Induction of mRNA activity for phenylalanine ammonia lyase (PAL) by L-α-aminooxy-β-phenylpropionic acid, a substrate analogue of L-phenylalanine, in cell suspension cultures of *Daucus carota* L

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Received 7 July 1982

Messenger RNA induction *Phenylalanine ammonia lyase* *L-α-aminooxy-β-phenylpropionic acid*
L-Phenylalanine *Deamination inhibitor* (Daucus carota L.)

1. INTRODUCTION

The deamination of phenylalanine to *trans*-cinnamic acid is catalyzed by phenylalanine ammonia lyase (PAL; EC 4.3.1.5). The amino-oxy analogue of L-phenylalanine, L-α-aminooxy-β-phenylpropionic acid (L-AOPP), a competitive inhibitor, interferes specifically with this deamination reaction [1,2]. Cells of *Daucus carota*, grown in suspension, respond to the application of L-AOPP (10^{-4} M) by a drastic and transient increase in their extractable PAL activity. This increase is the result of de novo synthesis of the enzyme [3]. The L-AOPP effect is highly specific, since neither growth nor protein content of the cells are affected by this compound. The inhibition of PAL leads to an accumulation of soluble phenylalanine [2]. L-AOPP has no effect on the extractable activity of subsequent enzymes in the general phenyl-propanoid metabolism [3].

Here we describe experiments which demonstrate a direct effect of a substrate analogue (L-AOPP) on the amount of translatable mRNA for an enzyme (PAL). The mRNA was identified by immunoprecipitation of the subunits of PAL (M_r 84 000) synthesized in a rabbit reticulocyte in vitro system.

2. MATERIALS AND METHODS

The carrot cells were propagated as in [2]. For the preparation of free polysomes, the cells were frozen in liquid nitrogen and homogenized in a mortar for 10 min. Free polysomes were isolated by centrifugation through a 1.5 M sucrose cushion and separated on a linear sucrose gradient (125–500 mg/ml) according to [4]. Polysomes used for in vitro translation were, in addition, centrifuged through the sucrose cushion and resuspended in 10 mM Tris–acetate buffer (pH 7.6) containing 5 mM magnesium acetate, 0.1 mM KCl, 14 mM 2-mercaptoethanol, 40% glycerol (v/v), 0.5% Triton X-100 (v/v) [6]. The suspension could be stored at −18°C for at least 2 months without considerable loss of activity.

Polyribosomal RNA was extracted by the chloroform/phenol method [5] with some modifications described in [7]. In vitro translation in a rabbit reticulocyte lysate (Amersham Buchler, Braunschweig) was performed according to [7]. If polysomes were used, the reaction mixture contained up to 100 μg RNA, whereas in the case of polysomal RNA up to 30 μg were present. The incubation was carried out at 30°C for 2 h; 20 μCi L-[^{35}S]methionine were present in each assay. The reaction was terminated according to [7]. The procedure of immunoprecipitation with a PAL-specific antibody and the analysis of the PAL subunits translated in vitro on SDS PAGE is described in [3].

3. RESULTS AND DISCUSSION

3.1. *Isolation of free polysomes*

Experiments were designed to determine the content of translatable mRNA for PAL in carrot

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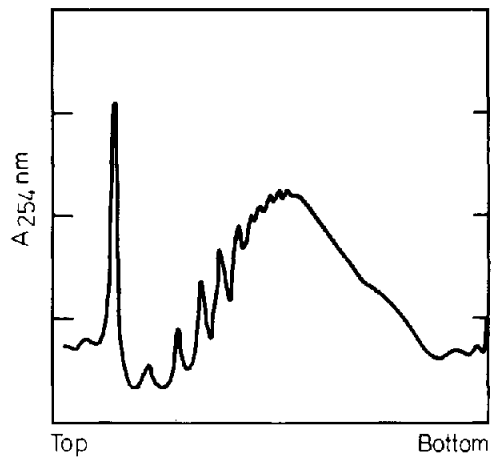

Fig.1. Sucrose density gradient sedimentation of polyribosomes from a cell suspension culture of *Daucus carota*. The cells were treated with L-AOPP (10^{-4} M). The compound was present in the culture medium from the outset of culturing and the polysomes were prepared after 84 h according to [4].

cells which were treated with L-AOPP, a competitive inhibitor of this enzyme. To test the quality of polysomes used for in vitro translations, they were fractionated in linear sucrose gradients. Fig.1 shows a polysomal pattern in the period of maximum PAL synthesis, 84 h after inoculation. L-AOPP was present in the culture medium from the beginning. In the samples layered on the gradients, A_{260}/A_{280} was 1.6–1.9. Preparations with ratios < 1.5 were not used for in vitro translation experiments.

3.2. *Messenger RNA activity in free polysomes from L-AOPP-treated cells*

In cells cultured in the presence of L-AOPP two maxima in extractable PAL could be observed. The first maximum, 12 h after inoculation into fresh culture medium, is caused by this dilution. Such a 'dilution effect' has been described for various systems [2,8]. With L-AOPP in the medium (10^{-4} M), a second, very marked maximum occurs 96 h after inoculation [2,3]. This 'super-induction' has also been observed in gherkin hypocotyls [9]. During 'super-induction' and during the 'dilution effect', free polysomes were prepared and checked for their capacity to drive polypeptide synthesis in a rabbit reticulocyte lysate. The [^{35}S]methionine-labeled translation products were immunoprecipitated with a PAL-specific antiserum, produced against purified PAL from parsley cells [10]. The dissolved precipitates were counted for ^{35}S-radioactivity. The results are shown in fig.2. Before the maximum of extractable PAL activity during the 'dilution effect', a maximum of the amount of translatable mRNA was observed. In the presence of L-AOPP a very high content of translatable mRNA was registered 84 h after the start of culturing. In the control, without L-AOPP, the mRNA activity was hardly detectable. The maximum of mRNA activity corresponds to the high rate of de novo synthesis of PAL under these conditions [3].

