Monovalent carboxylic ionophores inhibit transport of carbamoyl-phosphate synthetase I into mitochondria in Reuber hepatoma H-35 cells and cause accumulation of enzyme precursor

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Transport of the precursor for carbamoyl-phosphate synthetase I into mitochondria in Reuber hepatoma H-35 cells was inhibited by adding monensin or nigericin to the culture medium at a concentration of 0.5 μM, and the enzyme precursor accumulated, mainly in the cytosolic fraction. Accumulated precursor was degraded slowly with a half-life of more than 16 min. Valinomycin, nonactin, A23187, X-537A (lasalocid), bromo-lasalocid, and carbonyl cyanide *m*-chlorophenylhydrazone did not exhibit these effects at concentrations at which they did not inhibit protein synthesis of the cells.

Carbamoyl-phosphate synthetase I *Carboxylic ionophore* *Monensin* *Nigericin* *Reuber hepatoma H-35* *Mitochondrial enzyme*

1. INTRODUCTION

Carbamoyl-phosphate synthetase I (CPS-I) is one of the limited number of mammalian proteins for which transport into mitochondria is well documented. This enzyme is synthesized on cycloheximide-sensitive ribosomes [1] as a precursor (pCPS-I) with a larger M_r than the mature enzyme, and is then processed into the mature size either coincident with or immediately after transport into the mitochondrial matrix [2,3]. Authors in [4] reported that the processing of pCPS-I in liver explants was inhibited by a protease inhibitor, *p*-aminobenzamidine. In their experiment, newly synthesized pCPS-I was almost instantly degraded rather than accumulated in the cells [4]. Authors in [5] found that a fluorescent dye, rhodamine 123, inhibited the intake of pCPS-I into mitochondria of isolated hepatocytes. They observed accumulation of pCPS-I in the cells and found that this pCPS-I was slowly degraded with an apparent $t_{1/2}$ of 10 min [5].

Schatz and his associates found that the accumulation of precursor for several mitochondrial proteins of yeast was caused by an uncoupler of oxidative phosphorylation, CCCP [6,7], and developed conditions under which large amounts of precursors accumulate in yeast [8]. With animal cells, however, inhibition of intake of precursors into mitochondria by uncouplers or ionophores has not been reported. Monovalent carboxylic ionophores are known to interfere with the intracellular transport of secretory [9,10], plasma membrane [11], and lysosomal proteins [12] with protein synthesis of the cells preserved. We describe here inhibition of the transport of pCPS-I into mitochondria of Reuber hepatoma H-35 cells

Abbreviations: CPS-I, carbamoyl-phosphate synthetase I; pCPS-I, precursor for CPS-I; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride

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by monensin and nigericin. Accumulation of pCPS-I was observed mainly in the cytosolic fraction, and this pCPS-I was slowly degraded with an apparent $t_{1/2} > 16$ min.

2. MATERIALS AND METHODS

Monensin, valinomycin, and nonactin were obtained from Calbiochem. Nigericin, CCCP, and X-537A (lasalocid) were from Sigma. Bromolasalocid was a gift from Dr E. Racker (Cornell University). All other materials and chemicals were obtained as in [13].

The stock culture of Reuber hepatoma H-35 was grown in 60-mm culture dishes from NUNC (Denmark) as in [13]. For the preparation of cells for labeling with [^{35}S]methionine (Amersham), 0.2 ml of a cell suspension (5×10^4 cells/ml) was put into each U-shaped well of a 96-well microtest plate from NUNC and incubated at 37°C under a humidified atmosphere of 5% CO_2 and 95% air. After 4–5 days, when the monolayer of cells was confluent, the medium was changed to 50 μl of the preincubation medium containing methionine-free Eagle's minimal essential medium, 20 mM Hepes at pH 7.4 (instead of sodium bicarbonate) and the indicated concentration of ionophore, and incubated for 30 min at 37°C under air. Labeling of cells was initiated by changing the medium to 20 μl of a labeling medium in which [^{35}S]methionine was added to the preincubation medium at a concentration of 0.637 mCi/ml. After labeling at 37°C under air for the indicated period, the cells were lysed by adding 20 μl of a buffer containing 20 mM Tris–HCl, 4 mM EDTA, 2% Triton X-100, 0.2% SDS, and 2 mM PMSF (pH 7.4). Insoluble material was spun out from the cell lysate with a Beckman Microfuge B and the supernatant was frozen at −20°C before immunoprecipitation. For the chase of labeled cells, 60 μl of the chasing medium, containing 27 mM unlabeled methionine and 133 μg/ml of cycloheximide in addition to preincubation medium, was added at the end of the labeling period. After chasing the cells for the indicated period, the cells were lysed as described above.

