Antibacterial action of dipeptides containing an inhibitor of glucosamine-6-phosphate isomerase

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Several dipeptides, containing the *N*³-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid (FMDP) moiety linked to protein and non-protein amino acids, exhibited a strong growth-inhibitory and bactericidal effect against *Bacillus subtilis*. FMDP-dipeptides were efficiently transported into bacterial cells by a di-tripeptide permease and subsequently cleaved by intracellular Mn²⁺/Co²⁺-dependent peptidases. Cleavage rates [0.1–5.6 µmol min⁻¹ (mg protein)⁻¹] were about two orders of magnitude lower than transport rates [40–200 µmol min⁻¹ (mg dry wt)⁻¹]. The released FMDP inactivated glucosamine-6-phosphate (GlcN-6-P) isomerase, an enzyme catalysing the first committed step in a biosynthetic pathway leading to amino sugar-nucleotide precursors of bacterial peptidoglycan. Inhibition of GlcN-6-P isomerase precluded peptidoglycan biosynthesis and resulted in a strong bacteriolytic effect. Results of the studies on consequences of GlcN-6-P isomerase inhibition upon the action of FMDP-dipeptides provided evidence demonstrating that the lack of endogenous GlcN-6-P could be a reason for the triggering of bacterial autolysis. Peptides containing the inhibitors of GlcN-6-P isomerase are one of the very few antimicrobial agents known that exhibit both bactericidal and fungicidal effects.

Keywords: dipeptides, *Bacillus subtilis*, cell wall

INTRODUCTION

The enzymes participating in the biosynthesis of peptidoglycan have long been considered attractive targets for antibacterial agents. Most of the known, established 'cell-wall-acting' agents interfere in the biosynthesis, assembly and cross-linking of the peptide part of bacterial peptidoglycan. Among them the most notable examples are the β -lactam antibiotics and glycopeptides such as vancomycin and teicoplanin. The presence of non-protein amino acids, including D-amino acids and *meso*-2,6-diaminopimelic acid (*meso*-DAP) in the peptidoglycan structure, has pointed to the enzymes in-

volved in their formation as potential targets. Indeed, several alanine analogues, such as β -haloalanines (Boisvert *et al.*, 1986), L-aminoethylphosphonic acid (Atherton *et al.*, 1979), L-aminomethylphosphonic acid (Atherton *et al.*, 1982), β -chloro- α -aminoethylphosphonic acid (Vo-Quang *et al.*, 1986) and D-cycloserine (Neuhaus & Hammes, 1981) were found to inhibit alanine racemase, D-Ala:D-Ala ligase and D-amino acid transaminase. Some interesting inhibitors of various enzymes of the *meso*-DAP biosynthetic pathway have also been proposed (Girodeau *et al.*, 1986; Berges *et al.*, 1986; Bauman *et al.*, 1988). On the other hand, very few compounds interfering in the biosynthesis of the (GlcNAc-MurNAc)_n core are known. The antibiotic fosfomycin inhibits phosphoenolpyruvate:UDP-GlcNAc-3-enolpyruvyl transferase (Kahan *et al.*, 1974).

The biosynthetic pathway leading to eventual formation of the amino sugar core of bacterial peptidoglycan is initiated with formation of D-glucosamine 6-phosphate (GlcN-6-P) (Warren, 1972) upon the action of L-glutamine:D-fructose-6-phosphate aminotransferase

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Abbreviations: FMDP, *N*³-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid; Fru-6-P, D-fructose 6-phosphate; GlcNAc, N-acetyl-D-glucosamine; GlcN-6-P, D-glucosamine 6-phosphate; DAP, 2,6-diaminopimelic acid; Nva, L-norvaline; UDP-MurNAc-tripeptide, UDP-N-acetylmuramyl-L-Ala- γ -D-Glu-*meso*-DAP; UDP-MurNAc-pentapeptide, UDP-N-acetylmuramyl-L-Ala- γ -D-Glu-*meso*-DAP-D-Ala-D-Ala.

(hexose-isomerizing) (EC 2.6.1.16) known under the trivial name GlcN-6-P isomerase. This enzyme was previously reported to be inhibited by a number of glutamine analogues such as 6-diazo-5-oxo-L-norleucine, azaserine, albizziin and anticapsin (Chmara *et al.*, 1986; Badet *et al.*, 1987). Unfortunately, these inhibitors are not selective and can interact with some other enzymes utilizing L-glutamine as a substrate. On the other hand, a rationally designed glutamine analogue, N^3 -(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid (FMDP), appears to be a strong and selective inhibitor of GlcN-6-P isomerase from prokaryotic (Chmara *et al.*, 1986; Andruszkiewicz *et al.*, 1986) and eukaryotic organisms (Milewski *et al.*, 1985). FMDP inactivates the enzyme, forming a covalent bond with the Cys-1 residue localized in the glutamine-binding domain (Kucharczyk *et al.*, 1990). Although FMDP itself is poorly transported into microbial cells and thus not able to inhibit microbial growth at low concentrations, incorporation of this compound into an oligopeptide structure, according to the 'warhead delivery' concept, has afforded a series of FMDP-oligopeptides exhibiting high antimicrobial activity, especially against human-pathogenic fungi and Gram-positive bacteria (Andruszkiewicz *et al.*, 1987, 1990).

