Carrot cells detoxify *N*-phosphonoacetyl-L-aspartate by esterification

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Unlike bacterial and mammalian cells, carrot cells are able to tolerate *N*-phosphonoacetyl-L-aspartate (PALA), a potential inhibitor of pyrimidine biosynthesis, by detoxifying the compound. Anion-exchange chromatography showed that detoxified PALA was less negatively charged than PALA, and allowed detoxified PALA to be isolated. Incubation of detoxified PALA with a low-specificity carboxylic-ester hydrolase fully restored the ability to inhibit aspartate transcarbamoylase, the target enzyme, indicating that the detoxification involves the formation of carboxylic ester. G.l.c. analysis of the alcohol products of enzymic hydrolysis, and of their ratio to PALA, showed that the detoxification produced a mixture of mono- and di-carboxylic esters and of methyl and ethyl esters. The detoxification mechanism showed considerable specificity towards PALA, since the analogous carboxy groups of succinate were not modified in the same way. Succinate was depleted much more slowly, no succinate esters could be detected, and the presence of a 10-fold excess of succinate did not inhibit the esterification rate of PALA. The possible significance of these results is discussed.

INTRODUCTION

N-Phosphonoacetyl-L-aspartate (PALA) is a putative analogue of the transition-state intermediate of the action of aspartate transcarbamoylase (ATCase, EC 2.1.3.2), a key enzyme of the biosynthesis pathway of pyrimidine nucleotides *de novo*, and hence an important factor in cell division and growth. PALA has the following structure at pH 8:

It is a potent inhibitor of ATCases from *Escherichia coli* (Collins & Stark, 1971), mammalian tissue cultures (Swyryd *et al.*, 1974), wheat germ (Yon, 1984) and carrot (Cole & Yon, 1985), the inhibition (dissociation) constant invariably being in the nanomolar range. However, mammalian cell lines grown in the presence of low, but increasing, concentrations of PALA developed a resistance to the drug, the mechanism of which was traced to the overproduction of ATCase and related activities by gene amplification (Kempe *et al.*, 1976; Wahl *et al.*, 1979).

We set out to induce a similar PALA-induced enzyme overproduction in cultured carrot cells, but found instead an innate, highly efficient, mechanism for detoxifying the drug (Cole & Yon, 1985). In our initial report we described experiments indicating (a) that the detoxified product was incapable of inhibiting purified wheat ATCase, (b) that the detoxifying enzyme(s) were present throughout the culture cycle, i.e. were not induced, (c) that the detoxified product accumulated in the extracellular medium, and (d) that the phosphate/aspartate ratio in the product, as in PALA, was 1:1, i.e. the PALA skeleton appeared to be intact. In the present paper we describe the isolation and further characterization of detoxified PALA.

MATERIALS AND METHODS

Chemicals

Substrates and buffers were from Sigma Chemical Co. or BDH Chemicals and were of analytical-reagent grade where possible. Dowex ion-exchange resins and pig liver carboxylic-ester hydrolase were from Sigma Chemical Co. PALA was a gift from Dr. G. R. Stark (Imperial Cancer Research Laboratories, London, U.K.). G.l.c. supports were from Supelchem, Sawbridgeworth, Herts., U.K. Wheat-germ ATCase was purified by the method of Yon (1984) and assayed by the colorimetric procedure of Prescott & Jones (1969) as described by Yon (1972). Culture medium (Murashige & Skoog, 1962) was obtained from Flow Laboratories, Irvine, Ayrshire, U.K.

Cell culture

Suspension cultures of carrot (*Daucus carota* L. cv. Nantes) were established and propagated as described in Cole & Yon (1985).

Assay of phosphate, aspartate and PALA

Phosphate was assayed before or after ashing (to convert phosphonate into phosphate) as described by Ames & Dubin (1960). In the present paper phosphate assayable before ashing is called 'free phosphate', and phosphate assayable only after ashing is called 'derived phosphate'. Aspartate was determined colorimetrically by the method of Moore & Stein (1948), after release from PALA or its derivatives by hydrolysis in 6 M-HCl at 110 °C for 24 h. PALA was assayed by the inhibition of wheat-germ ATCase as described in Cole & Yon (1985).

