatus (Savant Instruments, Hicksville, NY,

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Abbreviations: p_4A, adenosine 5′-tetraphosphate; MurC, uridine diphosphate *N*-acetylmuramate:L-alanine ligase (ADP-forming) (EC 6.3.2.8); MurD, uridine diphosphate *N*-acetylmuramoyl-L-alanine:D-glutamate ligase (ADP-forming) (EC 6.3.2.9); MurNAc, *N*-acetylmuramoyl; PEI-cellulose, poly(ethyleneimine)-cellulose

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USA). Radioactive spots on chromatograms or electropherograms were detected by autoradiography (type R2 films, 3M, St. Paul, MN, USA) and quantitated with a radioactivity scanner (Tracemaster LB285, EG&G Wallac/Berthold).

2.3. Sodium borohydride reduction

MurC (0.57 nmol) was incubated for 60 min at 37°C in a 20-μl reaction mixture containing 0.1 M Tris-HCl, pH 8.6, 20 mM $MgCl_2$, 5 mM ATP and UDP-[^{14}C]MurNAc (2.86 nmol, 26.6 kBq). A control, in which the enzyme had been previously inactivated for 5 min at 100°C, was also performed. Reactions were quenched by addition of water (980 μl) and dimethyl sulphoxide (1.5 ml), and after cooling on ice for 5 min, $NaBH_4$ (5 mg) was added to each tube. After 16 h at room temperature, five volumes of water were added and the resulting solutions were lyophilised repeatedly to remove the dimethyl sulphoxide. The residues were taken up in water (500 μl) and treated with formic acid (50 μl) for 60 min to destroy the remaining borohydride. The pH was adjusted to 8.5 and the solutions were applied to Sephadex G-25 columns (50×5 mm) in order to remove borate ions. Elution was carried out at a flow rate of 60 μl min^{-1}. The radioactive fractions were pooled, lyophilised and hydrolysed (6 M HCl, 95°C, 16 h). The hydrolysates were evaporated and taken up with water, and the radioactive compounds were separated by high-voltage electrophoresis. The same procedure was essentially followed with MurD (0.60 nmol) and UDP-MurNAc-L-[^{14}C]Ala (3.83 nmol, 21.2 kBq), except that the concentration of $MgCl_2$ was 5 mM.

2.4. Chromatographic visualisation of p_4A

The reaction mixtures contained, in volumes of 10 or 20 μl: (i) for MurD, 0.1 M Tris-HCl, pH 8.6, 5 mM $MgCl_2$, 1 mM dithiothreitol, 12 μM MurD, 1 mM [γ-^{32}P]ATP (0.58 kBq μl^{-1}), and 100 μM UDP-MurNAc-L-Ala; (ii) for MurC, 0.1 M Tris-HCl, pH 8.6, 20 mM $MgCl_2$, 1 mM EDTA, 2 mM dithiothreitol, 36 μM MurC, 5 mM [γ-^{32}P]ATP (0.58 kBq μl^{-1}), and 1 mM UDP-MurNAc. Controls, in which the non-radioactive substrate or the enzyme was omitted, were also performed. The mixtures were incubated at 37°C, and 1-μl aliquots were removed. They were either spotted directly on silica gel plates, or mixed with 3 μl of 8 M urea prior to spotting on PEI-cellulose plates, or mixed with 99 μl of the HPLC elution buffer prior to injection.