In this paper we present the results of our studies on the factors limiting the growth-inhibitory activity and physiological consequences of the action of FMDP-dipeptides against *Bacillus subtilis*.

METHODS

Growth media. The following media were used. TSB, Tryptic Soy Broth (Difco). MBD, Minimal Broth Davis (Difco) supplemented with 0.1% each of glucose, Yeast Extract (Difco) and Casamino acids (Difco). MAD, MBD medium supplemented with 1.5% (w/v) Bacto Agar (Difco). CGPY, a rich growth medium containing (g l⁻¹): Na₂HPO₄, 6; NaCl, 3; NH₄Cl, 2; MgCl₂·6H₂O, 0.1; Na₂SO₄, 0.15; KH₂PO₄, 3; Bacto Peptone (Difco), 10; Yeast Extract, 0.1; pH 7.0. CWSM, a cell wall synthesis medium containing (g l⁻¹): KH₂PO₄, 6; K₂HPO₄, 6; NH₄Cl, 2; MgSO₄·7H₂O, 0.05; FeSO₄, 0.005; glucose, 2; uracil, 0.04; L-alanine, 0.005; L-glutamic acid, 0.12; meso-DAP, 0.05; Casamino acids, 1; Yeast Extract, 1; pH 7.0.

Micro-organism. *Bacillus subtilis* ATCC 6633 was used throughout the study. The cells were stored on MAD slants and transferred monthly.

Chemicals. FMDP and FMDP-peptides were synthesized in our laboratory, as described previously (Andruszkiewicz *et al.*, 1987). L-[2,3-³H]Alanine (sp. act. 2220 GBq mmol⁻¹), [5,6-³H]uridine (sp. act. 1295 GBq mmol⁻¹) and [U-¹⁴C]UDP-GlcNAc (sp. act. 7.4 GBq mmol⁻¹) were from Amersham. DL-[1-¹⁴C]Alanine (sp. act. 410 MBq mmol⁻¹) was from the Institute of Nuclear Research, Świerk, Poland. Penicillin G, vancomycin, D-cycloserine and 6-diazo-5-oxo-L-norleucine were from Sigma. Cloxacillin and flucloxacillin were from Beecham. All other chemicals were from Serva.

Estimation of antibacterial activity. MIC was determined by a serial dilution method in liquid MBD medium. Tubes containing serially diluted L-norvalyl-FMDP (Nva-FMDP) and control tubes, without the antibacterial agent, were inoculated with 5×10^6 c.f.u. ml⁻¹ of an overnight culture of *B. subtilis* in

MBD and then incubated for 18 h at 37 °C. MIC was defined as the lowest drug concentration preventing visible growth.

Disc diffusion assay. MAD medium (1 litre) was inoculated with 1 ml of the *B. subtilis* culture grown in TSB medium for 6 h at 37 °C. Aliquots (15 ml) of the seeded agar medium were poured into 100 mm Petri dishes. The FMDP-peptides were dissolved in 25 mM potassium phosphate buffer, pH 7.2, and the solutions were sterilized by filtration through 0.22 µm Millipore membrane filters. FMDP-peptide solutions (10 µl, containing 50 mmol FMDP-dipeptide) were adsorbed onto 13 mm Schleicher & Schuell discs and the discs were placed on the seeded agar surface in Petri dishes. For antagonism studies, discs saturated with solutions of different oligopeptides were co-applied with those containing FMDP-peptides. Plates were incubated at 37 °C for 18 h and growth inhibition zones were measured.

Uptake of peptides. *B. subtilis* cells, grown exponentially in MBD medium at 37 °C were harvested, washed with saline and resuspended in 50 mM potassium phosphate buffer, pH 7.0, containing 0.2% glucose to the final cell density corresponding to 0.3 mg dry wt cells ml⁻¹. Cell suspensions were preincubated at 37 °C for 10 min and peptides, previously dissolved in minimal aliquots of potassium phosphate buffer, were added to a final concentration of 100 µM. At that moment and at 10 s intervals, 2 ml samples of the cell suspension were withdrawn and filtered immediately through Whatman GF/C glass fibre filters under suction. Mixtures containing bacterial cells in phosphate buffer/glucose and 100 µM peptide in phosphate buffer/glucose were processed as negative and positive controls, respectively. Samples of the filtrate aliquots (1 ml) were taken for assay. The concentration of an oligopeptide remaining in filtrates was assayed by the colorimetric TNBS method (Milewski *et al.*, 1988).

Cleavage of dipeptides in cell-free extracts. *B. subtilis* cells grown overnight in MBD medium at 37 °C were harvested and washed with ice-cold saline. Wet cells were resuspended in small aliquots of 50 mM potassium phosphate buffer, pH 7.0, and subjected to ultrasonic disruption with a Branson sonifier. Cell wall debris was removed by centrifugation (15000 g, 4 °C, 20 min). Mixtures containing 100 µM dipeptide and the cell-free extract (1 mg protein ml⁻¹) in 50 mM potassium phosphate buffer, pH 7.0, were incubated at 37 °C. At zero time and at 10 min intervals, 0.5 ml samples of the incubation mixtures were withdrawn and taken for determination of free amino acids, using the Cd-ninhydrin method of Doi *et al.* (1981). In each case the results were corrected for a background level of free amino acids present in a control mixture containing the cell-free extract in phosphate buffer.

