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SPERMIDINE AND SPERMINE STIMULATE THE TRANSPORT OF THE PRECURSOR OF ORNITHINE CARBAMOYLTRANSFERASE INTO RAT LIVER MITOCHONDRIA

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SUMMARY: We have examined the effect of low molecular weight components of the transport mixture generally used for the import of rat liver pre-ornithine carbamoyltransferase by isolated rat liver mitochondria. These studies revealed that spermidine and spermine, at physiological concentrations, stimulate the transport of the precursor of ornithine carbamoyltransferase into mitochondria. This stimulatory effect of spermidine and spermine is concentration-dependent and is completely inhibited at higher than physiological concentrations (20 mM for spermidine and 4 mM for spermine). Magnesium ions, which also have a stimulatory effect, inhibit the stimulatory effect of spermidine.

Spermidine and spermine, which occur in the millimolar range in eukaryotic cells, affect a large number of cell functions. For example, it is well established that these polyamines play an important role in the regulation of ion transport by mitochondria (1-3). They also enhance the rates of DNA, RNA and protein biosynthesis, which is the reason for the frequent inclusion of spermidine in systems used for the study of protein synthesis "in vitro" (4,5).

It has been shown that the transport of pOCT[1] into mitochondria requires energized mitochondria, soluble cytosolic component(s) present in reticulocyte lysate (6-8) or rat liver[2], and low molecular weight components from the mixture used for the "in vitro" synthesis of protein precursors. Since isolated precursor had not been available until recently, the translation mixture used for synthesis, which frequently included spermidine, was added to the incubation medium for the "in vitro" transport of mitochondrial protein precursors.

Given the recent availability of purified rat liver pOCT (9), we tested the effect of the components of the translation mixture on the import of the precursor of OCT by isolated mitochondria. We found that spermidine, as well as

1. pOCT: precursor of ornithine carbamoyltransferase.
2. manuscript in preparation.

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spermine, stimulates the transport of pOCT into mitochondria. This fact had not been recognized, possibly, because as indicated, when the components of the synthesis mixture were added to the transport test system some polyamines were carried over. Documentation for the new stimulatory role for spermidine and spermine is presented in this paper.

MATERIALS AND METHODS

Materials

[^{35}S]methionine (>1000 Ci/mmol, 15 mCi/ml) was from The Radiochemical Center, Amersham.

Cell-free protein synthesis.

pOCT-mRNA of rat liver was transcribed and translated in a nuclease-treated rabbit reticulocyte lysate system as described elsewhere (10). The translation mixture (14.25 µl, pH 7.6) contained 22 mM Hepes, pH 7.6, 76 mM KCl, 0.6 mM magnesium acetate, 0.7 mM ATP, 0.3 mM GTP, 15 mM creatine phosphate, 1.5 mg/ml creatine phosphokinase, 2.2 mM dithiothreitol, 0.5 mM spermidine, about 20 µCi of [^{35}S] methionine, and a mixture of the remaining 19 aminoacids (27 µM each), 5 µl of nuclease-treated rabbit reticulocyte lysate and ca. 25 ng pOCT-mRNA (estimated from [^{32}P]UTP incorporation during transcription). Incubation was carried out at 30°C for 60 min. At that time, cycloheximide was added to the mixture to give a final concentration of 12 µg/ml.

General

"In vitro" import and processing of pOCT by isolated rat liver mitochondria, sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis, fluorography of dried gels and other methods related to this study were performed as described elsewhere (10). Oxygen consumption was measured polarographically with a Clark type oxygen electrode using an aliquot of the mitochondrial suspension equivalent to 4 mg of protein in 7 ml of the incubation medium (11).

RESULTS AND DISCUSSION

As previously reported by Miura et al. (7) the "in vitro" transport of pOCT into mitochondria requires factor(s) present in reticulocyte lysate as well as components of the translation mixture employed in the synthesis of pOCT. As shown in Fig. 1 the absence of the components of the translation mixture (lanes 1-2) decreases the binding of pOCT to mitochondria. Their presence results in a higher binding of pOCT (lanes 5-6) but they are not enough to permit transport, which requires also the presence of reticulocyte lysate (lanes 3-4). The different mobilities of the bands in lanes 2,4 and 6, with respect to lanes 1,3 and 5, are due to the different amount of mitochondrial protein added.