Analytical ion-exchange chromatography

A column (22 cm × 1.5 cm) of Dowex 1-X8 was equilibrated with 50 mM-Tris/HCl buffer, pH 8.5.

Samples (10 ml) of culture medium, initially containing 1 mM-PALA, were taken at various times after inoculation with carrot cells, and loaded on the column. Development was by a linear gradient (0–1 M) of NaCl (total volume 500 ml). Fractions (6 ml) were assayed for free and derived phosphate.

Preparative ion-exchange chromatography

A sample of detoxified PALA was prepared by a modified scale-up of the analytical ion-exchange method. The supernatant from a 250 ml 4-day culture, originally containing 70 mg of PALA, was loaded on a Dowex 1-X8 column (25 cm × 2.5 cm) equilibrated with 0.1 M-ammonium carbonate buffer, pH 8. The column was developed with a linear gradient (1 litre) of 0–1 M-NaCl in the same buffer. Fractions containing detoxified PALA were pooled and freeze-dried exhaustively to remove the volatile buffer. The yield of detoxified PALA was 48 mg.

Analysis of esterase-liberated alcohols

Portions (0.5 ml) of detoxified PALA were incubated with 50 units of esterase for 2 h. Protein was removed by gel filtration through a column (0.5 cm × 20 cm) of Sephadex G-10, and the included-volume fractions were pooled. Portions (5 µl) were taken for g.l.c. analysis. A Perkin-Elmer F11 gas chromatograph with flame-ionization detection was used. The glass column (2 m × 1.5 mm) was packed with GP80/100 Carbowax C/0.2% Carbowax 1500. The carrier gas was N₂ (20 ml/min) and the column temperature 120 °C. Chromatographic performance was optimized to separate a 5 µl mixture of ten aliphatic alcohols (C₁ to C₉). Chromatograms of esterase-liberated alcohols were run for 30 min to ensure that no alcohols higher than C₉ were present. Alcohols were identified and quantified by reference to butan-1-ol as internal standard. Control chromatograms included the extract before esterase treatment and the treated sample without internal standard. The gel-filtered extracts were also assayed for PALA and derived phosphate to enable the stoichiometry of esterification to be calculated.

Succinate determination

Concentrations of succinate in culture media were determined by g.l.c. after conversion into diethyl succinate. Dimethyl succinate (5 mM; 100 µl) was added as internal reference to 500 µl of all samples. To this was added 500 µl of ethanol containing 5% (v/v) HCl, followed by refluxing at 80 °C for 30 min and extraction into 200 µl of hexane with vigorous shaking. Portions of the hexane layer (1 µl) were taken for g.l.c. (Perkin-Elmer F11 instrument with flame-ionization detection). The glass column (1.8 m × 3 mm) was packed with 3% SP-2340 on 100–120-mesh Supelcoport with N₂ as carrier gas (20 ml/min) and run isothermally at 125 °C. Hexane extracts of blank media with and without internal standards were prepared as controls.

RESULTS

Ion-exchange chromatography

Any modification of PALA involving the phosphate or carboxylate groups might be expected to alter the net charge, and hence the ion-exchange behaviour. Ion-

Fig. 1. Ion-exchange chromatography of culture medium initially containing 1-mM-PALA

For experimental details see the text. —, NaCl gradient. Elution profiles are shown after (a) 0 h, (b) 1 h, (c) 3 h and (d) 6 h after inoculation with carrot cells. Phosphate detectable before (●) and after (○ and □) ashing is shown. The elution positions shown by arrows are for aspartate (1) and phosphonoacetate (2).