Purification of GlcN-6-P isomerase. Unless otherwise stated, all operations were performed at 4 °C. Buffers used were: buffer A, 25 mM potassium phosphate buffer, pH 7.5, 1 mM EGTA, 1 mM KCl, 1 mM DTT; buffer B, buffer A supplemented with 10 mM L-glutamine, 0.5 mM D-glucose 6-phosphate and 500 mM sucrose; buffer C, buffer A supplemented with 15 mM D-fructose 6-phosphate (Fru-6-P) and 500 mM sucrose. *B. subtilis* cells grown overnight in TSB medium at 37 °C were harvested, washed twice with ice-cold buffer A and resuspended in a small volume of buffer B. The dense cell suspension was subjected to sonic disruption with a Branson sonifier. The cell debris were removed by centrifugation (55000 g, 90 min). The supernatant (crude extract) was treated dropwise with a one-third volume of a 2% (v/v) solution of polymyxin sulfate in buffer B. The resulting suspension was centrifuged and the precipitate was discarded. The supernatant was diluted threefold with buffer B and applied to a

Cellex D column, equilibrated previously with buffer B. The column was washed with 100 ml buffer B and proteins were eluted with a 0–0.3 M KCl gradient in buffer B. Fractions containing GlcN-6-P isomerase activity were combined, diluted fourfold with buffer C and applied to a DEAE-Sepharose CL-6B column, previously equilibrated with buffer C. The column was washed with buffer C and then eluted with a 0–0.3 M KCl gradient in buffer C. Fractions containing GlcN-6-P isomerase activity were pooled. The partially purified enzyme exhibited a specific activity of $0.43 \mu\text{mol GlcN-6-P formed min}^{-1} (\text{mg protein})^{-1}$ and constituted 11% of protein present in the final preparation after the DEAE-Sepharose CL-6B column, as judged from densitometric SDS-PAGE analysis (not shown). The purity of this preparation was sufficient for kinetic studies since it was free from glutaminase and phosphoglucose isomerase activities. The enzyme stored in buffer C at 4 °C was relatively stable for 2–4 weeks.

Determination of GlcN-6-P isomerase activity. The standard incubation mixture contained 15 mM Fru-6-P, 5 mM L-glutamine, 1 mM DTT, 1 mM EGTA and appropriately diluted enzyme in 25 mM potassium phosphate buffer, pH 7.5. In kinetic experiments the concentration of one of the enzyme substrates was fixed and that of the other one was changed. Inhibitors were added if necessary. Samples were incubated at 37 °C for 30 min. The enzymic reaction was started by the addition of enzyme and was terminated by boiling for 1 min. The concentration of GlcN-6-P formed was determined by a modified Elson–Morgan procedure (Kenig *et al.*, 1976). One unit of enzyme activity is defined as the amount of enzyme catalysing the formation of $1 \mu\text{mol GlcN-6-P min}^{-1}$.

Inactivation of GlcN-6-P isomerase *in vitro*. Progress of time- and concentration-dependent inactivation of GlcN-6-P isomerase by FMDP was followed using the previously described method (Chmara *et al.*, 1986) applying a small column-centrifuge procedure of Penefsky (1977) for separation of the enzyme from the unbound inactivator.

Inactivation of GlcN-6-P isomerase *in vivo*. *B. subtilis* cells grown overnight in MBD medium were harvested, washed and resuspended in prewarmed fresh MBD medium to an OD_{660} of 0.5. After 30 min preincubation at 37 °C with shaking, inhibitors were added at appropriate concentrations. Incubation was continued for another 60 min. Samples of the cell cultures were rapidly cooled at time intervals, cells were harvested, washed with buffer A and resuspended in small volumes of ice-cold buffer B. Cells were disrupted by sonication, cell debris separated by centrifugation ($10\,000 g$, 4 °C, 10 min) and the crude extracts were passed through small Sephadex G-25 columns, equilibrated previously with buffer B. GlcN-6-P isomerase activity in protein-containing eluates was determined as described above.

Isolation of radioactively labelled cell wall precursors. *B. subtilis* cells grown exponentially in CGPY medium were washed and resuspended in CWSM medium containing $100 \mu\text{g chloramphenicol ml}^{-1}$ to the cell density of $5 \times 10^8 \text{ cells ml}^{-1}$. After 15 min preincubation at 37 °C, $148 \text{ kBq } [5,6\text{-}^3\text{H}]\text{uridine ml}^{-1}$ and an inhibitor at appropriate concentration were added. Incorporation was carried out for 30 min at 37 °C. The cells were harvested and the pellet was resuspended in a small volume of distilled water and heated for 15 min in a boiling water bath. After chilling in ice-cold water, cold 40% (w/v) TCA was added to the final concentration of 5%. The suspension was centrifuged, the pellet was discarded and 15 mg charcoal ($\text{ml supernatant}^{-1}$) was added. The suspension was gently shaken for 30 min and then pipetted into a small column (7 mm diam.). The non-adsorbed material was removed by five washings with 3 ml aliquots of distilled water.

The adsorbed material was eluted by four washings with 1 ml aliquots of ethanol/0.1 M ammonia (1:1, v/v) and these washings were combined. The solvent was removed under reduced pressure and the residue containing labelled cell wall precursors was dissolved in a small but defined volume of distilled water.

