Metabolism of DD-2,6-Diaminopimelic Acid by a Diaminopimelate-requiring Mutant of *Bacillus megaterium*

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A diaminopimelate-requiring mutant of *Bacillus megaterium* was shown to lack the enzyme *N*-acetyl-LL-diaminopimelate deacylase. By repeated subculture of this mutant, a second-stage mutant was derived that could grow slowly in minimal defined medium supplemented with DD-diaminopimelate alone. After growth in such medium, the peptidoglycan of the wall still contained mainly *meso*- and LL-diaminopimelate; radioactivity from DD-diamino-[¹⁴C]pimelate entered peptidoglycan (as diamino[¹⁴C]pimelate) and protein (as [¹⁴C]lysine) to similar extents.

INTRODUCTION

The DD-isomer of 2,6-diaminopimelic acid (A₂pm) accounted for about 7% of the total A₂pm present in acid-hydrolysates of walls of *Bacillus megaterium* NCIB 7581 (Day & White, 1977). This organism slowly took up DD-[¹⁴C]A₂pm during growth and most of the radioactivity incorporated into peptidoglycan was in the form of *meso*-[¹⁴C]A₂pm (Day & White, 1979), suggesting that DD- and *meso*-A₂pm are enzymically interconvertible.

In the present study, a diaminopimelate-requiring mutant of *B. megaterium* was grown with DD-A₂pm as the sole source of this amino acid. The *meso*- and LL-isomers of A₂pm still predominated in the peptidoglycan, and when DD-[¹⁴C]A₂pm was given, [¹⁴C]lysine was found in the protein, which implies that free *meso*-A₂pm must have been formed from the DD-A₂pm.

METHODS

Organisms. *Bacillus megaterium* NCIB 7581 and a diaminopimelate-requiring mutant of this strain (NCIB 11251; here called mutant SW1) have been described by Saleh & White (1976). A second-stage mutant, derived from mutant SW1 (see below) and called mutant SW2, could grow on DD-A₂pm as well as the other two isomers of A₂pm. All were maintained on slopes of nutrient agar or of medium FGB (see below) with synthetic A₂pm (100 mg l⁻¹) added for the mutants; incubation was at 37 °C overnight, and stock cultures were kept at 2 °C.

Media. All media were made with glass-distilled water. Medium FGB (Saleh & White, 1976) was used except where stated otherwise, with glycerol (1%, w/v) as the main source of carbon. Trisodium citrate dihydrate (20 mg l⁻¹) was generally added. Medium A (White, 1972) supplemented with biotin (20 µg l⁻¹) and sodium citrate (as above) was used in some experiments. Difco agar (1.5%, w/v) was added for solid media.

Conditions of growth. Organisms were grown at 37 °C in shaken flasks and growth was assessed from turbidity measurements as described previously (White, 1972).

Assays for isomers of A₂pm. The procedures described by Day & White (1979) were used.

Preparation of isomers of A₂pm. Unlabelled isomers were isolated as described by Day & White (1977). Isomers of [¹⁴C]A₂pm were separated essentially as described by Day & White (1979). To ensure the absence of *meso*-[¹⁴C]A₂pm, the DD-[¹⁴C]A₂pm was incubated with diaminopimelate decarboxylase for a second time, after the separation from LL-[¹⁴C]A₂pm by chromatography.

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Measurement of radioactivity. Samples in the scintillation fluid of Bray (1960) were counted by the channels ratio method in a Nuclear-Chicago apparatus (Isocap/300). Developed chromatograms were placed in contact with Kodirex X-ray film for appropriate times to locate labelled compounds. Radioactive areas were counted by cutting strips of the chromatograms into pieces and counting each piece.

Chromatography. Descending chromatograms on Whatman no. 1 paper were developed in solvent A (methanol/water/10 M-HCl/pyridine; 80:17.5:2.5:10, by vol.). This separates LL-A₂pm from the *meso*- and *DD*-isomers, which run more slowly and almost together (Rhuland *et al.*, 1955); lysine runs well in front of LL-A₂pm. Amino acids were detected by dipping the dried paper in ninhydrin (0.1 % in acetone), drying and then heating at about 80 °C for 5 min.

Isolation and hydrolysis of walls. The procedures of Day & White (1977) were used. Before assay of isomers of A₂pm in hydrolysates, the A₂pm was adsorbed on Dowex 50 and eluted with aqueous ammonia as described by Day & White (1977).

