CONFIRMATION OF UNUSUAL STEREOCHEMISTRY OF GLUTAMATE BIOSYNTHESIS IN CLOSTRIDIUM KLUYVERI

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Recently controversial reports on the stereochemistry of glutamate biosynthesis in Clostridium Kluyveri have appeared. The results were obtained with cell-free extracts [1-3] and in ^{14}C-labeling studies with growing cells [4,5]. In order to explain the conflicting findings different strains with opposite stereospecificities of citrate synthase have been postulated [5].

This note reports that glutamate biogenesis does not follow the usual (pig heart type [6]) stereochemical course in both ethanol-acetate and crotonate grown Clostridium kluyveri.

This organism is a strict anaerobe growing on synthetic media with ethanol, acetate and bicarbonate or crotonate and bicarbonate as the sole carbon substrates. The origin of carbon in amino acids was studied using ^{14}C-long time labeling techniques as previously described [7] with growing cultures (revived from dried cells, Boehringer Biochemica, Tutzing). The carbon substrate composition and the ^{14}C-labeling of the media are given in table 1. The amino acids of a hydrolysate (6N HCl, 110°, 24 hr) of the cell protein were first separated into acidic, neutral and basic fractions by paper electrophoresis (35 mM NH_4OAc-buffer, pH 6.0; 950 V/36 cm, 15 mA, 3 hr). The neutral amino acids were further fractionated by paper chromatography in phenol: water 8:2. Final purification of the amino acids was achieved by chromatography: aspartate, glutamate and alanine in n-butanol: acetic acid: water 10:2:5; glycine in phenol: water 8:2; proline in acetone: n-butanol: water: diethylamine 10:10:5:2 and arginine in tert.-butanol: methyl ethyl ketone: water: diethylamine 10:10:5:1. Whatman 3 MM paper was used exclusively. The amino acids were assayed by the ninhydrin reaction [8]. All amino acids were α-decarboxylated and aspartic acid was α- and β-decarboxylated by treatment with chloramine T [9]; glutamic acid was also γ-decarboxylated by the Schmidt reaction [10]. Radioactivities were determined in Tricarb Scintillation Spectrometers.

Table 1
The number of carbon atoms (n) in amino acids synthesized from ^{14}C-bicarbonate.

	A_3	B_2	S_2
	$n = \frac{\text{dpm}/\mu\text{mole amino acid}}{\text{dpm}/\mu\text{mole } ^{14}\text{C-bicarbonate}}$		
Aspartic acid	1.83	1.99	1.88
Glutamic acid	0.97	1.04	1.00
Proline	0.94	-	-
Arginine	1.83	2.06	-
Alanine	1.05	1.02	1.00
Glycine	0.98	0.96	0.93

Composition of growth media: A_3: ethanol 274 mM, acetate 104 mM, bicarbonate 30 mM; B_2: as A_3 supplemented with formate 20 mM; S_2: crotonate 50 mM, bicarbonate 30 mM. ^{14}C-bicarbonate was the labeled substrate in all experiments. A_3 and B_2 refer to experiments of which other data have already been published [7].

It was reported already from this laboratory [7] that the carbon substrates form distinct, non-mixing pools with constant specific radioactivities on ethanol-acetate media; the same was verified for crotonate cultures. Therefore the number of carbon atoms in a cell constituent derived from a labeled precursor can easily be calculated (*n*-value). *n* equals the specific radioactivity of a cell component divided by that of the labeled substrate. It was thus found that in ala-

Table 2
Labeling patterns of amino acids (radioactivity before decarboxylation = 100%).

		A_3	B_2	S_2
		% radioactivity		
Aspartate	C_1+C_4	100.0	96.0	98.0
	C_2+C_3	1.0	2.1	1.8
Glutamate	C_1	3.3	4.8	2.0
	C_2-C_5	102.5	102.0	105.5
	C_5	93.5	97.0	100.0
	C_1-C_4	0.4	1.3	0.8
Proline	C_1	9.2	-	-
	C_2-C_5	99.5	-	-
Arginine	C_1	9.0	-	4.5
	$C_2-C_5+C_5$	83.5	-	94.5
Alanine	C_1	96.7	103.7	104.0
	C_2+C_3	2.3	3.2	3.5
Glycine	C_1	98.4	-	-
	C_2	1.8	-	-

nine, glycine, glutamate and proline one carbon atom each, and in aspartate and arginine two carbon atoms each are derived from CO_2 (table 1).

The labeling patterns of the isolated amino acids (table 2) indicate that the carboxyl groups of alanine and glycine, both the α- and β-carboxyl groups of aspartate and the γ-carboxyl group of glutamate are synthesized from CO_2, while the α-carboxyl groups of all the amino acids of the glutamate family are formed solely from the two carbon substrates.

Carboxylation of pyruvate, formed from acetyl CoA and CO_2, leads to oxaloacetate, which is transformed to glutamate [1,11] via citrate by the action of citrate synthase, aconitase, isocitrate dehydrogenase, and glutamate dehydrogenase. If citrate synthase and aconitase have both usual or both unusual stereospecificities, the α-carboxyl groups of glutamate, proline and arginine will exclusively be synthesized from CO_2. Yet atypical stereospecificity of one of the two enzymes will make acetate the precursor of the α-carboxyl groups and CO_2 the precursor of the C_5 positions. As aconitase of Clostridium kluyveri has the normal specificity [1,2] the labeling patterns of glutamate, proline and arginine (table 2) definitely prove, that the citrate synthase of the studied organism has an unusual stereospecificity both in ethanol-acetate and in crotonate cells.

This finding is in excellent agreement with the results of Tomlinson [4] and of Gottschalk and Barker [1], who observed a 90% unusual stereochemistry of glutamate synthesis both in long-time labeling studies with whole cells and in cell-free extracts. It is not in agreement with the experiments of Ilse and O'Brien [3] who found in cell-free extracts an unexplained time variant change of stereospecificity and of Stern et al. [2], who obtained a 90% usual specificity. Our results are also in contradiction to the ^{14}C-labeling experiments with growing cultures of Stern and O'Brien [5]. These workers found that of the total radioactivity of glutamate isolated from 1-^{14}C-acetate grown cells 25% was in the α-carboxyl and 40% in the γ-carboxyl group, while the labeling pattern of aspartate was 25% each in the two carboxyl groups and 50% in the remainder of the molecule.

This latter finding led them to conclude, that $^{14}CO_2$ arises from 1-^{14}C-acetate by an unknown pathway. They further assumed that the cell-internal and the cell-external pools of bicarbonate are not in equilibrium. This assumption, crucial to the interpretation of their experiment, is not supported by a large series of investigations in our laboratory: there is no significant conversion of the C_1-carbon of acetate to CO_2, and the bicarbonate pools inside and outside the cells are in equilibrium. This is proved by the fact, that the calculation of *n*-values, based on the specific radioactivity of cell-external bicarbonate, gives unequivocal results for the carbon distribution in carbohydrates [12,13], in one-carbon units [7] and in amino acids (table 1). Unless the existence of two strains with differences in both the stereochemistry of glutamate biogenesis and in the equilibration of cell-internal and cell-external bicarbonate can be accepted, a more plausible explanation – in view of the difficulties to obtain pure cultures of Cl. kluyveri – for the differing results [1,2] must be seen in a contamination, which was already considered as a remote possibility by Stern's group [2].

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