Cell wall precursors containing labelled uridine were separated by two-dimensional paper chromatography to avoid the possibility of interference with other uridine derivatives, such as UDP and UTP. Paper chromatography was performed on Whatman 3MM paper. Aliquots containing labelled precursors were applied as small spots at the corner and the chromatograms were first developed with ethanol/1 M ammonium acetate, pH 7.5 (7:3, v/v) and then in the second dimension with isobutyric acid/1 M ammonia (5:3, v/v). The chromatograms were air-dried and cut into $2 \times 2 \text{ cm}$ squares which were counted for radioactivity.

Nucleotides were identified by comparing their location with appropriate standard precursors labelled with L- $[^3\text{H}]\text{alanine}$, isolated and purified as described by Lugtenberg *et al.* (1972), or with commercially available $[\text{U-}^{14}\text{C}]\text{UDP-GlcNAc}$. The bound N-acetylhexosamine content was determined by the Elson–Morgan reaction, according to the procedure of Ward (1975).

Estimation of the peptidoglycan synthesis rate. *B. subtilis* cells grown overnight in CGPY medium at 37 °C were harvested, washed and resuspended in prewarmed CWSM medium. Growth was performed at 37 °C with vigorous shaking until OD_{660} reached 0.5–0.6. Chloramphenicol was added to a final concentration of $100 \mu\text{g ml}^{-1}$ and incubation was continued. After 15 min, $19 \text{ kBq DL-}[1\text{-}^{14}\text{C}]\text{alanine ml}^{-1}$ and an inhibitor at appropriate concentration were added. Samples of the cell suspension (0.5 ml) were withdrawn and combined with 5 ml ice-cold 10% TCA. The mixtures were kept on ice for at least 15 min and then heated at 80 °C for 15 min to remove teichoic acids. Precipitates were separated by filtration through Whatman GF/C glass fibre filters, washed with ice-cold 10% TCA, ice-cold water and ethanol, air-dried and counted for radioactivity.

Bacteriolytic effect. *B. subtilis* cells grown exponentially in CGPY medium at 37 °C were washed and resuspended in prewarmed MBD medium to an OD_{660} of 0.15. Cell suspensions were incubated at 37 °C with vigorous shaking. Inhibitors at appropriate concentrations were added after 15 min preincubation and the growth rate was followed by measurement of OD_{660} . Samples of the cell suspensions were collected at time intervals and taken for inspection by scanning electron microscopy.

Scanning electron microscopy. Microbial cells were harvested centrifugation at $2000 g$ for 5 min, washed four times with sterile saline, fixed for 12 h in 0.25% glutaraldehyde, washed four times and resuspended in 5 ml sterile demineralized water. A drop of each suspension was air-dried on $10 \times 10 \text{ mm}$ glass slides. The slides were attached to specimen stubs, coated with pure gold in a high-vacuum SEM coating unit and observed in a Stereo Scan 200 Cambridge electron microscope at 30 kV.

Other methods. General peptidase activity was determined by TNBS/ Cu^{2+} described by Payne (1972). Protein concentration was measured using the method of Bradford (1976). SDS-PAGE was performed using the discontinuous method of Laemmli (1970). Radioactivity was measured in a Beckman LS 3801 scintillation counter. Spectrophotometric measurements were made in a Perkin Elmer Lambda Bio UV/Visible spectrometer.

RESULTS AND DISCUSSION

Transport, cleavage and antimicrobial activity of FMDP-dipeptides

Several FMDP-containing dipeptides were examined to determine factors affecting their *in vitro* activity against *B. subtilis*. All the compounds inhibited bacterial growth but data presented in Table 1 clearly demonstrate that their antibacterial potency was strongly dependent on the primary dipeptide sequence. Generally, X-FMDP dipeptides were more active than their FMDP-X counterparts. The most active compound, namely Nva-FMDP, exhibited an MIC of $1.6 \mu\text{g ml}^{-1}$ in a serial dilution test in liquid MBD medium. No growth inhibition was observed for FMDP alone. This compound was not transported into *B. subtilis* cells, thus confirming an opinion suggesting the strict specificity of amino acid permeases (Anraku, 1980). On the contrary, FMDP-dipeptides were rapidly taken up by the bacteria with initial velocities quite comparable to those of some model dipeptides composed of protein amino acids. Actually, the uptake was so rapid that after a 30–40 s

period of linear progress, a substantial slowing down was observed, obviously due to the substantially decreased dipeptide concentration in the medium. Uptake rates shown in Table 1 were therefore calculated from the data measured for the initial 30 s period and thus reflect the true initial velocities. Such high velocities of uptake of FMDP-dipeptides must mean that transport into cells is unlikely to be a factor determining the antibacterial activity of these compounds, despite differences in the uptake rate dependent on dipeptide constitution. On the other hand, cleavage of the dipeptides by peptidases present in the crude extract prepared from *B. subtilis* cells was about two orders of magnitude slower than their uptake. As is shown in Table 1, especially low rates were observed for FMDP-X dipeptides. Therefore, one may assume that the velocity of releasing of the active 'warhead' inside the cells upon the action of intracellular peptidases is a factor limiting the antibacterial activity of FMDP-dipeptides, although FMDP-Ala, FMDP-Nva and FMDP-Leu do not seem to follow this rule. This situation is different from that found previously for