3. Results

3.1. Reduction of the MurC and MurD acyl phosphates

Todhunter and Purich [16] used the borohydride reduction method [17] to identify the γ-glutamyl phosphate intermediate of the *E. coli* glutamine synthetase reaction by transforming it into the easily characterised alcohol derivative α-amino-δ-hydroxyvalerate. We adapted this procedure to MurC and MurD. Labelled substrate UDP-[^{14}C]MurNAc or UDP-MurNAc-L[^{14}C]Ala was incubated with ATP and the respective enzyme in order to form the acyl phosphate, which was then reduced by sodium borohydride. The resulting material was subjected to acid hydrolysis, theoretically yielding radioactive reduced muramic acid (2-amino-2-deoxy-3-*O*-[(*S*)-1-hydroxymethyl)ethyl]-D-glucose) for MurC, and radioactive L-alaninol for MurD. The radioactive amino alcohols were indeed clearly identified by comparing their electrophoretic migrations with those of unlabelled reference compounds revealed with ninhydrin (Fig. 1). With MurC the electropherogram of the reduced hydrolysed reaction mixture displayed three radioactive spots (Fig. 1A): muramic acid (−2.7 cm; 80% of the radioactivity spotted), an unidentified compound (−13 cm; 10%) and reduced muramic acid (−15 cm; 10%). Since only the two former spots were detected in the control with boiled MurC, it can be concluded that part of the substrate had been transformed into the alcohol derivative via the acyl phosphate. The intermediary spot, which was also present in an acid hydrolysate of UDP-[^{14}C]MurNAc alone (not shown), was undoubtedly a degradation product from muramic acid, which is known to be partially destroyed upon acid hydrolysis [18]. With MurD, three radioactive spots were also detected (Fig. 1B): alanine (−3.5 cm; 74% of the radioactivity spotted), an unidentified compound (−24 cm; 21%) and alaninol (−31 cm; 5%). However, only the alanine spot was present in the control with boiled MurD. Therefore, the procedure has given rise to the appearance of two radioactive products, one of them being alaninol. It was checked that alaninol is stable in the hydrolysis conditions used, thus rendering the possibility of the unknown spot to be a degradation

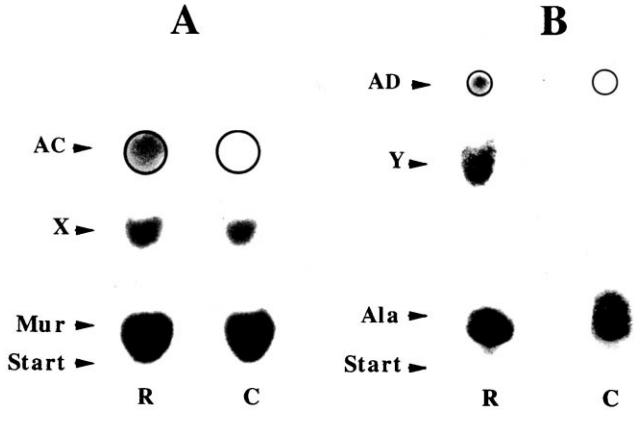


Fig. 1. Electrophoretic analysis of the sodium borohydride reduction experiments with MurC (A) and MurD (B). R, reaction; C, control with previously boiled enzyme; Mur, muramic acid; AC, reduced muramic acid; AD, alaninol; X, degradation product of muramic acid; Y, unidentified compound. The direction of migration is that of the anode. Circle, ninhydrin spot of the reference compound (reduced muramic acid or alaninol, 50 nmol) added to the sample prior to electrophoresis. For reasons of convenience, the two photographs are not at the same scale (see migration distances in Section 3).

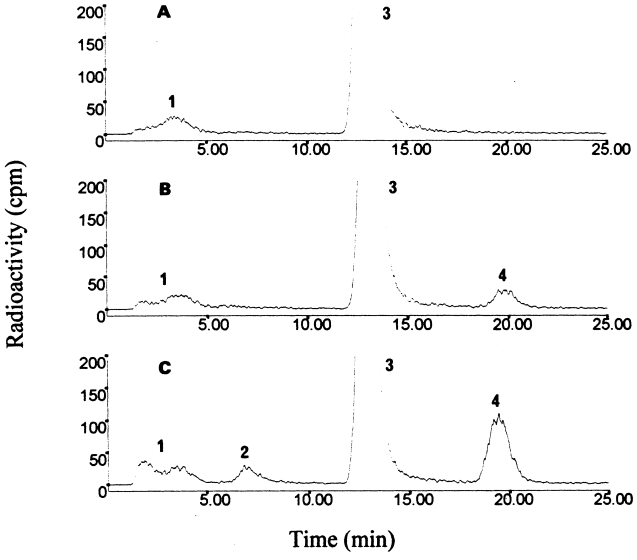


Fig. 2. Production of p_4A by MurD. The enzyme was incubated with [γ-^{32}P]ATP and UDP-MurNAc-L-Ala as described in Section 2, and the mixture was analysed by HPLC. A: [γ-^{32}P]ATP. B and C: Reaction mixture at $t = 10$ min and 90 min, respectively. Peaks: 1, early eluting compounds (inter alia, phosphate); 2, unidentified compound; 3, ATP; 4, p_4A.

