DISSIMILAR SEPARABLE FORMS OF L-PHENYLALANINE ACTIVATING ENZYME IN RAT HEART CYTOPLASM

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1. Introduction

It has become generally [1], although not universally [2], accepted that the Mg^{2+}-dependent activation of an individual amino acid, such as L-phenylalanine, by its specific amino acid:tRNA ligase proceeds through an obligatory two-step mechanism in which a tRNA-independent reversibility primary reaction forming enzyme-associated L-phenylalanyl adenylate, (a) is followed by transfer of the activated aminoacyl moiety to $tRNA^{phe}$ species, (b):

(a) L-phe + ATP + Enz $\overset{Mg^{2+}}{\rightleftharpoons}$ Enz· · ·L-pheAMP + PP_i

(b) Enz· · ·L-pheAMP + $tRNA^{phe}$ $\rightleftharpoons$ L-phe $tRNA^{phe}$ + AMP + Enz

Rouget and Chapeville [3–5] have described the isolation of two separable forms, E_I and E_{II}, of L-leucine:tRNA ligase of *E. coli*, where E_I is unable to catalyse the acylation of tRNA, but does carry out the L-leucine-specific partial reaction analogous to (a) above; E_{II} is apparently converted into E_I by proteolytic loss of a polypeptide segment of the enzyme. The occurrence of different molecular forms of these enzymes varying in their catalytic properties is obviously an important potential control mechanism for cellular protein synthesis.

As part of a general investigation of the cellular physiology of amino acid:tRNA ligases we have found that L-phenylalanine activating activity in rat heart cytoplasmic extracts occurs in two forms differing in catalytic mechanism and physical parameters such as molecular weight, heat stability and precipitability by salts. The larger enzyme catalyses reaction (a) above, whereas the smaller one is able to acylate $tRNA^{phe}$ (sum of reactions (a) and (b)), but is notably less active in the performance of reaction (a) as measured by L-phenylalanine-dependent ATP–PP_i exchange or by the formation of L-phenylalanine hydroxamate.

2. Materials and methods

Materials were obtained from the following sources: EGTA, inorganic pyrophosphatase (yeast) (Sigma); triethanolamine (Boehringer); ATP, PEP, pyruvate kinase, DTT, yeast tRNA (Miles Seravac); [U-^{14}C]L-phenylalanine (The Radiochemical Centre). Salt-free hydroxylamine was prepared by the method of Beinert et al. [6]

Rat heart fractions were obtained according to procedures suggested by Dr. R. Zak, Univ. of Chicago (personal communication). Freshly obtained heart mus was cut into small pieces, washed twice for 10 min in 1 vol of buffer I (0.1 M KCl, 5 mM $MgCl_2$, 5 mM EGTA, 5 mM sodium phosphate, pH 6.8) and homogenized in 10 vol of buffer II (0.25 M sucrose, 0.05 M KCl, 5 mM EGTA, 1 mM sodium phosphate, 5 mM $MgCl_2$, 1 mM DTT, pH 6.8) for 10 sec at half maximum speed with an Ultraturrax homogenizer. Particulate material was removed by centrifugation in the cold, first at 1500 *g* for 10 min, then at 30 000 *g* for 20 min and finally at 105 000 *g* for 100 min. Solid ammonium sulphate (50 g/100 ml) was added, with stirring over 15 min, to the ice-cold cytoplasmic fraction thus obtained. The resulting precipitate was recovered by centrifugation at 30 000 *g* for 20 min and dissolved in a minimum volume of buffer C (0.25 M sucrose, 0.05 M KCl, 50

mM triethanolamine, 1 mM DTT, 5 mM $MgCl_2$, 1 mM EGTA, pH 7.6). The solution was passed through a column of fine grade Sephadex G-50, (25 cm × 1 cm, void volume 6.0 ml) to remove amino acids and salts before storage at −180°.

The formation of [^{14}C]L-phenylalanyl:tRNA^{phe} was measured by mixing small amounts of enzyme at pH 7.6 with 0.1 M Tris-HCl, 0.01 M $MgCl_2$, 0.01 M KCl, 0.02 M ATP, 0.025 M PEP, 0.2 mM DTT, 10 μg pyruvate kinase, 0.08 mg deacylated yeast tRNA (prepared as described by Sarin and Zamenenik [7]), 10 μg inorganic pyrophosphatase and 0.5 μCi [^{14}C]L-phenylalanine (492 mCi/mmole), all in 0.1 ml. After incubation at 37°, trichloroacetic acid-precipitable radioactivity was determined on Whatman 3MM filters [8] by counting them in a Packard Tri-Carb liquid scintillation spectrometer in vials containing 5 ml Fluoralloy (Beckman Instruments) in toluene (1:10, v/v).

