FREE (*S,S*)-DIAMINOPIMELATE IS NOT AN OBLIGATORY INTERMEDIATE IN LYSINE BIOSYNTHESIS IN *CORYNEBACTERIUM GLUTAMICUM*

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Received 17 December 1978

1. Introduction

The organisation of enzymes into multi-enzyme complexes is a relatively simple way to promote the rapid and efficient channelling of material along specific metabolic pathways in vivo [1–4]. Direct evidence for such chanelling can in principle be obtained when an intermediate in the pathway possesses a C_2 rotational axis of symmetry. This is because diffusion through the cytoplasm would inevitably randomise an isotopic label originally present in only one of the homotopic groups, while a direct 'handing-on' process might prevent the rotation and preserve the original isotope labelling pattern. Such a stereochemical test for channelling was first proposed for squalene, as an intermediate in triterpene biosynthesis [5] but, in the cases examined, no conclusive evidence for 'handing-on' of squalene was found [6,7]. In contrast, the five-carbon chain in sedamine and related piperideine alkaloids appears to be derived from cadaverine that remains tightly enzyme-bound (perhaps via an imine linkage) [8]. The present report concerns the use of the same stereochemical approach to detect in vivo channelling in a primary metabolic pathway.

Lysine biosynthesis in bacteria and higher plants proceeds by way of (*S,S*)-diaminopimelate ((*S,S*)-DAP) [9], which obtains four of its carbon atoms from aspartate and the remaining three from pyruvate, as indicated in fig.1 [10]. If this intermediate were able to rotate about its axis of symmetry, label derived from [U-^{14}C]aspartate would become distributed over all seven carbons (pathway b, fig.1). Alternatively, (*S,S*)-DAP could be channelled directly from one enzyme to the next so that the two ends retain their individuality (pathway a, fig.1). The two ends would also remain distinct if (*S,S*)-DAP were covalently bound to the enzyme producing it (perhaps as an imine) and this covalent adduct were to serve as the substrate for the next enzyme in the pathway. The relative importance of pathways a and b can be determined by analysis of the distribution of label in the [^{14}C]lysine produced.

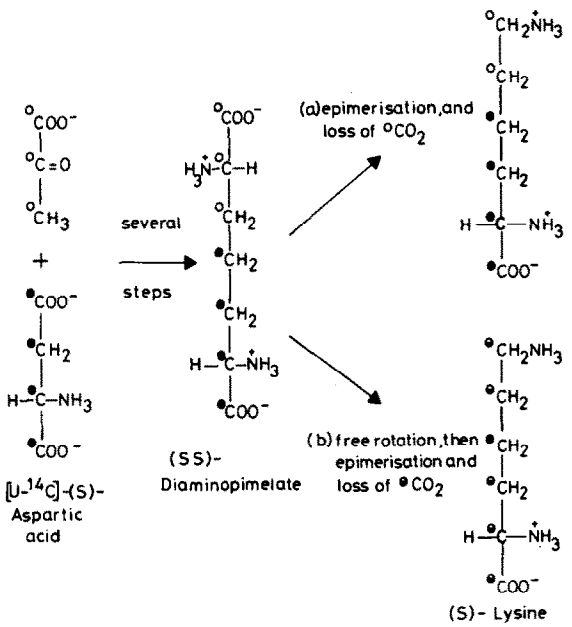

Fig.1. Predicted labelling patterns for lysine, assuming either (a) that the intermediate (*S,S*)-DAP does not rotate or (b) that (*S,S*)-DAP rotates freely before conversion to lysine. Relative specific activity at each carbon is denoted by: (•) 1.0; (⊖) 0.5; (○) 0.

2. Materials and methods

2.1. *Materials*

Corynebacterium glutamicum (*Micrococcus glutamicus*) ATCC 13287 was obtained from the American Type Culture Collection, Rockville, MD and maintained on nutrient agar (Difco). (*S*)-[U-^{14}C]aspartic acid (10 mCi/mmol) and (*R,S*)-[1-^{14}C]lysine monohydrochloride (10 mCi/mmol) were from the Radiochemical Centre, Amersham. Amberlite IRC-50 (H^+ form), analytical grade, was purchased from BDH, Poole and Dowex 50W-X8 (H^+ form) 200–400 mesh was from Cambrian Chem., Croydon.

2.2. *Incorporation of label into lysine*

C. glutamicum (homoser$^-$), which accumulates substantial quantities of lysine in the medium under appropriate growth conditions [11], was grown in nutrient broth (Difco) at 37°C, with vigorous shaking, until the stationary phase was reached. The cells (400 mg wet wt) were centrifuged, washed twice with 50 ml fermentation medium [11] and resuspended in 100 ml of this medium. After 22–24 h at 30°C, with vigorous shaking, [U-^{14}C]aspartic acid (75 μCi) was added. After a further 40 h the broth was heated and centrifuged, and [^{14}C]lysine recovered from the supernatant.

2.3. *Purification of [^{14}C]lysine*

The fermentation supernatant was diluted with water (200 ml) and percolated through an Amberlite IRC-50 column (1.5 × 15 cm), previously converted into the NH_4^+ form by washing with 2 M NH_4OH and then water. The column was washed extensively with water and the [^{14}C]lysine was eluted with 0.15 M NH_4OH. The eluate was acidified with 2 M HCl and evaporated to dryness. The crude lysine monohydrochloride was recrystallised (from ethanol–water) to constant specific radioactivity, in a final yield of 230–300 mg. Chemical and radiochemical purity was checked by thin-layer chromatography on cellulose thin-layer sheets, using *n*-butanol/water/glacial acetic acid 12:5:3 (v/v/v) as solvent (R_F 0.22). The chromatograms were autoradiographed with Kodirex film (Kodak) and sprayed with ninhydrin. Before degradation of the [^{14}C]lysine, it was mixed with 0.5 g inactive lysine monohydrochloride and recrystallised twice from ethanol–water.