Cells for the study of intracellular location of pCPS-I were harvested from the 60 mm culture dishes by scraping, and suspended in the preincubation medium without ionophores. After packing the cells by gentle centrifugation, cells corresponding to 2.5×10^5 were suspended in 50 μl preincubation medium with or without 1.67 μM monensin. After preincubation for 30 min at 37°C under air, 50 μl of a labeling medium containing 1.27 mCi/ml of [^{35}S]methionine in addition to preincubation medium was added. The cells were labeled for 15 min and were fractionated into cytosolic and particulate fractions as in [13].

Immunoprecipitation of pCPS-I and CPS-I from labeled cell lysates, SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and fluorography of dried gels were carried out as in [13].

3. RESULTS AND DISCUSSION

After preincubation with various concentrations of monensin or nigericin, Reuber hepatoma H-35 cells were incubated with [^{35}S]methionine in the presence of the same concentration of ionophore. Fig.1A depicts the effect of ionophores on [^{35}S]methionine incorporation into cell proteins. Slight inhibition was observed with monensin at 0.5 μM but about 50% of the activity was still present even with 5 μM monensin. The effect of nigericin was stronger than that of monensin, with almost complete inhibition of incorporation observed at 5 μM. The [^{35}S]methionine-labeled cell lysate was immunoprecipitated with anti-CPS-I and the immunoprecipitate analyzed with SDS–PAGE and fluorography (fig.1B). In the presence of $\geq$0.5 μM monensin, the processing of pCPS-I to CPS-I was inhibited. Although this effect of monensin could be an indirect effect through action on protein synthesis (fig.1A), this result more likely indicates that processing of pCPS-I in vivo requires energy, as interpreted from the effect of CCCP on the precursors for yeast F_1-ATPase [6]. In the case of nigericin, inhibition of the processing was observed at 0.5 μM (fig.1B), but at higher concentrations, radioactivity in pCPS-I could not be detected because of the inhibition of protein synthesis. The effects of other ionophores or uncouplers such as valinomycin (0.03 μM), nonactin (0.5 μM), A23187 (10 μM), X-537A (5 μM), bromo-lasalocid (5 μM), and CCCP (1 μM) were also studied. These reagents almost completely inhibited [^{35}S]methionine incorporation of cells at the above concentrations. At lower concentrations, these ionophores or un-

coupler did not noticeably inhibit the processing of pCPS-I (not shown).

To determine the intracellular location of accumulated pCPS-I, Reuber hepatoma H-35 cells were incubated with [^{35}S]methionine in the presence or absence of 1.67 μM monensin and fractionated into cytosolic and particulate fractions. As shown in fig.2, accumulation of pCPS-I was observed mainly in the cytosolic fraction. The precursor found in the particulate fraction in the presence of monensin may be due to either contamination from the cytosolic fraction or interaction of the precursor with a receptor site on the mitochondrial surface [14].

Stability of pCPS-I in Reuber hepatoma H-35 cells was studied by pulse-chase labeling in the presence or absence of monensin (fig.3). In the absence of monensin, labeled pCPS-I disappeared within 4 min as the result of processing into CPS-I.