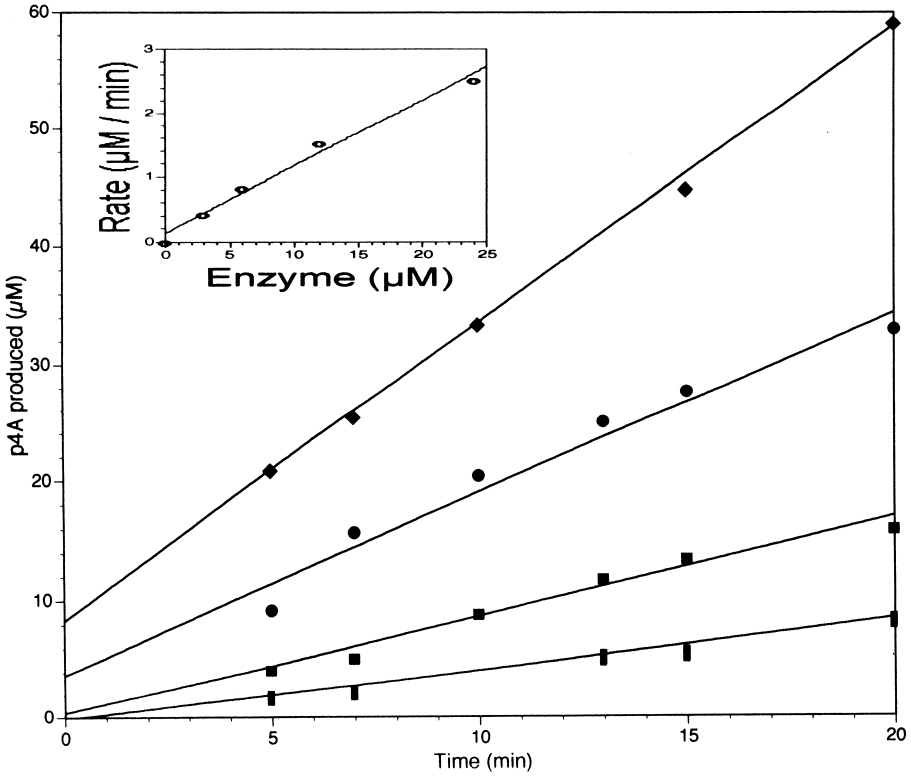


Fig. 3. Kinetics of formation of p_4A by MurD. The enzyme at different concentrations was incubated with [γ-^{32}P]ATP and UDP-MurNAc-L-Ala in the conditions described in Section 2. MurD concentrations were 24 μM (diamonds), 12 μM (circles), 6 μM (squares) and 3 μM (bars). The concentrations of p_4A indicated in ordinates take into account the double labelling of p_4A (see Section 3). Inset, plot of the rate of p_4A synthesis vs MurD concentration.

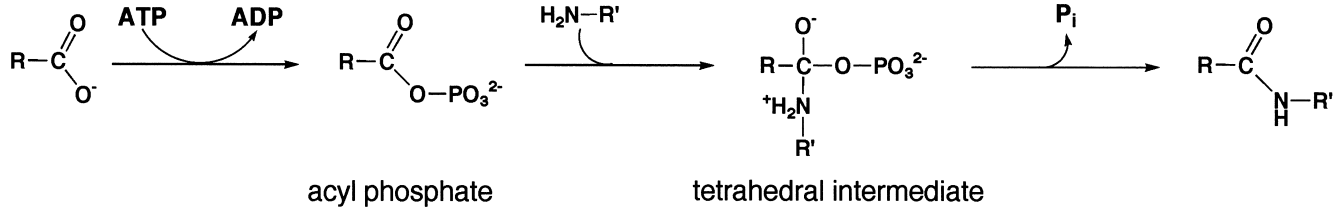
product unlikely. Owing to the minute amounts available, no further attempts were made to identify it.

