Superinduction of ornithine decarboxylase (ODC) by actinomycin D is due to stimulation of ODC mRNA translation

U. Margaretha Wallon[1,*], Lo Persson[2] and Olle Heby[3]

[1] *Department of Zoophysiology, University of Lund, S-223 62 Lund, Sweden,* [2] *Department of Physiology, University of Lund, S-223 62 Lund, Sweden* and [3] *Department of Zoophysiology, University of Umeå, S-901 87 Umeå, Sweden*

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Inhibition of transcription by treatment with actinomycin D caused superinduction of the ornithine decarboxylase (ODC) activity in Ehrlich ascites tumor cells. Experiments with cycloheximide ruled out the possibility that this superinduction was due to stabilization of ODC. Instead the ODC activity exhibited a more rapid turnover in the presence of actinomycin D ($t_{1/2}$=36 min) than in its absence ($t_{1/2}$=56 min). The superinduction was found to coincide with an increased rate of ODC synthesis, as determined by measuring the incorporation of [^{35}S]methionine into immunoreactive ODC protein. The steady-state level of ODC mRNA was unchanged, indicating an effect on the translational efficiency.

Ornithine decarboxylase; Superinduction; Actinomycin D

1. INTRODUCTION

Cells depend on an adequate intracellular polyamine content for their proliferation and differentiation [1,2]. Ornithine decarboxylase (ODC; EC 4.1.1.17), the first and rate-limiting enzyme in the polyamine biosynthetic pathway, has been the subject of many studies because of its interesting features and its high degree of regulation [3,4]. ODC is stimulated by a variety of agents [3] and exhibits a half-life that is shorter than that of most mammalian enzymes [5–7].

Changes in ODC activity may be due to regulation at the transcriptional, translational and/or post-translational level [3,4]. In an attempt to determine whether the changes in ODC activity caused by stimulation of cell growth might be due to changes in ODC mRNA turnover, transcription was blocked by actinomycin D both during a phase of increasing and a phase of decreasing ODC mRNA content [8]. No significant change in ODC mRNA turnover was seen [8], but the previously observed actinomycin D-mediated superinduction of ODC activity [9] was confirmed.

With the availability of an ODC cDNA [10] and a monospecific ODC antibody [11] it has become possible to further analyze the mechanism behind the superinduction of ODC. Thus, we now show that the superinduction caused by actinomycin D is mainly due to an increased rate of ODC mRNA translation, and is not a consequence of ODC protein stabilization.

Correspondence address: O. Heby, Department of Zoophysiology, University of Umeå, S-901 87 Umeå, Sweden

**Present address:* U.M. Wallon, Department of Radiation Oncology, University of Arizona Health Sciences Center, Tucson, Arizona 85724 U.S.A.

2. EXPERIMENTAL

2.1. *Materials*

Cell culture plastics and medium components were obtained from Gibco-Europe. Actinomycin D was purchased from Boehringer Mannheim, cycloheximide from Sigma, [^{32}P]dCTP, [^{35}S]methionine and Hybond-N from Amersham, and L-1-[^{14}C]ornithine from Du Pont-New England Nuclear. The cDNA (pSP64-ODC) encoding mouse ODC was a kind gift from Dr Chaim Kahana, the Weizmann Institute of Science, Rehovot, Israel.

2.2. *Cell cultures*

Ehrlich ascites tumor (Ehrlich Lettré Diploid; ELD) cells were routinely subcultured twice a week in a 1:1 mixture of Ham's F12 and Eagle's minimal essential medium supplemented with 0.2% bovine serum albumin and antibiotics (50 IU/ml penicillin and 50 μg/ml streptomycin). For the experiments, plateau phase cells were induced to proliferate by dilution in fresh medium. At 10 h of growth, actinomycin D was added to a final concentration of 10 μg/ml.

2.3. *ODC activity*

Cell pellets were stored at −70°C until analysis. Extracts were prepared by sonication in 0.1 M Tris-HCl (pH 7.5) containing 0.1 mM EDTA and 2.5 mM dithiothreitol (ODC-buffer). After centrifugation at 20000 × *g* for 20 min, aliquots of the supernatants were incubated with carboxyl-labeled ornithine in the presence of 0.1 mM pyridoxal 5′-phosphate and 0.5 mM L-ornithine [12].

2.4. *ODC mRNA content*

Pellets containing 5 × 10^6 cells were lysed in a buffer containing 4 M guanidinium isothiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% Sarkosyl and 0.1 M β-mercaptoethanol. Total RNA was isolated by the method of Chomczynski and Sacchi [13] and fracationated by formaldehyde-denaturing agarose gel electrophoresis. After blotting onto nylon membranes (Hybond-N) the RNA was hybridized to [^{32}P]dCTP-labeled pSP64-ODC insert. The relative amounts of ODC mRNA were measured by densitometry scanning of the autoradiographs.