Table 1. Growth-inhibitory activity, uptake and cleavage rates of FMDP-peptides by *B. subtilis*

FMDP-peptide	Inhibition zone diameter (mm)*	Uptake rate $\pm$ SD [nmol min^{-1} (mg dry wt) $^{-1}$] $\dagger$	Cleavage rate $\pm$ SD [nmol min^{-1} (mg protein) $^{-1}$] $\ddagger$
Gly-FMDP	21	40 ± 8	1.8 ± 0.25
Ala-FMDP	31	175 ± 15	3.7 ± 0.15
Val-FMDP	31	200 ± 18	5.2 ± 0.2
Leu-FMDP	30	185 ± 13	4.6 ± 0.25
Nva-FMDP	32	190 ± 8	5.6 ± 0.25
Met-FMDP	30	162 ± 10	5.1 ± 0.3
Phe-FMDP	25	124 ± 7	3.2 ± 0.15
Tyr-FMDP	23	135 ± 9	3.8 ± 0.25
Lys-FMDP	15	40 ± 9	0.1 ± 0.2
FMDP-Gly	16	68 ± 6	0.2 ± 0.1
FMDP-Ala	25	103 ± 14	0.8 ± 0.15
FMDP-Val	21	86 ± 8	0.9 ± 0.15
FMDP-Leu	25	105 ± 10	0.8 ± 0.2
FMDP-Nva	26	102 ± 7	0.7 ± 0.15
FMDP-Met	18	122 ± 5	0.4 ± 0.1
FMDP-Phe	19	88 ± 8	0.35 ± 0.05
FMDP-Tyr	20	96 ± 9	0.45 ± 0.05
FMDP	No zone	Not detected	—

* Growth-inhibitory activity was determined by a disc diffusion assay on MAD agar inoculated with an exponentially growing culture (5×10^6 c.f.u. ml^{-1}) of *B. subtilis*. Discs contained 50 nmol of an FMDP-dipeptide. Petri dishes were incubated for 18 h at 37 °C and inhibition zones were measured to an accuracy of ± 1 mm.

$\dagger$ Samples of *B. subtilis* cell suspensions incubated at 37 °C in 50 mM potassium phosphate buffer, pH 7.0, containing 0.2% glucose and 100 μM dipeptide were withdrawn at 10 s intervals and immediately filtered. The concentration of the dipeptide remaining in the filtrate was estimated by the TNBS procedure.

$\ddagger$ Mixtures containing 100 μM dipeptide and cell-free extract prepared from *B. subtilis* cells (1 mg protein ml^{-1}) in 50 mM potassium phosphate buffer, pH 7.0, were incubated at 37 °C. At zero time and at 10 min intervals, samples of the incubation mixtures were withdrawn and taken for the determination of free amino acids.

Fig. 1. Dependence of the pseudo-first order rate constants of GlcN-6-P isomerase inactivation on FMDP concentration. Inset: a double reciprocal plot of the data according to Dixon & Webb (1979).

Candida albicans cells, where the low transport rates strongly affected the anticandidal activity of FMDP-dipeptides (Milewski *et al.*, 1991).

A disc diffusion experiment was performed to identify a permease transporting the FMDP-dipeptides into *B. subtilis* cells. The diameter of the growth inhibition zone [32 mm found for the action of Nva-FMDP (50 nmol per disc)] was reduced to 21–24 mm when any of the dipeptides L-Leu-L-Ala, L-Ala-L-Ala and L-Met-L-Val or tripeptides L-Ala-L-Ala-L-Ala and L-Gly-L-Leu-L-Tyr in 500-fold molar excess was co-applied with the FMDP-dipeptide. No decrease of the growth inhibition zone was observed for the co-applied dipeptide D-Ala-D-Ala and a tetrapeptide (L-Ala)₄. Similar results were obtained for other FMDP-peptides, thus indicating that these compounds are transported into *B. subtilis* cells by a stereospecific peptide permease. The multiplicity of the oligopeptide transport systems in *Escherichia coli* and *C. albicans* is well documented (Payne, 1980; McCarthy *et al.*, 1985; Milewski *et al.*, 1988). Gram-positive bacteria have not been extensively studied in this respect; however, the presence of an oligopeptide permease and a di-tripeptide permease in *B. subtilis* has been reported (Perego *et al.*, 1991; Mathiopoulou *et al.*, 1991). Since we found that the tetrapeptide (L-Ala)₄, unable to compete with Nva-FMDP in the disc diffusion test, was actually transported to *B. subtilis* cells at a rate of 70 nmol min⁻¹ (mg dry wt)⁻¹, it seems very likely that the uptake of (Ala)₄ is mediated by the oligopeptide permease and that of FMDP-dipeptides by the di-tripeptide permease.

Cleavage of FMDP-dipeptides by peptidases present in the crude extract was inhibited by 50% in the presence of 12 mM EDTA, but its velocity nearly doubled in the presence of 1 mM MnCl₂ or CoCl₂. When we separated

the components of the *B. subtilis* crude extract on the DEAE-cellulose column, eluted with a linear 0–0.5 M KCl gradient, three peaks showing Mn²⁺/Co²⁺-dependent dipeptidase activity, as determined using the method of Payne (1972), were found (details not shown). Two activities eluted at lower ionic strength were able to cleave FMDP-dipeptides and several dipeptides composed of protein amino acids. The third activity, eluted at a higher KCl concentration was specific for Gly-X dipeptides, including Gly-FMDP. These results confirm the multiplicity of intracellular dipeptidases in *B. subtilis* demonstrated by Desmond *et al.* (1975).