Taking into account the radioactivity of the spots of reduced muramic acid and alaninol and assuming a quantitative yield for reduction, it was calculated that the amounts of acyl phosphates at $t = 60$ min were 0.50 and 0.32 mol mol^{-1} enzyme for MurC and MurD, respectively.

3.2. Formation of p_4A by MurD

When a mixture of MurD, [γ-^{32}P]ATP and UDP-MurNAc-L-Ala was analysed by ion-pairing reverse-phase HPLC, a new radioactive peak appeared at 19 min (Fig. 2). It was absent in controls lacking UDP-MurNAc-L-Ala or enzyme, as well as in a mixture containing 1 mM D-Glu. It was not present when UDP-MurNAc-L-[^{14}C]Ala and cold ATP were used, nor was it destroyed by 0.1 M HCl, 2 M NaOH or 0.8 M hydroxylamine, thereby excluding its identification as UDP-MurNAc-L-alanyl phosphate. Its UV absorption spectrum, recorded on-line, was characteristic of adenine, not uracil, nucleotides. This compound, which was detected by TLC on silica gel (R_F 0.22) and on PEI-cellulose (R_F 0.20) as well, had the same chromatographic properties as commercial p_4A. Its production, together with that of a stoichiometric amount of radioactive ADP, was also observed with [8-^{14}C]ATP instead of [γ-^{32}P]ATP.

Experiments were carried out to measure the kinetics of the reaction and to elucidate its mechanism. p_4A formation from [γ-^{32}P]ATP was linear up to 20 min (Fig. 3); the rate was proportional to the enzyme concentration (Fig. 3, inset). However, in the experiment with on-line UV spectrum recording, we noticed that the ratio p_4A/ATP calculated from the ^{32}P radioactivity was 1.7-fold that calculated from the integration of the A_{262} peaks. We therefore measured the rates of p_4A formation from [γ-^{32}P]ATP and [8-^{14}C]ATP and found that the former was 2.2-fold faster than the latter (Fig. 4). We thus concluded that a molecule of [^{32}P]p_4A synthesised in these conditions contained two ^{32}P atoms and originated from two [γ-^{32}P]ATP molecules. From all these data a rate constant of 0.129 ± 0.017 min^{-1} was deduced for p_4A synthesis.



Scheme 1.

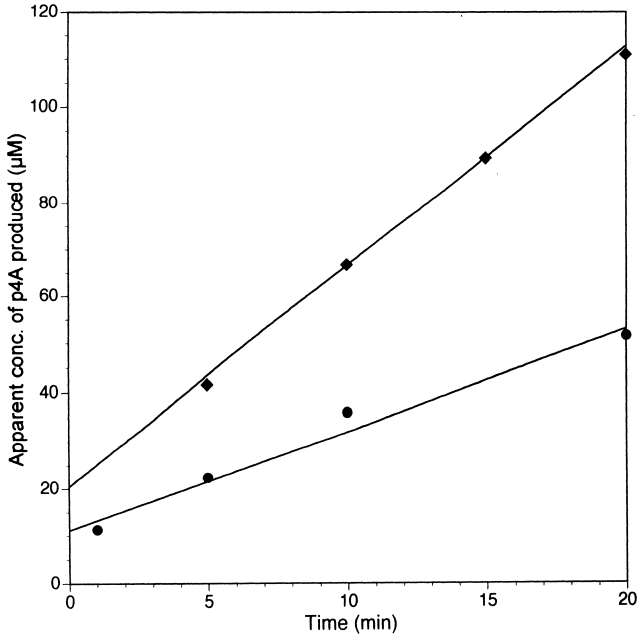


Fig. 4. Apparent rates of formation of [^{32}P]p_4A and [^{14}C]p_4A. MurD (24 μM) was incubated with UDP-MurNAc-L-Ala and [γ-^{32}P]ATP (♦) or [8-^{14}C]ATP (●). The calculated rates are 4.61 μM min^{-1} and 2.09 μM min^{-1}, respectively.

When MurC was incubated with [γ-^{32}P]ATP and UDP-MurNAc, no p_4A formation could be detected.