Measurements of L-phenylalanine-dependent ATP–[^{32}P]PP_i exchange was effected according to Calendar and Berg [9].

The formation of L-phenylalanine hydroxamate was determined as described by Loftfield and Eigner [10], excepting that carboxymethyl-cellulose paper (CM82, Whatman Products, W. and R. Balston Ltd., Maidstone, Kent, England) was used instead of the strong cation-exchanger SA-1 to separate the product from unreacted radioactive L-phenylalanine. Development in 0.005 M sodium phosphate, pH 6.2 gave the best results. The radioactive product, detected by scanning the dried (110° for 10 min) chromatogram in a Nuclear-Chicago Actigraph III gas-flow counter, was cut out and counted in a liquid scintillation counter as described above. The hydroxamate could be eluted from the paper with 0.01 M Tris-HCl, pH 8.0.

Thin-layer chromatography was performed on silica gel-G precoated sheets (Eastman Kodak Co., Rochester, N.Y. 14650, USA) by development in n-butanol:formic acid:water (75:15:10, v/v/v) [11]. Protein concentrations were determined colorimetrically [12].

3. Results and discussion

Because of the suspected presence of a separate phenylalanine:tRNA ligase in mitochondria [13], the enzyme activity in the nonparticulate fraction was selected for study. The crude ammonium sulphate preparation exhibited activity in all 3 assays chosen, catalysing the formation of L-phenylalanyl tRNA^{phe}, the formation of L-phenylalanine hydroxamate as well as a L-phenylalanine-stimulated ATP–PP_i exchange.

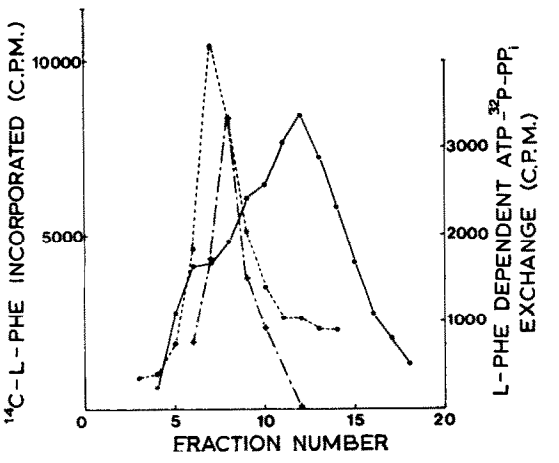

Fig. 1. Differential sedimentation of phenylalanine-activating enzyme in a sucrose density gradient. Crude enzyme fraction (0.15 ml, 2 mg protein dissolved in buffer C without sucrose) was layered on 4.8 ml of a 3–15% sucrose density gradient in buffer C. Centrifugation at 180 000 *g* for 6 hr in the cold was carried out in the SW65 L Ti rotor in an L4 ultracentrifuge (Beckman Instruments). Fractions of 0.2 ml were collected by puncturing the bottom of the tube. Assays described in Methods, were carried out as follows: (●—●—●) acylation of tRNA with [^{14}C]L-phenylalanine; (○- - ○- - ○), formation of [^{14}C]L-phenylalanine hydroxamate; (+ - - +) L-phenylalanine-dependent ATP–PP_i exchange.

We were initially interested in the possible occurrenc of amino acid:tRNA ligase complexes [14] and subjected the crude preparation to sucrose density gradient analysis by the method of Martin and Ames [15]. The activity involved in the formation of phenylalanine hydroxamate and phenylalanine-stimulated ATP–PP_i exchange migrated significantly further than the broad peak of phenylalanine-tRNA^{phe} formation, which gave a slight shoulder in the region of the other activity (fig. 1). With the use of the marker enzymes catalase (244 000 daltons), and yeast alcohol dehydrogenase (150 000 daltons), it was possible to fix the approximate molecular weights of the two enzymes at 194 000 and 150 000 daltons, respectively. The result was iden-