Interaction of the inhibitor with an intracellular target

With regard to GlcN-6-P isomerase of different origin, FMDP was previously found to be a stronger inhibitor than its structural analogue, present as the C-terminal amino acid of a dipeptide antibiotic A19009 (Chmara *et al.*, 1986). The partially purified GlcN-6-P isomerase from *B. subtilis* was also inhibited by this amino acid. Kinetic analysis of this process revealed that the inhibition was competitive with regard to L-glutamine ($K_i = 4 \times 10^{-7}$ M) and uncompetitive with regard to Fru-6-P (graphs not shown). The low K_i/K_M ratio (1.25×10^{-3}) reflects the high affinity of FMDP to the L-glutamine binding site of the enzyme and an uncompetitive mode of inhibition in respect of Fru-6-P indicates that the inhibitor binds predominantly to the [enzyme:Fru-6-P] complex.

Incubation of *B. pumilus* GlcN-6-P isomerase with FMDP at different concentrations, in the absence of L-glutamine, caused time- and concentration-dependent inactivation of the enzyme. When the natural logarithm of enzyme residual activity, determined after separation of the enzyme protein from an excess of unbound inhibitor by enforced gel filtration, was plotted versus

Table 2. Inactivation of GlcN-6-P isomerase in *B. subtilis* cells by FMDP-peptides *in vivo*

Exponentially growing *B. subtilis* cells were treated with FMDP-dipeptides. At time intervals sample cells were harvested, washed and broken. Cell-free extracts were passed through small Sephadex G-25 columns and GlcN-6-P isomerase activity was determined in eluates. The enzyme activity in cell-free extracts prepared from control cells (not treated with FMDP-dipeptides) was practically constant for 60 min at 7.2 ± 0.2 nmol GlcN-6-P min⁻¹ (mg protein)⁻¹.

Peptide (μM)	Percentage inhibition of GlcN-6-P isomerase activity after:		
	10 min	30 min	60 min
Gly-FMDP (2)	23	44	62
Nva-FMDP (2)	52	83	Bacteriolysis
Nva-FMDP (10)	85	Bacteriolysis	Bacteriolysis
FMDP-Ala (2)	15	26	45

incubation time, a set of straight lines was obtained (not shown). Kinetic analysis of this inactivation, shown in Fig. 1, revealed a hyperbolic dependence of apparent pseudo-first order rate constants, k_{app} , on FMDP concentration. On the other hand, when k_{app}^{-1} was plotted against the reciprocal of FMDP concentration, a straight line was obtained with a non-zero intercept. This behaviour is consistent with the idea of formation of a reversible [enzyme:inhibitor] complex before irreversible modification of the enzyme, according to the scheme:

Steady-state treatment of this process yields the equation (Dixon & Webb, 1979):

$$1/k_{app} = K_I/k_2 \times 1/[I] + 1/k_2$$

where an inactivation constant, $K_I = k_{-1} + k_1/k_2$, reflects the affinity of an inactivator to its binding site and k_2 is the rate constant for the irreversible modification. We found these values to be $K_I = 1.42 \times 10^{-6}$ M and $k_2 = 0.586 \text{ min}^{-1}$. The well-known, commercially available glutamine analogue, 6-diazo-5-oxo-L-norleucine, showed much lower affinity to the *B. subtilis* GlcN-6-P isomerase active site ($K_I = 75 \text{ } \mu\text{M}$).

Inactivation of GlcN-6-P isomerase could also be demonstrated under *in vivo* conditions when suspensions of *B. subtilis* cells were incubated with FMDP-dipeptides and the enzyme activity was determined in crude extracts prepared from the harvested cells. Low molecular mass components were removed from the extracts by gel filtration before determination of GlcN-6-P isomerase activity. The results of this experiment are summarized in Table 2. Concentrations of FMDP-dipeptides used in this experiment were rather low, since

Fig. 2. Influence of Nva-FMDP on incorporation of DL-[1-¹⁴C]-Ala into peptidoglycan of *B. subtilis* cells. □, Control; ●, 1 μM Nva-FMDP; ■, 5 μM Nva-FMDP; ▲, 1 μM Nva-FMDP + 1 mM GlcNAc; ▼, 1 μM Nva-FMDP + 10 mM GlcNAc. Bars indicate SD (three replicates). *B. subtilis* cells grown exponentially in CWSM medium were treated for 15 min with 100 μg chloramphenicol ml⁻¹ and then labelled with 19 kBq DL-[1-¹⁴C]alanine ml⁻¹ in the presence or absence of Nva-FMDP. Samples of the cell suspension (0.5 ml) were withdrawn, treated with ice-cold 10% TCA for 15 min and then heated at 80 °C for 15 min to remove teichoic acids. Precipitates were separated by filtration through Whatman GF/C glass fibre filters, washed with ice-cold 10% TCA, ice-cold water and ethanol, air-dried and counted for radioactivity.

at higher concentrations the immediate lysis of bacteria precluded successful harvesting of the cells. Nevertheless, data presented in Table 2 clearly demonstrate that GlcN-6-P isomerase present in *B. subtilis* cells