Isolation of peptidoglycan and material dissolved by trypsin (protein). The slightly modified procedure (Wilkinson & White, 1973) of Park & Hancock (1960) was used to isolate peptidoglycan and protein (material dissolved by trypsin) from organisms grown with [¹⁴C]A₂pm. Radioactivity was measured in the supernatant liquid left after each step in the treatment, and in the acid-hydrolysate of the final solid residue (peptidoglycan).

Assay of *N*-acetyl-LL-diaminopimelate deacylase. *Bacillus megaterium* 7581 and mutant SW1 were grown at 37 °C in medium A1, with synthetic A₂pm (100 µg ml⁻¹) added for both organisms; the wild-type was also grown in the absence of A₂pm. Bacteria were harvested during the exponential growth phase, washed and crude extracts prepared as for the purification of diaminopimelate epimerase (White *et al.*, 1969). The deacylase was measured as described by Sundharadas & Gilvarg (1967), except that formation of A₂pm was usually determined by the method of Work (1957) as follows: samples (100 µl) from the enzymic assay mixture were added to tubes containing acetic acid (0.8 ml) at room temperature, and then the ninhydrin reagent (0.1 ml) was added. Absorbance at 440 nm was measured after incubating the tubes at 37 °C for 90 min. Assay mixtures without enzyme, or without substrate, or with A₂pm in place of substrate were incubated as controls. *N*-Acetyl-LL-diaminopimelate and lysine gave no colour in this assay.

Accumulation of *N*-acetyl-LL-diaminopimelate by mutant SW1. Organisms were harvested after incubation for 3 d at 37 °C in medium A1 plus synthetic A₂pm (40 mg l⁻¹). With this limiting concentration of A₂pm, the stationary phase had been reached after 1 d. Pool amino acids were extracted with 0.25 M-perchloric acid as described by Tempest *et al.* (1970). *N*-Acetyl-LL-diaminopimelate in this extract was recognized by its behaviour in the colorimetric assay of Sundharadas & Gilvarg (1967), which involves heating at 100 °C with acidic ninhydrin. Maximum absorbance (at 420 nm) is produced after 20 min with *N*-acetyl-LL-diaminopimelate, A₂pm or lysine, but whereas A₂pm and lysine give about 90 % of maximum absorbance after heating for only 5 min, *N*-acetyl-LL-diaminopimelate gives only 14 to 25 % of the maximum in this time. The pool extract contained material which gave a peak on the amino-acid analyser trace near the position of α -amino-adipic acid (as does authentic *N*-acetyl-LL-diaminopimelate) but no A₂pm was present. After heating the pool extract at 105 °C for 18 h in 6 M-HCl, the peak near α -aminoadipate had disappeared and a peak in the position of A₂pm was found.

Isolation of a second-stage mutant able to grow on *DD*-A₂pm. A large inoculum of mutant SW1 (about 10⁷ colony-forming units) was plated on medium FGB containing *DD*-A₂pm (100 µg ml⁻¹). After incubation at 37 °C for 3 d several very small colonies appeared. Ten of these were subcultured on to the same medium. Only three continued to grow. After subculturing each of these five times on the same medium all three were separately transferred to liquid medium FGB containing *DD*-A₂pm (100 µg ml⁻¹). Only one culture grew within 2 d; these organisms were called mutant SW2.

Chemicals. *N*-Acetyl-LL-diaminopimelate was prepared from LL-A₂pm as described by Ward (1975). Sources of A₂pm are given by Day & White (1979).

RESULTS

Identification of the block in synthesis of A₂pm in mutant SW1

Since mutant SW1 did not require methionine or threonine and grew in medium FGB supplemented with LL-A₂pm, the block in the synthesis of this amino acid must presumably occur at one (or more) of the five enzymic reactions that lead to the conversion of L-aspartic β -semialdehyde into LL-A₂pm (see Ward, 1975). The frequency of reversion of mutant SW1 (to regain ability to grow on medium FGB without A₂pm after overnight incubation at 37 °C) was 1 in 10⁸ colony-forming units plated, which is consistent with a single point mutation causing the block in synthesis of A₂pm.