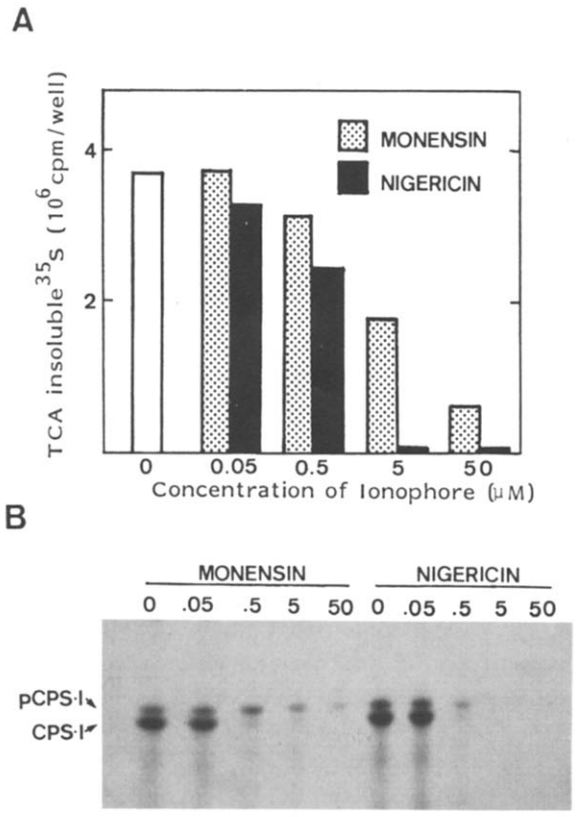

Fig.1. Effect of monensin and nigericin on the synthesis of the precursor (pCPS-I) and the mature form (CPS-I) of carbamoyl-phosphate synthetase I in Reuber hepatoma H-35 cells. Reuber hepatoma H-35 cells (5×10^4) in a U-shaped well of a 96-well microtest plate were preincubated with methionine-free, Hepes buffered Eagle's minimum essential medium containing the indicated concentrations of monensin or nigericin. After preincubation, cells were labeled with [^{35}S]methionine (0.637 mCi/ml) for 30 min at 37°C and the cells were lysed with a solution containing detergents. (A) Trichloroacetic acid insoluble radioactivity of cells was measured. (B) Cell lysate corresponding to 1.25×10^4 cells was immunoprecipitated and analyzed by SDS–PAGE and fluorography as described in section 2. The numbers at the top of the lanes indicate the concentration of ionophores in μM. A separation gel of 5% acrylamide was used for the electrophoresis.

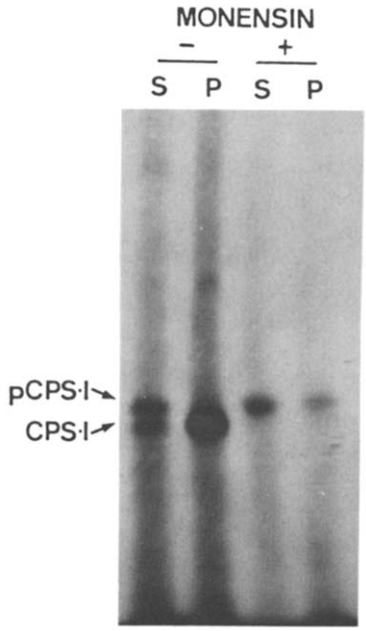

Fig.2. Subcellular location of the precursor (pCPS-I) and the mature form (CPS-I) of carbamoyl-phosphate synthetase I in the presence or absence of monensin. Suspensions of Reuber hepatoma H-35 cells (2.5×10^5 cells/50 μl) were preincubated in the presence or absence of 1.67 μM of monensin as in fig.1 and labeled for 15 min by adding 50 μl preincubation medium containing 1.27 mCi/ml of [^{35}S]methionine. The labeled cells were fractionated into cytosolic (S) and particulate (P) fractions with digitonin, as described in section 2. Both fractions corresponding to 6.25×10^4 cells were immunoprecipitated and analyzed by electrophoresis and fluorography. A separation gel of 4% acrylamide was used for electrophoresis, and the lower part of the fluorogram is shown.

