Vol. 99, No. 3, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1981 Pages 789-795

MUTANT CELL LINES RESISTANT TO AZETIDINE CARBOXYLIC ACID: QUANTITATIVE AND QUALITATIVE DIFFERENCES IN PYRROLINE-5-CARBOXYLATE SYNTHASE ACTIVITY

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Received February 26,1981

SUMMARY: Mutant Chinese hamster lung fibroblasts were selected that are resistant to the proline analog L-azetidine-2-carboxylic acid. Resistance in the two mutant cell lines is associated with two distinct alterations in pyrroline-5-carboxylate synthase, the enzyme that catalyzes the proline biosynthetic step leading from glutamic acid to pyrroline-5-carboxylate. In one mutant cell line, pyrroline-5-carboxylate synthase specific activity is increased 30-fold over the level in control cells. In the other mutant line, pyrroline-5-carboxylate synthase activity is not increased, but the enzyme has become insensitive to inhibition by ornithine and proline.

INTRODUCTION

L-Azetidine-2-carboxylic acid is a naturally occurring analog of proline that is toxic to mammalian cells (Fig. 1) (1). It is incorporated into protein in place of proline, where its steric properties alter protein structure and lead to cytotoxicity (2). When cultured cells are exposed to mutagens and then placed in medium containing AZCA, mutant lines can be isolated that are resistant to its toxic effects (3,4).

Resistance to AZCA theoretically could arise from any of several possible changes in the cellular pathways illustrated in Fig. 2. For example, the intracellular AZCA concentration could be reduced either by decreased activity of the proline/AZCA transport system as described in E. coli (5), or by accelerated AZCA degradation as occurs naturally in an Agrobacterium (6). Al-

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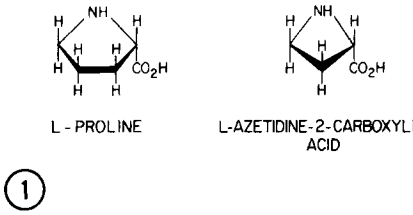
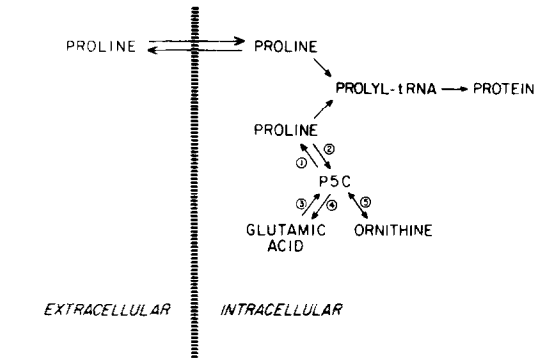
Abbreviations: AZCA, L-azetidine-2-carboxylic acid; P5C, pyrroline-5-carboxylate; MEM, Eagle's minimum essential medium; FCS, fetal bovine serum; OAB, o-aminobenzaldehyde.

0006-291X/81/070789-07$01.00/0



(1)



(2)

Fig. 1 Comparison of the structural formulas of L-proline and the analog L-azetidine-2-carboxylic acid.

Fig. 2 The pathways of proline metabolism in mammalian cells. The numbers indicate the proline synthetic and degradative enzymes: 1, P5C reductase; 2, proline oxidase; 3, P5C synthase; 4, P5C dehydrogenase; and 5, ornithine aminotransferase.

ternatively, the intracellular proline concentration could be raised by increased synthesis of proline from glutamic acid or ornithine. Finally, a qualitative change in prolyl-tRNA synthetase could lead to greater specificity in binding with proline, the apparent mechanism of AZCA resistance in a number of plant species (7).

Based on isotopic tracer studies in intact mammalian cells, it has been suggested that AZCA resistance results from alterations in the reaction catalyzed by P5C synthase (3,4). This enzyme or enzyme complex catalyzes the conversion of glutamate to P5C in a reaction that requires ATP and NADPH (Fig. 2) (8). Precise characterization of the mechanism underlying AZCA resistance was not possible because P5C synthase activity could not be directly measured in cell-free homogenates.

We have isolated mutants of Chinese hamster lung fibroblasts that are capable of growing at concentrations of AZCA that are lethal to non-resistant cells. Using a recently developed radioisotopic assay for P5C synthase activity (8), we have identified two AZCA-resistant mutants with distinct alterations in P5C synthase.