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2.5. *ODC turnover*

Cells were grown for 10 h in fresh medium and then for 1 h in the absence or presence of actinomycin D (10 μg/ml). The half-life of ODC was determined by measuring the decline in enzyme activity after addition of cycloheximide (50 μg/ml) to the cultures.

2.6. *ODC synthesis*

Cells were collected by centrifugation during the early period of superinduction, i.e. 30 min after the addition of actinomycin D. They were resuspended in growth medium lacking methionine and preincubated for 5–10 min at 37°C in the absence or presence of actinomycin D. Then [^{35}S]methionine (10 μCi/ml) was added and the cells were incubated for an additional 25 min. The incorporation of radiolabel into protein was terminated by cooling the cells on ice and adding excessive amounts of cold methionine. Cell pellets were sonicated in ODC-buffer. After centrifugation at 20000 × *g* for 20 min, aliquots of the supernatant (all containing equal amounts of acid-insoluble radioactivity) were incubated with a polyclonal, monospecific antibody raised against mouse ODC [11]. The ODC-antibody-complex was collected by precipitation with bacterial protein A adsorbent, and washed with 10 mM Tris-HCl (pH 7.5), containing 0.1 mM EDTA, 0.5 mM dithiothreitol, 0.1% bovine serum albumin, 0.1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS) and 0.1% Tween 80. Immunoreactive proteins were separated by SDS-polyacrylamide gel electrophoresis and visualized by fluorography. [^{14}C]Methylated proteins (Amersham) as well as [^{3}H]difluoromethylornithine-labeled mouse ODC [14] were used as molecular mass markers.

3. RESULTS

When cells are stimulated to grow and proliferate their ODC activity increases rapidly [1,2]. In a previous study we have shown that this increase is correlated with an increase in cellular ODC mRNA content, and

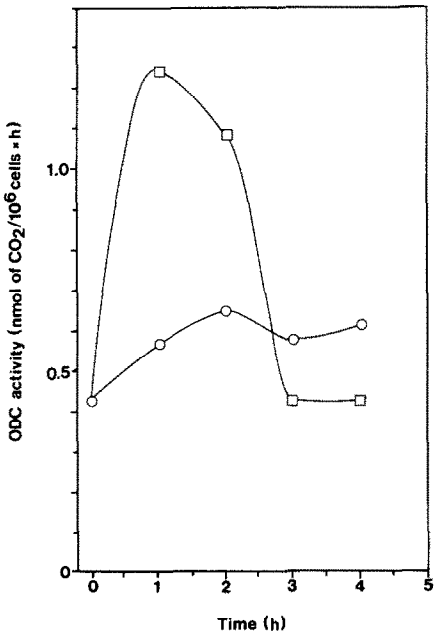

Fig. 1. Effect of actinomycin D on the ODC activity of ELD cells. At 10 h after seeding, the cell cultures were supplemented with actinomycin D to yield a final concentration of 10 μg/ml. Cell samples were removed at 1 h intervals and analyzed for ODC activity using a radioisotope assay. (□) Actinomycin D-treated cells; (○) untreated cells. The data points represent the means of 6 experimental series.

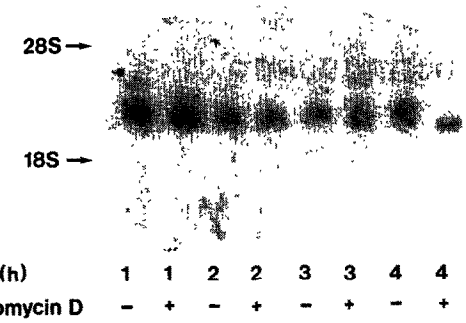

Fig. 2. Effect of actinomycin D on the ODC mRNA content of ELD cells. At 10 h after seeding, the cell cultures were supplemented with actinomycin D to yield a final concentration of 10 μg/ml. Cells grown in the absence (−) or presence (+) of actinomycin D (AMD) were removed at 1 h intervals and subjected to Northern blot analysis. The positions of 18S and 28S rRNA are indicated.

that the turnover of the message remains constant during the period of induction [8]. Actinomycin D, which was used to inhibit transcription in the study, was found to cause superinduction of ODC activity (Fig. 1). Thus, a transient, approximately 3-fold increase in ODC activity was seen within one hour of the actinomycin D addition. There was no significant change in ODC mRNA content during the experimental

