Studies on the Regulation of the Branched Chain Amino Acyl-tRNA Synthetases of the Fungus Neurospora crassa

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Summary. The specific activities of the branched chain amino acyl-tRNA synthetases from the cytosolic and mitochondrial fractions of N. crassa were low in dormant conidia and increased during germination, reaching a maximum 8 h after inoculation. This stage of development is characterised by high rates of many other cellular activities.

The increases in activity of synthetases of both cytosol and mitochondria are inhibited by cycloheximide indicating that they are synthesized on cytoplasmic ribosomes. The mitochondrial synthetases show a stimulation of their specific activity when mitochondrial RNA and protein synthesis are inhibited by either ethidium bromide or chloramphenicol suggesting that a mitochondrial translation product regulates the synthesis of the mitochondrial synthetases.

The activities of amino acyl-tRNA synthetases are dependent on energy production. When respiration is uncoupled from oxidative phosphorylation, synthetase specific activities decrease although the activities of other mitochondrial enzymes like NADH-dehydrogenase increase. This phenomenon suggests that more than one mechanism regulates the synthesis of mitochondrial proteins which are formed on cytoplasmic ribosomes.

The synthesis of branched chain amino acyl-tRNA synthetases of Neurospora is neither repressed by their cognate amino acids, nor is there inhibition by the precursors of these amino acids, as has been observed in other amino acyl-tRNA synthetases of various organism including Neurospora.

Key words: Amino acyl-tRNA synthetases – Development – Nuclear-mitochondrial interactions – Neurospora.

Introduction

The amino acyl-tRNA synthetases are among the most complex enzymes known. The reactions catalysed by these enzymes involve three specific substrates: amino acid, tRNA, and ATP. The enzymes are highly specific for each cognate amino acid and for one or more multiple species (isoacceptors) of tRNA for that amino acid.

Multiple forms of these enzymes for a particular amino acid have been reported in prokaryotes (Rouglet and Chapeville 1971; Kisselev and Baturina 1972; Yem and Williams 1973) and eukaryotes. Some of these may be attributed to the presence of species unique to mitochondria (Barnett et al. 1967; Buck and Nass 1969; Boguslawski et al. 1974) and chloroplasts (Burkard et al. 1970; Reger et al. 1970), however, there have also been reports describing multiple forms that function in the cytosol (Kull and Jacobson 1969; Favorova and Kisselev 1970; Cowles and Key 1973).

In prokaryotes, the synthesis of amino acyl-tRNA synthetases is regulated by a “repression” mechanism in which the free cognate amino acid and the amino acyl-tRNA have an undefined role (Williams and Neidhardt 1969; McGinnis and Williams 1971; Coleman and Williams 1974). This type of control is also described for the valyl and methionyl-tRNA synthetases of yeast (Ehresmann et al. 1971; Surdin-Kerjan et al. 1973).

Some of these enzymes are also inhibited in vitro by precursors of their cognate amino acids (Nazario 1967; Williams et al. 1973; Yem and Williams 1971) and by ADP and AMP (Brenner et al. 1970).

Very little is known about the regulation of the amino acyl-tRNA synthetases in fungi. The existence of defined developmental stages, e.g., germination, characterized by shifts of protein synthesis from low to high levels, makes them attractive experimental systems for these studies.
In the present paper, the patterns of regulation of synthesis and activity of the branched chain amino acyl-tRNA synthetases during the germination of the conidia of the fungus *Neurospora crassa* are described.

**Materials and Methods**

**Strain**

The *Neurospora crassa* wild type LSDT-A was used throughout this investigation (Harding et al. 1970).

**Growth Conditions**

Minimal medium N (Vogel 1956) was used, supplemented as indicated in the text.

Myelia to be used for the extraction of cytosolic and mitochondrial enzymes were grown in fluted 21 flasks containing 11 of medium. The flasks were inoculated with a known amount of conidia and shaken at 160 cycles/minute. All cultures were harvested and washed 3 times with cold, sterile water by filtration on Buchner funnels using Whatman No. 1 filter paper. Prior to washing, 1 ml of suspension was used to calculate the percentage of germination after counting 500 cells under a Zeiss light microscope.

The washed conidial or mycelial pad was thoroughly pressed between sheets of filter paper towels using a roller. The wet pad was weighed and 3 samples were removed and used for dry weight, total protein and free amino acid determinations. The dry weight was obtained after heating a piece of the pad in an oven at 65°C for 24 h.