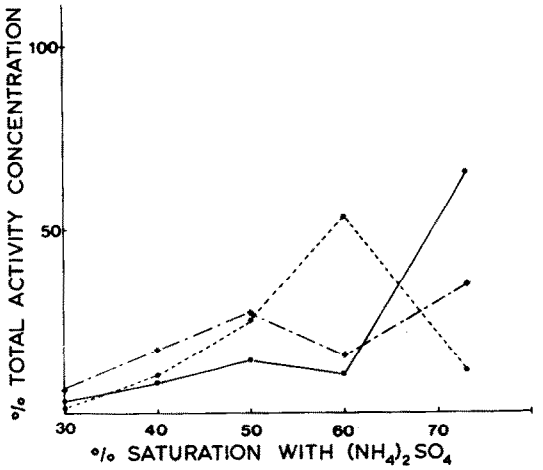

Fig. 2. Differential salt fractionation of phenylalanine activating enzymes. A high-speed supernatant fraction (11 mg/ml protein) was prepared from 6 rat hearts as described in Methods, except that the homogenization was carried out in 2 vol of buffer II. Solid ammonium sulphate was added slowly, with stirring, to give the required degrees of saturation at 0°. Precipitates were collected and dissolved in minimal vol of buffer C before dialysis for 16 hr against 500 vol of buffer C in the cold. Activities and protein concentrations were determined as described in Methods. (•—•—•), acylation of tRNA with [^{14}C]L-phenylalanine; (o - - o - - o), formation of [^{14}C]L-phenylalanine hydroxamate; (+ - - - +) protein; all these values are given as % of the total.

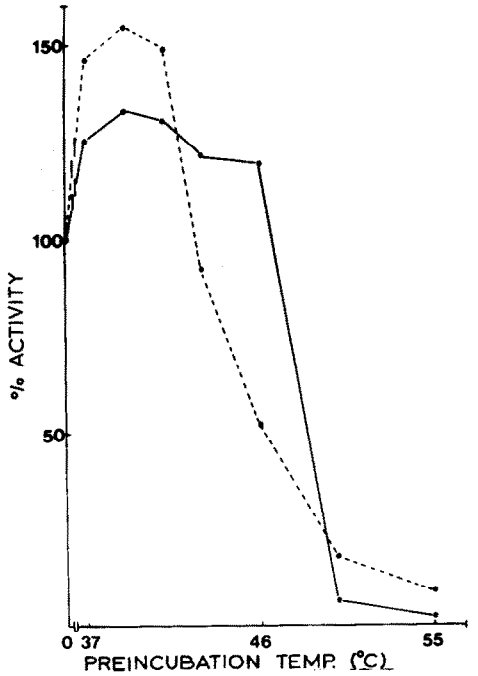

Fig. 3. Effects of preincubation at different temperatures on phenylalanine-activating enzymes. Soluble fractions prepared as described in Methods (12 mg/ml protein) were preincubated without additions at the temperatures indicated before being cooled to 4°. Assays were performed as described in Methods. Protein concentrations were identical in all samples. Activities are expressed as % of activity found in samples preincubated at 0°. (•—•—•), aminoacylation of tRNA with [^{14}C]L-phenylalanine; (o - - o - - o), formation of [^{14}C]L-phenylalanine hydroxamate.

tical when muscle tRNA prepared by phenol–SDS extraction [16], was used for the assays.

Further evidence for two molecular forms of phenylalanine activating enzyme was obtained by differential ammonium sulphate fractionation of the crude preparation. The form catalysing acylation of tRNA precipitated between 60–70% saturation with the salt, while the other activity was maximally recovered at 50–60% saturation (fig. 2).

The temperature stability of the two forms also differed (fig. 3). Both activities were stimulated by preincubation at temperatures between 39° and 41° when compared with samples of enzyme not preincubated, but the shapes of the 2 curves given by the activities in hydroxamate and aminoacyl tRNA formation were different at these and higher temperatures.

In order to exclude the possibility that we were detecting an enzyme activity unrelated to phenylalanine carboxyl activation for protein synthesis, the hydroxamate product of the large enzyme's activity was subjected after elution to hydrolysis in 1 N HCl at 100° for 60 min. The radioactivity now migrated to the front on CM-cellulose paper chromatography, together with marker phenylalanine, and also co-chromatographed with this amino acid on thin-layer chromatography.

The results reported here have been a superficial resen blance to those of Rouget and Chapeville [3–5] excepting that the enzyme able to activate the amino acid but not to transfer it to tRNA, is larger than the other form, and unlikely to be proteolytically derived from the "complete" enzyme. Also the latter appears to show little L-phenylalanine-stimulated ATP–PP_i exchange and does not form hydroxamate readily. It

thus resembles the spermine-stimulated aminoacylation reaction catalysed by isoleucine tRNA and valine tRNA ligases from *E. coli* [17, 18], which is believed to proceed without the intermediary formation of aminoacyl adenylate able to react with PP_i or hydroxylamine. It is possible that the two forms described here may be inter-convertible or that they represent special arrangements of subunits [19]. Further purification of the two enzymes will clarify the enzymological situation, and its possible cell-metabolic significance.

Acknowledgements

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