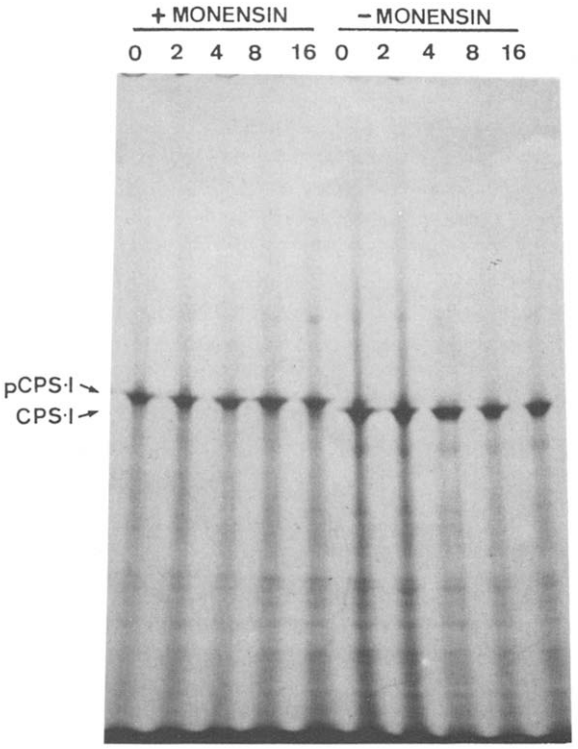

Fig.3. Chasing the labeled cells in the presence or absence of monensin. A monolayer of Reuber hepatoma H-35 cells in a 96-well microtest plate with U-shaped wells was labeled for 15 min after preincubation as in fig.1. At the end of the labeling period, 60 μl medium containing 27 mM unlabeled methionine and 133 μg/ml of cycloheximide with and without 1.67 μM monensin was added. The numbers at the top of lanes indicate the time of chasing in min. After the indicated chasing period, the cells were lysed and the radioactivity in the precursor (pCPS-I) and the mature form (CPS-I) of carbamoyl-phosphate synthetase I was analyzed as in fig.1. A separation gel of 5% acrylamide was used for the electrophoresis.

When the processing was blocked by monensin, pCPS-I became very stable and only a slight decrease of radioactivity in pCPS-I was observed during the 16 min of chasing. This result is in clear contrast to that of authors in [4], who reported that pCPS-I was almost instantly degraded in liver explants when the processing was blocked with *p*-aminobenzamidine. It may be that damage to the hepatocyte in their liver explants caused this difference. Authors in [5] estimated an apparent $t_{1/2}$ of 10 min for the degradation of pCPS-I in isolated hepatocytes when the processing was inhibited by rhodamine 123. The degradation in their experiment seems to be faster than that in our experiment.

Because our result suggested that monensin can be used to accumulate large amounts of pCPS-I, the incorporation of [^{35}S]methionine into pCPS-I in the presence of monensin was examined for a longer period. However, the accumulation of radioactive pCPS-I was limited because the course of the incorporation into pCPS-I declined within 15 min, when incorporation into CPS-I in the absence of monensin was still linear (fig.4). This result cannot be due to the viability of the cells, because [^{35}S]methionine incorporation into total cell proteins was linear even in the presence of monensin (not shown). The most likely explanation for this limited amount of accumulated pCPS-I is degradation. However, the results presented in fig.3 indicate that the degradation has a $t_{1/2}$ of more than 16 min. On the other hand, fig.4 suggests the decline of [^{35}S]methionine incorporation into pCPS-I within 15 min. Analysis of these

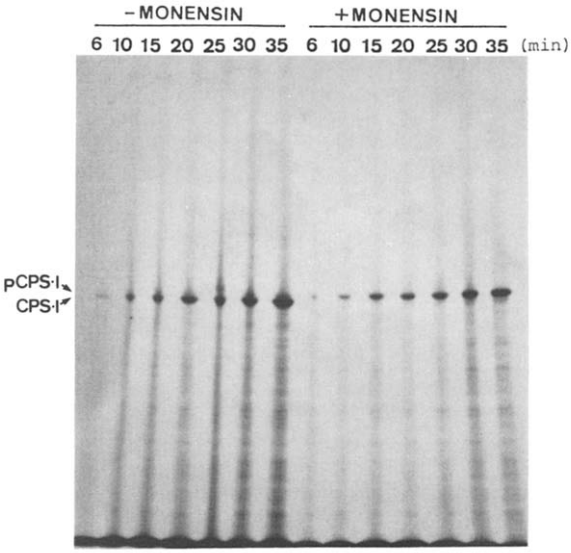

Fig.4. Course of the synthesis of the precursor (pCPS-I) and the mature form (CPS-I) of carbamoyl-phosphate synthetase I in the presence or absence of monensin. A monolayer of Reuber hepatoma H-35 cells was labeled for the indicated times in the presence or absence of 1.67 μM monensin as in fig.1. Radioactivity in pCPS-I and CPS-I was detected as in fig.1. A separation gel of 5% acrylamide was used for electrophoresis.

results with the type of kinetics developed in [15] indicates that this decline of incorporation is too early to explain our results by degradation of pCPS-I alone. Although more detailed experiments are needed, this interpretation suggests the operation of some additional mechanism(s) in the regulation of the pCPS-I level in Reuber hepatoma H-35 cells.

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