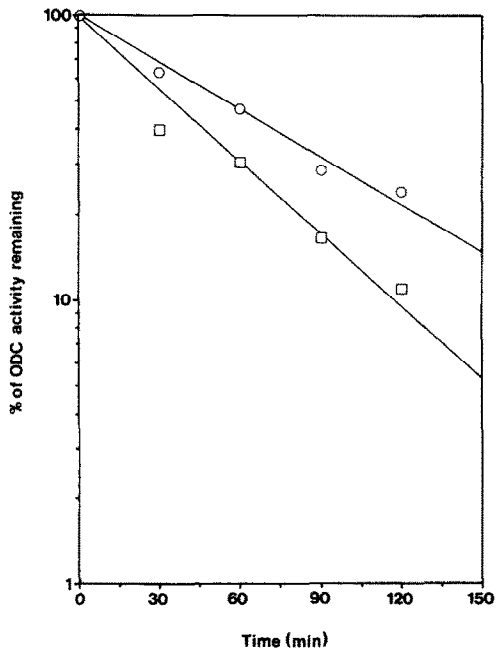

Fig. 3. Effect of actinomycin D on the turnover of ODC activity in ELD cells. At 10 h after seeding, the cell cultures were supplemented with actinomycin D to yield a final concentration of 10 μg/ml. After treatment with actinomycin D for 1 h, the cultures were supplemented with cycloheximide to yield a final concentration of 50 μg/ml. Cell samples were then removed at 30 min intervals and were analyzed for ODC activity. The data are given in percent of the ODC activity in actinomycin D-treated (□) and in untreated (○) cells at the time of cycloheximide addition. The estimated (least-square-fits) half-life for ODC activity in actinomycin D-treated cells was 36 min as compared to 56 min in untreated cells.

period (Fig. 2), which is consistent with its relatively low turnover rate [8,15,16].

The possibility that ODC superinduction was due to stabilization of the enzyme was analyzed by measuring the turnover of ODC activity in actinomycin D-treated and in untreated cells after addition of cycloheximide. Rather than stabilizing the enzyme, actinomycin D was found to stimulate its turnover (Fig. 3). Thus, the half-life of ODC activity was 56 min in the control cells and 36 min in the actinomycin D-treated cells.

To determine whether the superinduction of ODC activity was caused by stimulation of ODC mRNA translation, the rate of incorporation of [^{35}S]methionine into immunoreactive ODC protein was measured. As seen in Fig. 4, cells treated with actinomycin D exhibited a significantly higher rate of ODC synthesis than control cells. This difference in ODC synthesis was roughly 3-fold, as determined by densitometry.

4. DISCUSSION

The phenomenon of superinduction by actinomycin D has been observed for many enzymes, including ODC, and in a number of different cell systems [17–22]. The explanation for this paradoxical increase in enzyme content in the absence of RNA synthesis is not clear. It has been suggested that actinomycin D reduces the rate of degradation of the enzyme [18].

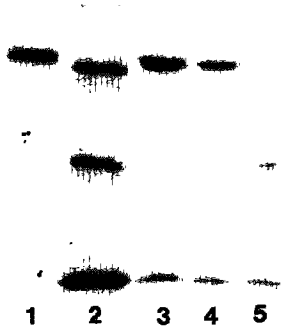

Fig. 4. Effect of actinomycin D on the rate of synthesis of ODC protein in ELD cells. At 10 h after seeding, cell cultures were supplemented with actinomycin D to yield a final concentration of 10 μg/ml. The cells were pulse-labeled with (^{35}S)methionine and the incorporation of radioactivity into ODC was determined as described in Materials and Methods. Lane 1, pure mouse kidney ODC, labeled with (^{3}H)difluoromethylornithine; 2, ladder representing proteins with a molecular mass of 69, 46 and 30 kDa (from top to bottom); 3, cells treated with actinomycin D for 1 h and labeled with [^{35}S]methionine during the final 25 min; lane 4, control cells grown in the absence of actinomycin D for 11 h and labeled with (^{35}S)methionine during the final 25 min; 5, control cells (treated as described for 'Lane 4'), non-immune rabbit serum.

However, this explanation is not consistent with the present data for ODC superinduction. Instead, actinomycin D treatment caused an increase in the turnover of the enzyme.

Hölttä et al. [22] recently found that actinomycin D treatment caused a slight (50%) superinduction of ODC activity in c-Ha-*ras*-transformed NIH 3T3 cells, which was accompanied by a corresponding increase in ODC mRNA content. This finding is consistent with the hypothesis that a labile repressor is involved in the regulation of message stability [19,20]. Since there was no change in ODC mRNA content in ELD cells treated with actinomycin D, we had to find another explanation for the superinduction, the latter being of greater magnitude than that of *ras*-transformed cells.

By measuring the incorporation of [^{35}S]methionine into immunoreactive ODC protein we revealed that actinomycin D treatment stimulates the synthesis of ODC protein. The fact that this increase equalled the degree of superinduction, indicates that stimulation of ODC mRNA translation may be the sole mechanism behind the observed superinduction.

The fact that actinomycin D has been shown to cause superinduction in wheat germ extracts and in reticulocyte lysates [23], also suggests that this compound interacts with the translational machinery. One possible site of action could be the hairpin loops in the 5′ leader of the ODC mRNA. The high free energy of stabilization of the leader appears to be the reason for the poor translatability of this message, evident from polysome profiles and in vitro translation studies [24–26]. Conceivably, actinomycin D may contribute to the melting of the secondary structure, thus facilitating ODC mRNA translation.

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