METHODS

Selection of AZCA-Resistant Mutants: The Chinese hamster lung cells were originally obtained from the American Type Culture Collection (CCL 16) and cloned by plating 10^2 cells in 60 mm petri dishes in MEM with 10% FCS (Gibco). Several clones were isolated and a single clone (designated CHL-8) was used in all subsequent experiments. The CHL-8 cells were mutagenized by incubating confluent monolayers (1.6×10^6 cells per 100 mm petri dish) in Earle's balanced salt solution containing 10^{-2} M ethylmethane sulfonate for 3 hours. The monolayers were rinsed, cultured in nonselective medium (MEM + 10% FCS) for 8 days and then switched to the selective medium (MEM + 10% FCS + 0.4 mM AZCA) (Sigma). After 4 weeks of culture in the selective medium (changed every 5 days), six clones were isolated and transferred to new culture dishes in nonselective medium. Two weeks later these cells were returned to medium supplemented with 0.4 mM AZCA. Two clones (AZCA-1 and AZCA-4) survived these procedures and have subsequently been maintained in medium containing 0.4 mM AZCA. Following isolation, the CHL-8, AZCA-1 and AZCA-4 cell lines were stored at -196° C for approximately 12 months prior to being thawed and grown for enzyme studies. For these experiments the CHL-8 and Don cells (obtained separately from American Type Culture Collection, CCL 16) were grown in MEM + 10% FCS without antibiotics, while the AZCA-1 and AZCA-4 cells were grown in similar medium supplemented with 0.4 mM AZCA. All cell lines were tested and were negative for mycoplasma.

Enzyme Assays: The activity of P5C synthase in cell preparations was measured as previously described (8). In brief, 200 to 400 µgm of cell protein was incubated with 0.75 µmol [^{14}C(U)]L-glutamic acid (2.7 Ci/mol) (New England Nuclear), 1.25 µmol ATP, an ATP regenerating system (3.63 µmol creatine phosphate and 2 Units creatine phosphokinase), 0.1 µmol NADPH, an NADPH regenerating system (2.5 µmol DL-isocitrate and 0.9 Units isocitrate dehydrogenase), 6.25 µmol $MgCl_2$, 0.25 µmol 2-mercaptoethanol, 50 µmol L-proline, 1.03 µmol OAB (Sigma), and 0.1 M potassium phosphate buffer (pH 7.4) in a final volume of 0.25 ml. After incubation for one hour at 37°C, the reaction was terminated with 0.05 ml 6N HCl containing 12.5 µmol OAB.

During the incubation, glutamic acid is converted to P5C. Further reduction of P5C to proline is prevented through the inhibition of P5C reductase by proline (9) and through the formation of a covalently-linked, dihydroquinazolinium complex between OAB and P5C (10). This OAB-P5C complex can be separated from other labeled compounds by cation exchange resin column chromatography as previously described (8), and P5C formation from glutamic acid can thus be quantified by liquid scintillation spectrometry.

Ornithine aminotransferase (11), P5C reductase (12), and P5C dehydrogenase (13) activities were determined with previously published radioisotopic methods. Protein was determined with a modification of the method of Lowry et al. (14).

RESULTS

From a total of 8×10^6 mutagenized CHL-8 cells, we observed six colonies of cells surviving in medium with AZCA. Of these six, three could not be subcultured, one was lost to infection and two (AZCA-1 and AZCA-4) were successfully isolated and maintained. The AZCA-1 and AZCA-4 lines grow at nearly normal rates in medium containing 0.4 mM AZCA, a concentration that is lethal to both the CHL-8 and Don cell lines.

Table 1. The Activity of P5C Synthase in Chinese Hamster Lung Fibroblasts

Cell Line	P5C Synthase Activity (nmol/h/mg protein)
CHL-8	1.40 ± 0.16
Don	3.37 ± 0.56
AZCA-1	81.6 ± 10.1
AZCA-4	3.47 ± 0.39

Data represent mean ± standard error for at least 6 independent determinations.

The activities of P5C synthase are depicted in Table 1. P5C synthase specific activity in the AZCA-1 line is approximately 30-fold higher than in the two control lines. In the AZCA-4 line, however, P5C synthase activity is similar to control.

We have previously shown that ornithine is a potent and specific inhibitor of P5C synthase (15). In the presence of 0.75 mM ornithine, P5C synthase activity in CHL-8 and Don cells is reduced by approximately 50 percent (Table 2). In contrast, AZCA-4 activity is unaffected by the addition of ornithine, and AZCA-1 activity is intermediate in sensitivity (25 percent inhibition).