Table 3. Accumulation of nucleotide precursors of peptidoglycan in *B. subtilis* cells treated with Nva-FMDP and other antibiotics

B. subtilis cells suspended in CWSM medium containing 100 μg chloramphenicol ml⁻¹ were labelled with 148 kBq [5,6-³H]uridine ml⁻¹ for 30 min at 37 °C in the presence or absence of cell-wall-acting antibiotics. Then the cells were harvested and broken by consecutive boiling and treatment with 40% TCA. Labelled nucleotide precursors present in supernatants obtained after removal of cell debris were adsorbed on charcoal and recovered by elution with ethanol/0.1 M ammonia (1:1, v/v) mixture. The bound N-acetylhexosamine content was determined by the Elson-Morgan reaction. Labelled precursors were separated by two-dimensional paper chromatography and nucleotides were identified by comparing their location with appropriate standard precursors. Results presented in the table are the means of three independent determinations ± SD. ND, Not detected.

Treatment (μg ml ⁻¹)	Bound N-acetylhexosamine (μmol g dry wt ⁻¹)	Precursor accumulated (c.p.m. per spot ± SD)		
		UDP-GlcNAc	UDP-MurNAc-tripeptide	UDP-MurNAc-pentapeptide
Control	1.8 ± 0.1	368 ± 30	91 ± 18	214 ± 22
Nva-FMDP (4)	<0.1	18 ± 11	ND	ND
D-Cycloserine (4)	25.3 ± 0.3	375 ± 41	2148 ± 53	234 ± 18
Cloxacillin (4)	28.4 ± 0.3	412 ± 32	586 ± 17	2218 ± 41
Vancomycin (4)	39.6 ± 0.2	332 ± 19	348 ± 43	3865 ± 120

Fig. 3. Bacteriolysis of *B. subtilis* cells. (a) Bacteriolysis induced by Nva-FMDP and a protective effect of GlcNAc. □, Control; ■, 0.2 μM Nva-FMDP; ▲, 1 μM Nva-FMDP; ▼, 5 μM Nva-FMDP; ●, 25 μM Nva-FMDP; △, 5 μM Nva-FMDP + 1 mM GlcNAc; ○, 5 μM Nva-FMDP + 10 mM GlcNAc. (b) Bacteriolysis induced by Nva-FMDP and some other antibiotics. □, Control; ○, 2 μM Nva-FMDP; ▲, 2 μM penicillin G; ▼, 2 μM cloxacillin; ■, 0.4 μg vancomycin ml⁻¹. (c) Effect of chloramphenicol on bacteriolysis induced by Nva-FMDP. □, Control; ■, 5 μM Nva-FMDP added at zero time; ●, 100 μg chloramphenicol ml⁻¹ added at zero time and 5 μM Nva-FMDP added after 30 min; ▼, 100 μg chloramphenicol ml⁻¹ added at zero time and 5 μM Nva-FMDP added after 15 min; ▲, 100 μg chloramphenicol ml⁻¹ and 5 μM Nva-FMDP added at zero time.

treated with FMDP-dipeptides underwent progressive, irreversible inactivation, since the gel filtration did not restore the original enzyme activity. The progress of this inactivation was much slower for FMDP-Ala. Since these compounds and other FMDP-X dipeptides were shown to be much more slowly hydrolysed by the intracellular peptidases than their structural isomers (Table 1), data presented in Table 2 provide some evidence that only free FMDP can inactivate the *B. subtilis* GlcN-6-P isomerase. This is contrary to the situation found previously for the *C. albicans* enzyme, where the FMDP-X dipeptides were able to inactivate the enzyme, although the rate of this process was rather low (Milewski *et al.*, 1991). On the other hand, the relatively high antibacterial activity of FMDP-Ala, FMDP-Nva and FMDP-Leu indicates that at least some intact FMDP-peptides can inhibit bacterial GlcN-6-P isomerase.

Physiological consequences of GlcN-6-P isomerase inactivation

GlcN-6-P isomerase is the first unique enzyme in a biosynthetic pathway leading to eventual formation of

UDP-GlcNAc which, in turn, is a precursor of the main structural component of bacterial peptidoglycan. Therefore, one would expect that FMDP-peptides should strongly affect peptidoglycan biosynthesis.

When *B. subtilis* cells were treated with 4 μg Nva-FMDP ml⁻¹ for 30 min, a dramatic depletion in the intracellular pool of bound *N*-acetylhexosamines was observed (Table 3). On the other hand, the action of cloxacillin and vancomycin, i.e. antibiotics acting at later stages of the peptidoglycan biosynthetic pathway, induced substantial accumulation of *N*-acetylhexosamine derivatives. Two-dimensional paper chromatography enabled separation and identification of accumulated, radioactively labelled precursors. UDP-MurNAc-tripeptide (UDP-*N*-acetylmuramyl-L-Ala-γ-D-Glu-*meso*-DAP) and UDP-MurNAc-pentapeptide (UDP-*N*-acetylmuramyl-L-Ala-γ-D-Glu-*meso*-DAP-D-Ala-D-Ala) accumulated in cells treated with cloxacillin and vancomycin – antibiotics that interfere in the assembly of bacterial peptidoglycan. The action of D-cycloserine, an inhibitor of alanine racemase, caused accumulation of UDP-MurNAc-tripeptide only, but no nucleotide precursors accumulated upon treatment with Nva-FMDP.