Because of the relative ease of preparing the substrate and doing the assay, the first enzyme measured in extracts of mutant and wild-type was *N*-acetyl-LL-diaminopimelate deacylase. This enzyme was present in the wild-type and had the same activity [$5 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$] whether the organisms were grown with or without $A_2\text{pm}$, but the enzyme was not detected in three separate extracts of mutant SW1. The mutant extract did not inhibit or enhance the deacylase activity of wild-type extract when equal volumes of extracts of wild-type and mutant (each about $10 \text{ mg protein ml}^{-1}$) were mixed before assay. The mutant extract did contain diaminopimelate decarboxylase [$14 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$; assayed manometrically as described by White, 1971], but this activity was no higher than in the parent strain and was measurable only when pyridoxal phosphate was present in the assay system. No lysine (or $A_2\text{pm}$) was detected on chromatograms (solvent A) of deacylase assay mixtures (which did not contain pyridoxal phosphate) after incubation with mutant extracts, and there was no disappearance of $A_2\text{pm}$ when this amino acid and mutant extract were added to a deacylase assay mixture lacking the normal substrate. Hence, decarboxylation or other metabolism of $A_2\text{pm}$ (the product assayed when measuring the deacylase) by the mutant extract did not account for the failure to detect the enzyme in mutant SW1.

Cultures of mutant SW1 were grown at 37°C in medium A1 and A2 with a limiting concentration ($40 \mu\text{g ml}^{-1}$) of synthetic $A_2\text{pm}$. No *N*-acetyldiaminopimelate was detected in culture filtrates at any stage up to 2 d in the stationary phase, even when the filtrates were concentrated about 10-fold by evaporation. However, *N*-acetyldiaminopimelate was found in the free amino acid pool of late-stationary phase organisms (see Methods), when the proportion of revertants (independent of $A_2\text{pm}$) was still negligible; its concentration was about 0.02 mM , but it was absent from the pool at earlier stages of growth. The presence of this metabolic intermediate shows that mutant SW1 must contain all the enzymes needed for its synthesis, so that, of the enzymes concerned in formation of $A_2\text{pm}$, only the deacylase is absent from the mutant.

Properties of mutant SW2

Mutant SW2 (see Methods) grew in defined medium supplemented with any one of the isomers of $A_2\text{pm}$, though growth was quicker and heavier when *meso*- or LL- $A_2\text{pm}$ was supplied (Fig. 1). The organisms (as judged by phase-contrast microscopy) resembled the wild-type and mutant SW1, and their appearance was unaltered by growth with DD- $A_2\text{pm}$. It is very unlikely that mutant SW2 was really growing on *meso*- or LL- $A_2\text{pm}$ present as impurities in the DD- $A_2\text{pm}$. From its optical rotation and other properties (Saleh & White, 1976), the DD- $A_2\text{pm}$ must have been at least 95% (w/w) pure. No LL-isomer could be detected in the DD- $A_2\text{pm}$ by paper chromatography, and growth of mutant SW1 with DD- $A_2\text{pm}$ ($400 \mu\text{g ml}^{-1}$) was very sparse, whereas mutant SW2 grew to $0.5 \text{ mg dry wt ml}^{-1}$ when the same DD- $A_2\text{pm}$ was supplied at $100 \mu\text{g ml}^{-1}$.

Metabolism of DD- $A_2\text{pm}$

Walls of *B. megaterium* 7581 and of mutants SW1 and SW2 were isolated after growth with various supplements to the medium and assayed for the three isomers of $A_2\text{pm}$ (Table 1). In every case the *meso*-isomer remained predominant and LL- $A_2\text{pm}$ was also present. Even when mutant SW2 was grown with DD- $A_2\text{pm}$, this isomer constituted only a small part of the total $A_2\text{pm}$ in the wall.

To trace the fate of DD- $A_2\text{pm}$, medium FGB containing DD- $A_2\text{pm}$ was inoculated with mutant SW2, incubated at 37°C , and DD- $[^{14}\text{C}]A_2\text{pm}$ was added when the medium had just become turbid. When growth stopped, the bacteria were harvested and peptidoglycan and material dissolved by trypsin (i.e. derived from protein) were isolated and hydrolysed with acid (see Methods). Radioactivity was measured at each step in the isolation of peptidoglycan and in the hydrolysates (Table 2). Far more radioactivity was incorporated by the organisms than could possibly have been present as contaminating LL- or *meso*- $[^{14}\text{C}]A_2\text{pm}$. A

Fig. 1. Requirement for A_2pm by mutants SW1 and SW2. Organisms were grown in medium FGB at 37 °C with different concentrations of *meso*- or *DD*- A_2pm and turbidity was measured at intervals. Growth of organisms after 21 h (—) or after 45 h (---): Δ , mutant SW1 with *meso*- A_2pm (the response of mutant SW2 to *meso*- A_2pm was similar); $\blacksquare$, mutant SW1 with *DD*- A_2pm ; $\circ$, mutant SW2 with *DD*- A_2pm . The responses of both mutants to *LL*- A_2pm resembled their responses to the *meso*-isomer.