The P5C synthase assay contains OAB in order to increase the recovery of P5C and prevent inhibition by proline (8). We have previously observed that if OAB is not added, less P5C is recovered and the reaction is markedly inhibited by high concentrations of L-proline (8). To determine whether AZCA-resistant mutants had altered sensitivity to proline, we measured P5C synthase activity in the presence and absence of OAB. In all cases, the reaction

Table 2. The Effect of Ornithine on P5C Synthase Activity

Cell Line	Inhibition by 0.75 mM Ornithine (percent control)
CHL-8	50.1 ± 3.1
Don	45.6 ± 0.7
AZCA-1	75.5 ± 4.4*
AZCA-4	96.0 ± 6.1**

Data represent mean ± standard error for 3 independent determinations.
*Significantly less inhibition than CHL-8, $P<0.05$
**Significantly less inhibition than CHL-8, $P<0.01$

Table 3. The Effect of Proline on P5C Synthase Activity

Cell Line	P5C Synthase Activity (nmol/h/mg protein)		Inhibition by Proline (percent control)
	With OAB	Without OAB	
CHL-8	1.22 ± 0.20	0	0
AZCA-1	76.84 ± 13.57	14.62 ± 4.22	21.8 ± 8.4
AZCA-4	3.60 ± 0.85	4.27 ± 0.78	122.0 ± 8.5

Data represent mean ± standard error for 3 independent determinations.

was still terminated with HCl containing OAB to allow chromatographic separation of [^{14}C]P5C. As indicated in Table 3, P5C formation by extracts of both the CHL-8 and AZCA-1 cells is strongly inhibited by proline in the absence of OAB. In contrast, P5C synthase activity in homogenates from AZCA-4 cells is actually slightly increased by proline on removal of OAB.

The activities of other proline pathway enzymes are summarized in Table 4. Ornithine aminotransferase activity is similar in all cells. P5C reductase and P5C dehydrogenase activities are similar to control lines in homogenates of AZCA-1 cells and decreased by approximately 50 percent in AZCA-4 cells. Proline oxidase activity is not present in cultured fibroblasts (16).

DISCUSSION

We have isolated two mutant lines of Chinese hamster lung fibroblasts that are resistant to the cytotoxic effects of the proline analog AZCA. Resistance to AZCA appears to result from two distinct alterations in the proline biosyn-

Table 4. Activities of Proline Metabolic Enzymes in Chinese Hamster Cells

Cell Line	Ornithine Aminotransferase	P5C Reductase	P5C Dehydrogenase
	nmol/h/mg protein		
CHL-8	138.4 ± 9.7	407.3 ± 21.9	54.2 ± 4.2
Don	146.3 ± 11.4	452.8 ± 32.6	45.0 ± 1.4
AZCA-1	105.8 ± 11.2	503.3 ± 53.6	56.9 ± 5.7
AZCA-4	139.2 ± 15.3	285.8 ± 25.1*	25.2 ± 2.6*

Data represent mean ± standard error for 3 independent experiments.
*Significantly less than CHL-8, $P<0.05$

thetic step leading from glutamic acid to P5C. In the AZCA-1 line, the specific activity of P5C synthase is increased approximately 30-fold over the activity in control cells and appears to be slightly less sensitive to inhibition by ornithine and proline. The activities of the other proline synthetic and degradative enzymes are similar to control.

In contrast, resistance to AZCA in the AZCA-4 line is not associated with an increase in P5C synthase activity. Instead, P5C synthase is completely insensitive to inhibition by ornithine and proline, a change that presumably leads to accelerated proline biosynthesis. The activity of P5C dehydrogenase, which oxidizes P5C to glutamic acid, also is decreased in the AZCA-4 cells and may contribute to AZCA resistance by sparing P5C for proline synthesis.

Further studies of these mutant, AZCA-resistant cells should provide new information about both biochemical and genetic organization of the enzymes of proline metabolism. The high levels of P5C synthase activity in AZCA-1 cells make them a valuable source of enzyme for studies on subcellular localization and enzyme mechanism. Further characterization of AZCA resistance in the AZCA-4 cells should offer insight into the physiologic importance of regulation by ornithine and proline. Finally, karyotype analysis and selection of cell lines with greater resistance to AZCA should provide information about the genetic events leading to AZCA resistance.

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