4. Discussion

Acyl phosphate intermediates can be identified as stable derivatives to which they have been converted [16,19]. In the present work, the alcohol derivatives originating from the chemical reduction of the acyl phosphates for MurC and MurD were detected, thereby firmly establishing the formation of such compounds. The possibility that these acyl phosphates are off-pathway, dead-end adducts is ruled out by the observation that isotope exchange reactions occur when the amino acid substrate (L-Ala for MurC, D-Glu for MurD) is present [4,6].

In spite of the similarity of mechanism (Scheme 1), a clear difference between the two reactions appeared with the observation of p_4A synthesis only by MurD. We showed that this synthesis was strictly dependent on the presence of enzyme, ATP and UDP-MurNAc-L-Ala and that it was accompanied by a stoichiometric production of ADP. Furthermore, p_4A originating from [γ-^{32}P]ATP possessed two ^{32}P atoms. All these data lead us to propose the following mechanism involving UDP-MurNAc-L-alanyl phosphate:

$$\mathrm{ATP} + \mathrm{UDP-MurNAc-L-Ala} \leftrightarrow \mathrm{ADP} + \mathrm{UDP-MurNAc-L-Ala-P}$$

$$\mathrm{UDP-MurNAc-L-Ala-P} + \mathrm{ATP} \leftrightarrow \mathrm{UDP-MurNAc-L-Ala} + p_4\mathrm{A}$$

the overall reaction being:

$$2\mathrm{ATP} \leftrightarrow \mathrm{ADP} + p_4\mathrm{A}$$

In this mechanism, UDP-MurNAc-L-Ala and ATP first yield the acyl phosphate and ADP, which is released and replaced by a second ATP molecule. Then, the acyl phosphate undergoes the nucleophilic attack by the γ-phosphoryl of newly bound ATP. The result is the formation of p_4A and the regeneration of UDP-MurNAc-L-Ala, which is available for a second turnover.

The linear fits for the curves of Figs. 3 and 4, at least for the highest enzyme concentrations, clearly displayed positive values at $t = 0$. This can be explained by the initial formation of an acyl phosphate burst [8,20]. The burst amplitudes for the two upper lines of Fig. 3 were 8.3 and 3.8 μM, respectively; this corresponded to 35% and 31% of the respective enzyme concentrations, values which are nearly identical to the one found in the borohydride reduction experiment (0.32 mol alaninol mol^{-1} MurD). These data support the involvement of the acyl phosphate in the synthesis of p_4A.

Further evidence in favour of this mechanism was brought by an experiment carried out in single-turnover conditions, i.e. with a [γ-^{32}P]ATP concentration (10 nM) much lower than that of the enzyme (12 μM). If ATP binding is not the limiting step of the reaction and if all the ATP molecules are bound to the enzyme, each ATP molecule should be consumed to produce one molecule of acyl phosphate, which could not yield p_4A since no more ATP would be available. As a matter of fact, no p_4A synthesis could be detected in these conditions, the only product observed being $^{32}P_i$, presumably arising from the hydrolysis of the acyl phosphate.

p_4A has been discovered in commercial preparations of ATP and in several biological extracts; it is produced in vitro, apparently as a by-product, by several ATP-utilising enzymes (see [21,22] and references therein). To the best of our knowledge, it is the first time that its synthesis by a synthetase of the peptidoglycan metabolism is elicited. The absence of detectable p_4A when the amino acid substrate (D-Glu) is present is explained by the much higher rate (400 min^{-1} [10]) of aminolysis versus that (0.129 min^{-1}) of p_4A formation. It is probably not produced in vivo under normal growth conditions since the intracellular concentration of D-Glu is in the millimolar range [23].

The reason for the difference between MurC and MurD regarding p_4A synthesis is currently unknown. It is possible that the active site of MurD is more flexible than that of MurC, allowing the binding of an ATP molecule while the ATP-binding site is partially occupied by the phosphoryl moiety of UDP-MurNAc-L-alanyl phosphate. The comparison of the 3D structures of MurD, which has recently been solved [24], and of MurC, which is not yet available, might help clarify this point.

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