Table 1. Proportions of isomers of A_2pm in walls of *B. megaterium* 7581 and mutant strains

Walls of *B. megaterium* 7581 and the mutants SW1 and SW2 were isolated after growth of the organisms in medium FGB with the supplements indicated (each at 100 $\mu g\ ml^{-1}$). Isomers of A_2pm were measured enzymically (see Methods). The colour produced in the assay for residual *DD*- A_2pm was abnormal (slightly brown rather than yellow) because of the large excess of lysine present. Hence, the actual proportion of *DD*- A_2pm in the walls is probably smaller than given in this Table.

Organism	Total A_2pm [$\mu g\ (mg\ wall)^{-1}$]	Isomers of A_2pm present (% of total A_2pm)		
		<i>meso</i> -	<i>LL</i> -	<i>DD</i> -
<i>B. megaterium</i> 7581 (grown without A_2pm)	72	60	18	21
<i>B. megaterium</i> 7581 (grown with <i>meso</i> - A_2pm)	93	69	18	13
Mutant SW1 (grown with <i>meso</i> - A_2pm)	93	68	18	14
Mutant SW2 (grown with <i>meso</i> - A_2pm)	91	70	20	11
Mutant SW2 (grown with <i>DD</i> - A_2pm)	72	65	18	17

preliminary experiment showed that relatively little of the ^{14}C taken up could be extracted by cold trichloroacetic acid (i.e. was present in the free amino acid pool); most ^{14}C was present in peptidoglycan and in protein. The identities of labelled compounds in the hydrolysates were determined by chromatography and autoradiography. In the peptidoglycan the only labelled compounds found were isomers of A_2pm (*LL*- and *meso*-/*DD*-) and in the protein fraction only radioactive lysine and traces of A_2pm were found. The proportions of the individual isomers of [^{14}C] A_2pm were not determined in the peptidoglycan, but it seems reasonable to suppose that they were about the same as given in Table 1 (for unlabelled walls), because autoradiography showed that less *LL*-[^{14}C] A_2pm than *meso*-[^{14}C] A_2pm had been formed, and the presence of [^{14}C]lysine showed that free *meso*-[^{14}C] A_2pm had also been produced; this isomer is the only substrate that is attacked by diaminopimelate decarboxylase to give lysine (Work, 1962).

Similar experiments were done with mutant SW2 grown with *meso*-[^{14}C] A_2pm and with

Table 2. Uptake of radioactivity and its distribution during fractionation of the organisms after growth of mutants SW1 and SW2 with [^{14}C]A₂pm

Cultures were grown at 37 °C in shaken flasks containing 30 ml medium FGB with synthetic [^{14}C]A₂pm or *meso*-[^{14}C]A₂pm (each at 100 $\mu\text{g ml}^{-1}$) or in 10 ml medium FGB with DD-[^{14}C]A₂pm (50 $\mu\text{g ml}^{-1}$). The total uptake of A₂pm was measured as the difference between the radioactivity added and that left in the culture filtrate when the organisms were harvested. Counts measured during fractionation were corrected to the same initial specific activity [520 d.p.m. ($\mu\text{g A}_2\text{pm})^{-1}$] for each experiment.

	SW1 + synthetic [^{14}C]A ₂ pm	SW2 + <i>meso</i> -[^{14}C]A ₂ pm	SW2 + DD-[^{14}C]A ₂ pm
Total uptake of A ₂ pm [μg (mg dry wt organisms) $^{-1}$]	79	55	53
Percentage (w/w) of added [^{14}C]A ₂ pm taken up	77	48	48
Radioactivity recovered in fractions [corrected d.p.m. (mg dry wt organisms) $^{-1}$]			
Cold trichloroacetic acid extract	7700	2350	ND
Ethanol extract	1900	895	892
Hot trichloroacetic acid extract			
Water wash			
Material dissolved by trypsin (protein)	11 000* (33 μg lysine)	6090* (18 μg lysine)	4750* (14 μg lysine)
Water wash	400	213	478
Hydrolysed residue (peptidoglycan)	10100† (19 $\mu\text{g A}_2\text{pm}$)	9610† (18 $\mu\text{g A}_2\text{pm}$)	7490† (14 $\mu\text{g A}_2\text{pm}$)

ND, Not determined.