Fig. 4. Scanning electron micrographs of *B. subtilis* cells. (a) Untreated; (b) treated with 5 µM Nva-FMDP for 30 min. Bars, 20 µm.

Inhibition of peptidoglycan biosynthesis by Nva-FMDP was confirmed in another experiment where exponentially growing bacterial cells were labelled with DL-[1-¹⁴C]-alanine. The incorporation was carried out in the presence of chloramphenicol to protect the cells from lysis and prevent incorporation of the label into cell proteins. Data presented in Fig. 2 show that Nva-FMDP, even at a concentration as low as 1 µM, effectively inhibited the incorporation of the radioactive label into peptidoglycan. The presence of 1 mM GlcNAc partially reversed the inhibitory effect and 10 mM GlcNAc prevented it almost completely.

One of the consequences of Nva-FMDP action on *B. subtilis* cells was a fast induction of bacteriolysis. As shown in Fig. 3(a), in the presence of 1 µM Nva-FMDP cells continued to grow for about 30 min, but then a decrease in the OD of the cell suspension, indicating lysis, was observed. The time to lysis became shorter at higher concentrations of Nva-FMDP, but it was never shorter than 15 min, which corresponds to half the generation time for *B. subtilis* cells growing exponentially in MBD medium. It should be mentioned that

while the lysis induced by Nva-FMDP at lower concentrations (1–2 µM) was followed by partial re-growth, observed after 120–360 min, no such phenomenon was observed at Nva-FMDP concentrations ≥ 5 µM. In the presence of 1 mM GlcNAc the time to lysis induced by 2 µM Nva-FMDP was prolonged to 90 min, while 10 mM GlcNAc protected the cells against lysis.

The bacteriolysis caused by Nva-FMDP was compared to that induced by other cell-wall-acting antibiotics. Fig. 3(b) shows that the bacteriolytic effect of 2 µM Nva-FMDP is comparable to that caused by cloxacillin and more pronounced than that exhibited by penicillin G.

Lysis of *B. pumilus* cells induced by FMDP-dipeptides could be prevented by chloramphenicol, an antibiotic that inhibits protein biosynthesis in bacteria. However, in this case the time of addition was an important factor. As is shown in Fig. 3(c), 100 µg chloramphenicol ml⁻¹ added simultaneously with Nva-FMDP had no effect. On the other hand, addition of this antibiotic 15 min prior to Nva-FMDP almost completely prevented lysis and only growth inhibition was observed. According to current views, the bacteriolytic action of compounds inhibiting peptidoglycan biosynthesis is a consequence of induction of endogenous autolytic enzymes (Tomasz, 1979; Kitano & Tomasz, 1979). It is therefore assumed that the primary effect of action of these antibacterial agents is growth inhibition and only the triggering of autolytic, cell-wall-degrading enzymes is responsible for the bactericidal effect (Rogers & Forsberg, 1971). Our experiment provided evidence confirming this assumption in the case of the action of an agent inhibiting the earliest possible step in the peptidoglycan biosynthetic pathway.

Bacteriolysis of *B. subtilis* cells induced by Nva-FMDP was confirmed by scanning electron microscopy. Cells treated with the agent were collapsed and a massive amount of unidentified debris was observed (Fig. 4b), in contrast to normally shaped control cells (Fig. 4a). Similar changes have been noted using optical microscopy for glucosamine mutants of *B. subtilis* (Freese *et al.*, 1970).

The complete reversibility of Nva-FMDP-induced growth inhibition and lysis points at GlcN-6-P isomerase as being the only enzyme important for the bacterial growth and viability that was inhibited upon the action of the FMDP-dipeptide. Such selectivity is surprising in light of the fact that GlcN-6-P isomerase is a member of the amidotransferase family, comprising several enzymes transferring an amino group from the γ -amido function of L-glutamine to different acceptors (Zalkin, 1993). FMDP, being a glutamine analogue, could theoretically interact with other amidotransferases. Indeed, another glutamine analogue, 6-diazo-5-oxo-L-norleucine (DON) has been reported to show a pleiotropic effect. The bacteriostatic effect exerted by this agent could be prevented only by the simultaneous presence of GlcNAc and inosine, thus indicating that GlcN-6-P isomerase and any amidotransferase(s) involved in the purine biosynthetic pathway were targeted

by DON (Coggin & Martin, 1965). The selectivity of FMDP is therefore worth mentioning.

The following final conclusions based on the results of our studies presented above can be drawn. (1) The action FMDP-peptides against *B. subtilis* comprises three stages. (i) Rapid active transport into the cells, probably mediated by a di-tripeptide permease. (ii) Intracellular cleavage by any of the three Mn^{2+}/Co^{2+} -dependent peptidases. The rate of this process, much lower than that of uptake, seems to be the main factor determining the antibacterial activity of FMDP-peptides. (iii) Irreversible modification of GlcN-6-P isomerase by the released FMDP. The 'warhead' release is indispensable for its effect as an enzyme inactivator. (2) Irreversible blockage of GlcN-6-P isomerase results in a cessation of the intracellular pool of nucleotide precursors of peptidoglycan biosynthesis and triggers bacteriolysis. (3) GlcN-6-P isomerase is the only intracellular target important for cell growth and viability that is inhibited by FMDP.

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