**Transfer RNA Preparation**

Transfer RNA (tRNA) was prepared from *Neurospora* by phenol using the method of Weeks and Gross (1971). With this method the yield (mg tRNA/g wet weight mycelia) was 0.1%-0.2% and the average ratio \( \frac{A_{260}}{A_{280}} \) was 1.9-2.1. The tRNA stored at \(-20^\circ\)C was stable for at least one year.

**Enzyme Extractions**

Washed conidial and mycelial pads were ground in a cold mortar using 2 times the wet weight of acid-washed sand and 5 times the wet weight of 0.01 M potassium phosphate buffer, pH 7.8, containing 0.01 M \( \beta \)-mercaptoethanol and 0.25 M sucrose (buffer A).

The homogenate was centrifuged twice at 1,500 \( \times \) g for 10 min to remove sand, nuclei and cell debris, and then at 9,700 \( \times \) g for 30 min to sediment the crude mitochondrial pellet. The supernatant was centrifuged at 34,800 \( \times \) g for 30 min and then recentlyrifuged at 100,000 \( \times \) g for 1 h. The top two-thirds of the supernatant was removed, protamine sulfate (1 mg/10 mg protein) was added to the supernatant to precipitate nucleic acids, and centrifuged twice at 20,000 \( \times \) g for 30 min; glycerol was added to the supernatant to a final concentration of 40%. The preparation was kept at \(-20^\circ\)C and was stable over a three month period. The WC preparation was similar to S and P.

**Assay of Amino Acyl-tRNA Synthetases**

The formation of amino acyl-tRNA was assayed by measuring the incorporation of the labeled amino acid into TCA-insoluble amino acyl-tRNA after incubation of the enzyme preparation with amino acid, ATP and tRNA (Loftfield 1971). For measuring isoleucine, valine and leucine accepting activity of tRNA catalyzed by enzymes of cytosol or mitochondria, the following assay was performed. Each reaction mixture contained 0.1 ml tRNA (100 \( A_{260} \)/ml) and 0.05 ml of an incubation mixture containing Tris-HCl, magnesium acetate, ATP, dithiothreitol, bovine serum albumin, and \( ^{14} \)C-amino acid (see Table 1 for optimal assay conditions). The reaction mixture was preincubated at 37°C for 3 min, and the reaction initiated by the addition of 0.05 ml (0.1 mg/ml) of cytosolic enzymes or 0.1 ml (0.2 mg/ml) of mitochondrial enzyme. After 10 min of incubation 0.1 ml portions of the mixture were removed and placed on 2.4 cm diameter Whatman No 3 filter paper discs. The discs were dried in a stream of hot air for 1 min and plunged into cold 5% trichloroacetic acid (10 ml/filter). They were washed free of residual, nonspecific radioactivity as described by Bollum (1968). After drying at room temperature the discs were placed in glass scintillation vials containing 10 ml scintillation fluid (1 M toluene, 4 g PPO and 0.05 g POPOP). The samples were counted in a Beckman LS-100 liquid scintillation counter at an efficiency of 70%.

The enzyme preparations free of nucleic acids were further purified by passing them through a G-25 Sephadex column (1 \( \times \) 30 cm) equilibrated with 0.005 M potassium phosphate buffer, pH 7.8, containing 0.01 M \( \beta \)-mercaptoethanol. The enzymes under these conditions were extremely labile, loosing most of their activity in 48-72 h in the presence of 40% glycerol at \(-20^\circ\)C.

Specific activity is expressed in units per mg of protein 1 unit being defined as the amount of enzyme required to catalyze the attachment of 1 \( \mu \)mole of amino acid to the appropriate tRNA per hour under the conditions employed.

**Hydroxyapatite Chromatography**

Enzymes extracted from cytosolic, mitochondrial or whole preparation were applied to a 1.5 x 23 cm hydroxyapatite column (Clarkson Chem. Co.) previously equilibrated with 0.005 M potassium phosphate buffer pH 7.8, containing 0.01 M \( \beta \)-mercaptoethanol. A linear gradient of potassium phosphate buffer pH 7.8 containing 0.01 M \( \beta \)-mercaptoethanol was applied (0.005-0.25 M, total volume 2 l). The gradient was monitored by measuring the refractive index of the eluate with a Bausch and Lomb, Abbé 3-L refractometer. The flow rate was 0.45 ml per minute and 9 ml fractions were collected. The fractions were kept no more than 72 h at 0°C.

**Assay of Glucose-6-Phosphate Dehydrogenase**

Glucose-6-phosphate dehydrogenase activity was used as a marker to monitor contamination of the P preparation. Activity was