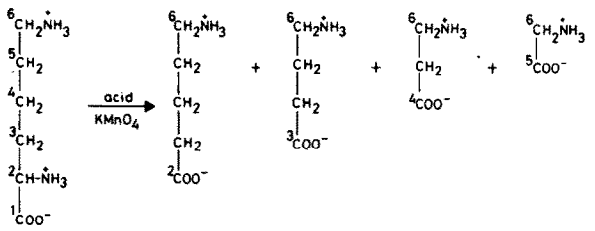

Fig.2. Degradation scheme for determination of the labelling pattern in [^{14}C]lysine.

2.4. *Degradation of [^{14}C]lysine*

The degradation was by a single-step method [12] using acidified potassium permanganate at room temperature. The four amino acid fragments obtained are shown in fig.2 with the carbon skeleton numbered to emphasise the original position in lysine. The amino acids were cleanly separated by chromatography on a Dowex 50 column (pyridinium form) (1.5 × 80 cm) essentially as in [12], except that glycine, β-alanine and 4-aminobutyric acid were further purified by sublimation in vacuo at 50–100°C below their respective melting points. Scintillation counting was performed, in quadruplicate, on weighed amounts of crystalline amino acid dissolved in 1 ml water, to which 10 ml scintillation fluid was added. The scintillation fluid contained Triton X-100/BDH liquid scintillator in toluene 1:2 (v/v). The values reported in table 1 are arithmetic means. In a control experiment, (*R,S*)-[1-^{14}C]lysine (50 μCi) was added to the fermentation instead of [^{14}C]aspartic acid, and the lysine recovered and degraded as before. The analysis (data not shown) confirmed that $\geqslant$ 99% of the ^{14}C label was still at C-1.

3. Results and discussion

The results in table 1 demonstrate that incorporation of label from [U-^{14}C]aspartic acid into lysine occurs with only partial randomisation. Evidently free (*S,S*)-DAP is not an obligatory intermediate in this transformation. The available evidence suggests that lysine biosynthesis in *C. glutamicum* does occur by the diaminopimelate pathway. For example, a lysine-requiring mutant has been isolated that specifically lacks DAP-decarboxylase activity and accumu-

Table 1
Distribution of ^{14}C in lysine derived from [U-^{14}C]aspartic acid

Amino acid fragment	Specific radioactivity of each fragment × 10^{-3} (dpm μmol^{-1})			Specific radioactivity relative to lysine					Calculated *r* values		
				Predicted for no randomisation	Predicted for complete randomisation	Found					
	1	2	3			1	2	3	1	2	3
Lysine	2.99	2.34	5.24	100	100	100	100	100	–	–	–
δ-Aminopentanoic acid	2.42	1.87	4.17	75	85.7	80.9	79.9	79.6	55.1	45.8	43.0
γ-Aminobutyric acid	1.83	1.36	3.06	50	71.4	61.2	58.1	58.4	52.3	37.9	39.3
β-Alanine	1.27	0.94	2.04	25	57.1	42.4	40.2	38.9	54.1	47.3	43.2
Glycine	0.42	0.28	0.63	0	28.6	14.1	12.0	12.0	49.3	42.0	42.0

r is the apparent percentage of (*S*,*S*)-DAP molecules able to rotate before further conversion into lysine

lates both *meso*-(*R*,*S*)- and (*S*,*S*)-DAP in the growth medium [13,14]. The possibility still exists that this bacterium uses a hitherto unknown variant of the normal pathway, but it is worth examining an alternative explanation: the two enzymes handling (*S*,*S*)-DAP (probably a de-acylase and the epimerase that converts it into *meso*-DAP) could interact directly within a multi-enzyme complex, so that the symmetrical intermediate is never generated but is held in covalent linkage until the second enzyme has acted upon it. The present data would also be consistent with the existence of (*S*,*S*)-DAP as a non-covalently bound intermediate that is transferred and reacts, within a complex, faster than it can rotate. From the specific radioactivity of each purified fragment it is possible to calculate the percentage *r* of (*S*,*S*)-DAP molecules apparently able to rotate before further conversion into lysine. In each of three experiments, *r* was only ~50%, so the 'handing-on' process is clearly an efficient one, if this explanation is correct.

The good agreement between experimental values for *r* from all the fragments derived from the same lysine parent strengthens confidence in the degradation procedure, and makes it unlikely that breakdown of the labelled precursor and subsequent incorporation by way of pyruvate can account for these results. A useful check on incorporation via pyruvate is provided by the specific radioactivity at C-4 in lysine. The operation of this indirect pathway should yield lysine containing 28.3% of its radioactivity at this position, and this value is independent of the extent of randomisation at the DAP stage. The observed percentages of radioactivity at C-4 were 28.3, 28.2 and 26.9, respectively. The experiment with [1-^{14}C]lysine shows that breakdown and resynthesis of lysine is negligible under the conditions used.

Channelling of (*S*,*S*)-DAP in other bacteria and in higher plants has not been explicitly tested for, but there is some evidence that DAP is a free intermediate in lysine biosynthesis in barley [15], while for *E.coli* there are two brief (and mutually contradictory) reports [16,17]. There is no other evidence for the existence of multi-enzyme complexes in this biosynthetic pathway. The present results have shown that free (*S*,*S*)-DAP is not an obligatory intermediate in lysine biosynthesis in intact bacterial cells. If *C. glutamicum* does use the normal diaminopimelate pathway to lysine, then the (*S*,*S*)-DAP must be channelled between two directly-interacting enzymes.

Acknowledgement

I gratefully acknowledge the technical assistance of Mrs C. Cotter.

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