Analysis of the effect of the components of the translation mixture on the transport of pOCT into mitochondria revealed that 0.5 mM spermidine, concentration used in the "in vitro" synthesis of pOCT (see Methods), had a stimulatory

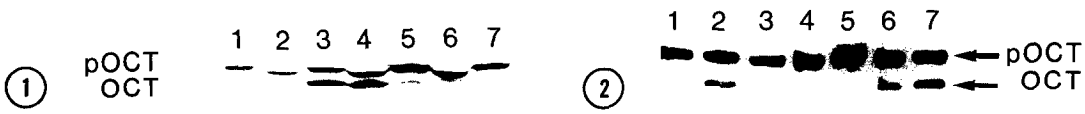

FIGURE 1. Effect of translation mixture components and of rabbit reticulocyte lysate on the transport of pOCT into isolated mitochondria

Rat liver pOCT was synthesized by translation of specific mRNA using rabbit reticulocyte lysate in the presence of [^{35}S]methionine, for 60 min. at 30°C. 2 µl of the translated mixture (ca. 36,000 cpm of pOCT) were incubated in a final volume of 100 µl with 200 µg or 400 µg of mitochondrial protein and additions as indicated. Lane 1: Rabbit reticulocyte lysate (2.9 mg of protein) and 200 µg of mitochondrial protein. Lane 2: As lane 1 but the amount of mitochondrial protein was 400 µg. Lane 3: Rabbit reticulocyte lysate (2.9 mg of protein), components of the translation mixture (except [^{35}S] methionine, pOCT-mRNA and lysate) at the concentration indicated in Methods and 200 µg of mitochondrial protein. Lane 4: As lane 3 but 400 µg of mitochondrial protein. Lane 5: As in lane 3 minus rabbit reticulocyte lysate. Lane 6: As lane 5 but 400 µg of mitochondrial protein. Lane 7: pOCT synthesized "in vitro". The gel was dried and treated for fluorography.

FIGURE 2. Spermidine stimulates the import of pOCT into mitochondria.

2 µl of the cell-free translated mixture (ca. 50,000 cpm of pOCT) were incubated in a final volume of 100 µl with 400 µg of mitochondrial protein, 20 µl of rabbit reticulocyte lysate (145 mg/ml of protein) and other additions as indicated. Lane 1: No additions. Lane 2: 0.5 mM spermidine. Lane 3: 0.7 mM ATP, 0.3 mM GTP, 15 mM creatine phosphate and 1.5 mg/ml creatine phosphokinase. Lane 4: 2.2 mM dithiothreitol. Lane 5: 27 µM aminoacid mixture. Lane 6: 0.6 mM magnesium acetate and 76 mM potassium chloride. Lane 7: The translation mixture components (as indicated in Fig. 1). The gel was dried and treated for fluorography.

effect on this process (Fig. 2, lane 2). The other components, at the same concentrations as in the translation mixture, apparently have no effect on the transport of pOCT: ATP, GTP, creatine phosphate, creatine phosphokinase (lane 3); dithiothreitol (lane 4) and aminoacid mixture (lane 5); however, as indicated by Miura et al. (7) potassium plus magnesium ions are effective (lane 6).

Further experiments to establish the effect of higher concentrations of spermidine and spermine, a related polyamine, on the stimulation of the transport of pOCT into mitochondria were carried out. As illustrated in Fig. 3, lanes 5-7 and Fig. 4A, lanes 4-6, spermidine, at concentrations in the physiological range (4), had a stimulatory effect on the "in vitro" transport of pOCT into mitochondria. Spermine (Fig. 3, lane 2 and Fig. 4A, lanes 9-10) was also active. Increasing concentrations of magnesium acetate augmented the stimulatory effect on pOCT transport (Fig. 3, lanes 8-10) as indicated by Miura et al. (7). However, spermidine and magnesium together had an antagonistic effect on pOCT transport (Fig. 3, lanes 3,4). A similar effect has been described in mitochondrial respiration (12), and in phosphate and in calcium transport by mitochondria (1,3).

As shown in Fig. 4B the stimulation of these polyamines on pOCT transport is concentration-dependent (maximum effect for spermine around 1 mM and 4 mM for