* Nearly all of this radioactivity was present as lysine (specific activity half that of [^{14}C]A₂pm, i.e. 338 d.p.m. μg^{-1} after correction).

† Nearly all of this radioactivity was present as A₂pm. Proportions of A₂pm and lysine in other fractions were not determined.

mutant SW1 grown with synthetic [^{14}C]A₂pm (Table 2). Again, [^{14}C]A₂pm was the only major radioactive component of the peptidoglycan, and [^{14}C]lysine alone was found in the protein.

The amounts of A₂pm taken up (as measured by disappearance of radioactivity from the medium) in supporting growth of 1 mg dry wt of mutant SW2 were 55 μg and 53 μg with the *meso*- and DD-isomers, respectively. Day & White (1979) calculated that about 25 μg A₂pm and 25 μg lysine might be needed for growth of 1 mg dry wt of *B. megaterium* organisms. The amounts of these two amino acids actually incorporated by mutant SW2 are fairly close to this estimate.

DISCUSSION

The A₂pm-requiring mutant (SW1) of *B. megaterium* isolated by Saleh & White (1976) lacks the enzyme *N*-acetyl-LL-diaminopimelate deacylase. This enzyme was detected in the wild-type (grown without A₂pm) with an activity just enough to produce LL-A₂pm (assuming 60 μg is needed for growth of 1 mg of new organisms, see Results) at a rate sufficient to allow the observed doubling time of 90 min in medium A1 at 37 °C. Growth of the wild-type with A₂pm did not repress the enzyme, and so it is very unlikely that the enzyme was absent from mutant SW1 as a consequence of the presence of A₂pm in the medium.

No previous reports have been made of DD-A₂pm being effective as a bacterial growth factor. The mutant SW2 grew more slowly with this isomer than with *meso*- or LL-A₂pm, presumably because of a slower rate of uptake of the DD-isomer, or because of a slow rate of isomerization of DD-A₂pm to *meso*-A₂pm, which is needed as a component of peptidoglycan and as a precursor of lysine. The rate of this isomerization for the observed doubling

time with DD-A₂pm (4 h) would only need to be 1.8 nmol min⁻¹ (mg protein)⁻¹. Preliminary attempts (unpublished) to detect conversion of DD-A₂pm to *meso*-A₂pm with extracts of mutant SW2 have been unsuccessful, perhaps because of the very low enzymic activity that the organisms may have.

The wild-type organisms (*B. megaterium* 7581) can slowly take up DD-A₂pm during growth (Day & White, 1979). The mutant SW1 also probably takes up some DD-A₂pm when it grows on racemic (i.e. LL- and DD-) A₂pm. Saleh & White (1976) found that culture filtrates of this mutant, after growth had stopped on limiting amounts of racemic A₂pm, always contained less residual DD-A₂pm than had originally been added to the medium in the racemate.

Certain amino acids may be enzymically altered after incorporation into peptidoglycan precursors (see review by Schleifer *et al.*, 1976). Such a mechanism for production of the DD-A₂pm that is in the wall of *B. megaterium* is unlikely (Day & White, 1979). Finding [¹⁴C]lysine in the protein after growth of mutant SW2 with DD-[¹⁴C]A₂pm proves that free *meso*-A₂pm can be formed from DD-A₂pm. There is no unambiguous proof (Day & White, 1979) that free *meso*-A₂pm can be converted to free DD-A₂pm. However, it seems uneconomical for organisms to have a separate system to catalyse this reaction, which (by analogy with the known reversibility of the enzyme LL-diaminopimelate 2-epimerase; White *et al.*, 1969) might simply be the reversal of the DD-A₂pm to *meso*-A₂pm reaction.

Miss Lorna Young prepared *N*-acetyl-LL-diaminopimelate and extracted and assayed this compound from mutant SW1. Some of the assays for *N*-acetyl-LL-diaminopimelate deacylase were done by Mr M. C. Evans. Amino acid analysis was done by Mrs Linda Gibb (Department of Biochemistry, University of Sheffield). We are very grateful to all these people.

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