exchange chromatography of PALA and related compounds was investigated by using a column of Dowex 1-X8 developed with a 0–1 M gradient of NaCl. Fig. 1 shows the chromatographic profile of free and derived phosphate at 0, 1, 3 and 6 h after inoculation of PALA-containing medium with carrot cells. In a separate experiment the elution positions of aspartate and phosphonoacetate were also determined (shown by arrows). The 0 h profile shows the original PALA (1 mM) being eluted at 0.85 M-NaCl (around fraction 72). Subsequent times show a diminution of PALA and the increase of a phosphorus-containing compound eluted at about 0.75 M-NaCl (around fraction 61). At 6 h no PALA remains and all the phosphorus can be accounted for in the new peak. Free phosphate (eluted at 0.4 M-NaCl, around fraction 34) is present at all times,

originating from the culture medium (Murashige & Skoog, 1962).

The net charges of PALA, phosphonacetate, phosphate and aspartate (-4 , -3 , -2 and -1 respectively) agree with their order of elution. From its elution position, detoxified PALA is clearly less negatively charged than PALA. This suggests that detoxification involves the carboxy or phosphate groups. The broadness of the peak of detoxified PALA may be indicative of a mixture of compounds, as is confirmed below.

Characterization of detoxified PALA

A sample of detoxified PALA was prepared from a 48 h culture by ion-exchange chromatography (see the Materials and methods section). Acid hydrolysis (2 M-HCl at 100°C) for 20 min of a portion (5 mg) of detoxified PALA did not restore the ability to inhibit ATCase. The hydrolysate did not contain sugars, as judged by the phenol/ H_2SO_4 method of Dubois *et al.* (1956), confirming that glycosylation was not the mechanism of detoxification.

To test for esterification of the carboxy groups, the detoxified compound was treated with a wide-specificity esterase (pig liver carboxylic-ester hydrolase, EC 3.1.1.1). The incubation of $10\text{ }\mu\text{mol}$ of detoxified PALA with 10 units of esterase (1 unit hydrolyses $1\text{ }\mu\text{mol}$ of ethyl butyrate/min at 25°C at pH 8) for 24 h resulted in complete restoration of PALA, as judged by derived phosphate content and ability to inhibit ATCase. To confirm this result, the stored detoxified PALA fractions from Fig. 1 were also esterase-treated, with recovery of the expected amount of PALA in each case.

Analysis of ester groups

Esterase treatment, apart from regenerating PALA, also releases the alcohol component(s) of the ester. Alcohol products were identified and quantified by g.l.c. (Fig. 2). The only alcohol products detected were methanol and ethanol, in the ratio 1:14. When compared with the starting material, the ratio (total alcohols)/phosphorus was found to be 1.65:1. Taken together, these indicate a predominance of diethyl PALA, i.e. PALA esterified with ethanol at both carboxy groups. However, since the ratio is less than 2:1, the carboxy groups are incompletely esterified.

Detoxified PALA taken from a culture at different ages (Fig. 1) was similarly esterase-treated, and the alcohols present, and their ratios to phosphorus, were determined. The results are presented in Table 1; for completeness, the result for the large-scale (48 h) sample is included. The methyl ester/ethyl ester ratio is fairly constant, with a preponderance of ethyl esters (about 93%) at all times. However, the alcohol/phosphorus ratio increased with culture age, up to 1.65:1 after 48 h.

Specificity of the detoxification mechanism

Since PALA is synthetic, carrot cells would not be expected to have a specific PALA-esterifying mechanism. Moreover, the detoxifying enzymes are a normal component of the cells, i.e. are not induced by PALA (Cole & Yon, 1985). They might be expected, therefore, to have quite wide specificity, i.e. wide enough to include PALA. To test this hypothesis, the effects of succinate, which has the same dicarboxylate structure as PALA, were explored.

Fig. 2. G.l.c. analysis of standard and esterase-generated alcohols

For experimental details see the text. (a) Standard C_1 to C_5 alcohols; peaks 1, 2 and 8 are methanol, ethanol and butan-1-ol respectively. (b) Alcohols liberated from detoxified PALA by esterase treatment. (c) As for (b), but spiked with butan-1-ol as internal standard.