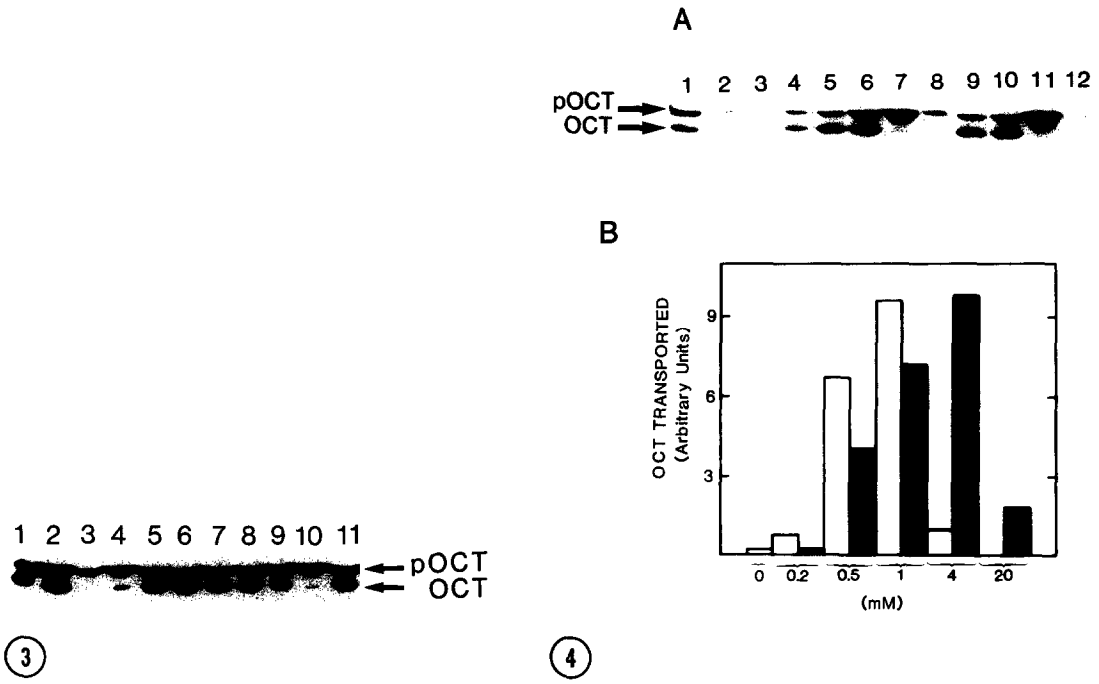

FIGURE 3.Effect of different concentrations of spermidine and magnesium acetate on the transport of pOCT into mitochondria.

Lane 1: pOCT synthesized "in vitro" and [^{14}C]-labeled OCT. Lanes 2-11: 2 µl of the cell-free translated mixture (ca. 35,000 cpm of pOCT) were incubated in a final volume of 100 µl with 400 µg of mitochondrial protein, 20 µl of rabbit reticulocyte lysate (145 mg/ml) and additions as follow. Lane 2: 1 mM spermine. Lane 3: 2 mM spermidine and 1.5 mM magnesium acetate. Lane 4: 2 mM spermidine and 0.75 mM magnesium acetate. Lane 5: 4 mM spermidine. Lane 6: 2 mM spermidine. Lane 7: 1 mM spermidine. Lane 8: 3 mM magnesium acetate. Lane 9: 1.5 mM magnesium acetate. Lane 10: 0.75 mM magnesium acetate. Lane 11: The translation mixture components (as indicated in Fig. 1). The gel was dried and treated for fluorography.

FIGURE 4.Effect of different concentrations of spermidine and spermine on the transport of pOCT into mitochondria.

PANEL A: 2 µl of the translated mixture (ca. 25,000 cpm of pOCT) were incubated in a final volume of 100 µl with 400 µg of mitochondrial protein, 20 µl of rabbit reticulocyte lysate (145 mg/ml) and other additions as indicated. Lane 1: The translation mixture components (as indicated in Fig. 1). Lane 2: No additions. Lanes 3-7: 0.2, 0.5, 1, 4 and 20 mM spermidine, respectively. Lanes 8-12: 0.2, 0.5, 1, 4 and 20 mM spermine, respectively. The gel was dried and treated for fluorography.
PANEL B: The transport of pOCT into mitochondria, presented in Panel A, was quantitated by measuring densitometrically the radioactive band corresponding to mature OCT. White bars correspond to spermine and black bars to spermidine, the value at 0 mM correspond to both polyamines.

spermidine). Complete inhibition was seen at higher concentrations of spermine and spermidine, 4 and 20 mM, respectively.

Our results show that spermidine and spermine, at physiological concentrations, stimulate the transport of pOCT into isolated mitochondria. It has been suggested that polyamines stabilize mitochondrial membranes (13) and

protect the oxidative phosphorylation (14,15). To clarify the observed effect of these polyamines on the transport of pOCT into mitochondria, we have measured the ADP/O ratio of mitochondria in the presence of spermidine from 0 to 20 mM, as described in methods (11); we have found no significant changes in the values of ADP/O ratio (data not shown).

It has been reported that signal sequences of mitochondrial protein precursors are needed for their transport, and that the amphipatic nature of these leading peptides could be responsible of the recognition and/or the transport into mitochondria (16,17). Also it is known that proteins and/or phospholipids of the mitocondrial membranes could be implicated in the different steps of the transport (18,19). Since the amphipatic nature of the polyamines has been related with the transport of cations through mitochondrial membranes (5), and has been reported that they are able to interact with components of the mitochondrial membranes (proteins and phospholipids) (4), it seems plausible that polyamines could influence the transport of protein precursors through mitochondrial membranes. As in other stimulatory effects due to spermidine and spermine, the mechanism by which these polyamines stimulate the transport of pOCT is unknown. The mechanism of action of other factors related with the transport of pOCT, such as potassium and magnesium ions, has not been established either. A major question to be clarified is whether the protein transport stimulated by polyamines requires the previous uptake of these compounds by mitochondria, or if they occur concomitantly, as in the transport of phosphate ion into mitochondria (1,2).

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