Analysis of the immunoprecipitates on SDS–PAGE revealed that the products synthesized in vitro with polysomes from carrot cells do not migrate in a distinct band which would correspond to a PAL subunit of 84 000 M_r. With polysomes from parsley cells, translated in a wheat germ system,

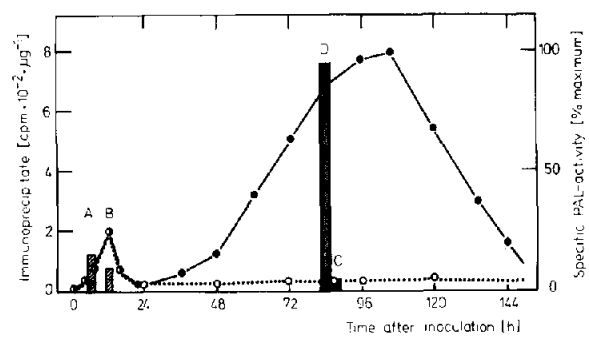

Fig.2. Immunoprecipitation of [^{35}S]methionine-labeled products synthesized in a rabbit reticulocyte lysate. Polysomes extracted from L-AOPP treated and untreated cells after various culture periods were utilized to drive in vitro translation. The final concentration of [^{35}S]methionine in the assay medium was 0.5 mCi/ml. The labeled products were precipitated with PAL-specific antiserum. The data are expressed as cpm in the immunoprecipitate/µg RNA in the assay (left scale): (A) polysomes prepared after 6 h (without L-AOPP); (B) polysomes prepared after 12 h (without L-AOPP); (C) polysomes prepared after 84 h (without L-AOPP); (D) polysomes prepared after 84 h (with L-AOPP; 10^{-4} M). The time course of extractable PAL activity is expressed as % of the maximum specific activity (25 pkat/mg): (●—●) PAL activity of the control without L-AOPP; (○---○) in the presence of 10^{-4} M L-AOPP.

similar observations were made [6]. Therefore, the data in fig.2 represent the total immunoprecipitable products synthesized in the rabbit reticulocyte system.

3.3. *Identification of mRNA for PAL in L-AOPP-treated cells*

Polysomes were utilized as a source of mRNA. The RNA was extracted following [5]. The mRNA was translated in a rabbit reticulocyte lysate with [^{35}S]methionine as a tracer and the products were precipitated with a PAL-specific antiserum. The products were separated on SDS–PAGE (fig.3). With mRNA extracted from polysomes of L-AOPP-treated cells only one distinct band of 84 000 M_r was present on the gels which corresponds to the subunit of PAL. In the control, without L-AOPP, no products corresponding to this molecular species mRNA were detectable on the gels. In all experiments, near the marker dye (bromophenol blue), there were low M_r products which could not be removed by appropriate washing procedures. These results demonstrate that the 'super-induction' of PAL triggered by L-AOPP is regulated at the level of translatable mRNA. The high content of mRNA leads to a de novo synthesis of the enzyme described in [3].

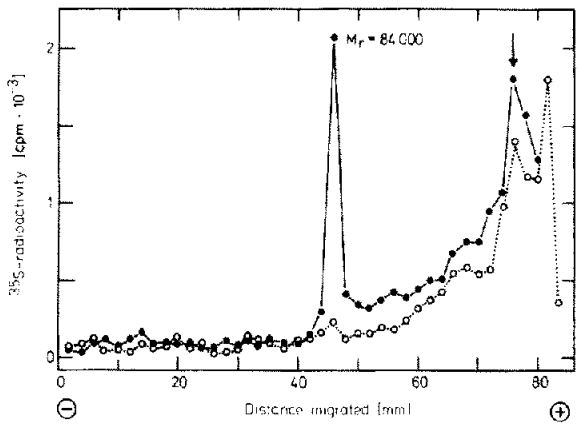

Fig.3. SDS–PAGE of PAL synthesized in a rabbit reticulocyte lysate. RNA extracted from polysomes prepared 84 h after inoculation was utilized to drive protein synthesis in vitro. The products were precipitated with PAL-specific antiserum and analyzed by SDS–PAGE (7.5% polyacrylamide). The gel slices (2 mm) were counted for ^{35}S-radioactivity: (•——•) 10^{-4} M L-AOPP; (○---○) control without L-AOPP. The arrow indicates the position of the marker dye (bromophenol blue). For M_r determinations see [3].

The inhibition of PAL by L-AOPP is highly specific. Here, it should be emphasized that the extractable activity of the subsequent enzymes of the general phenylpropanoid pathway (*trans*-cinnamic 4-hydroxylase and hydroxycinnamate : CoA ligase) are not affected by L-AOPP [3]. These results suggest that PAL synthesis is regulated independently, at the level of translatable mRNA. In parsley cells these enzymes are regulated concomitantly [11]. Our experiments with L-AOPP demonstrate that at least in carrot cells the enzymes of the general phenylpropanoid metabolism are not necessarily regulated together with the key enzyme.

ACKNOWLEDGEMENTS

We thank Professor Hahlbrock (Freiburg) for providing us with PAL-specific antiserum and Professor Amrhein (Bochum) for a gift of L-AOPP. We also thank Dr. Schröder (Köln) for valuable help during mRNA extraction from polysomes. The skillful technical assistance of Christiane Holderied is gratefully acknowledged. This work was supported by the Deutsche Forschungsgemeinschaft.

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