Table 1. Stoichiometry of esterification at various times after inoculation of a PALA solution with carrot cells

For experimental details see the text.

Culture time (h)	Methyl ester/ethyl ester ratio	Extent of esterification (mol of alcohol/mol of derived phosphate)
1	1:13	1.05:1
3	1:12.5	1.2:1
6	1:13	1.4:1
48	1:14	1.65:1
(large-scale preparation)		

The rate of depletion of PALA was measured with PALA initially at 1.0 and at 0.1 mM, both in the absence and in the presence of succinate (1 mM). In the absence of succinate, PALA was depleted as described previously (Cole & Yon, 1985). Succinate had no effect on the rate of detoxification of PALA, even when it was present in 10-fold molar excess. In a parallel experiment, the rate of depletion of succinate (initially 1 mM) was measured in the absence and in the presence of 1 mM-PALA. Succinate was removed from the medium much more slowly than PALA (half-times of 28 h and 3 h respectively), the rate being unaffected by the presence of PALA. No succinate esters were found in the medium over the entire course of the experiment (36 h).

DISCUSSION

This paper describes experiments indicating that the detoxification of PALA by carrot cells involves esterification of one or both of the carboxy groups of PALA by ethanol or methanol. It is known from competitive-inhibition experiments (Davies *et al.*, 1970; Grayson *et al.*, 1979) that both carboxy groups are involved in the binding of the substrate L-aspartate, and of inhibitory analogues of this substrate, to the target enzyme, ATCase. Thus esterification of even one carboxy group would detoxify PALA. The alcohol/phosphorus ratio in detoxified PALA increases with time, and eventually exceeds 1:1, indicating the esterification of both carboxy groups on a proportion of the PALA molecules. A substantial fraction of the final product appears to be the diethyl ester of PALA; methyl esters account for only about 7% of all the carboxy groups esterified. However, even after long periods of incubation (up to 48 h) the alcohol/phosphorus ratio remains less than 2:1, i.e. carboxy groups are incompletely esterified. Although other interpretations are possible, we favour the hypothesis that the monoethyl (or monomethyl) ester is formed first, and accumulates in the extracellular medium, but may later re-enter the cell for further esterification (see discussion in Cole & Yon, 1985). As a result, the extracellular medium probably contains a mixture of the eight possible ester products involving ethanol and methanol; however, their individual proportions have not been determined.

Succinate retains the structure of the dicarboxylate moiety of PALA, but lacks the *N*-phosphonoacetyl moiety. The detoxification mechanism shows a considerable specificity towards PALA, as judged by the failure of a 10-fold excess of succinate to inhibit or otherwise interact with PALA detoxification, and by differences in the rate and nature of succinate depletion from the medium. These differences may be attributed (a) to recognition by the esterifying enzyme(s) of the *N*-phosphonoacetyl moiety, the main structural difference between succinate and PALA, or (b) to differences in the rate of transport and/or metabolism (other than esterification) between succinate and PALA.

Since PALA is a synthetic product, it is difficult to suggest what the 'normal' function of this esterification

system might be. Doubtless many esterification reactions occur in plant cells, which are renowned for the diversity of their secondary metabolites; the fact that the substrate specificity of one of these reactions happens to include PALA may be entirely fortuitous. On the other hand, this may be a significant detoxification mechanism for a class of toxins as yet unknown. Although plant cells have been shown to be resistant to a variety of growth-inhibiting drugs and herbicides, the mechanisms of resistance usually involve reduced uptake (Cocking *et al.*, 1974), gene amplification leading to overproduction of the target enzyme (Amrhein *et al.*, 1983; Borg *et al.*, 1984; Smart & Amrhein, 1987) or a combination of both effects (Shimamoto & Nelson, 1981; Cella *et al.*, 1984). To our knowledge, no esterification mechanisms have been previously proposed. However, as more plant systems are challenged with new drugs and herbicides, other mechanisms, including esterification, may emerge as protective devices.

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