A TEST FOR THE ACTIVE INTERMEDIATE IN THE AMINOACYLATION OF $tRNA^{Phe}$ FROM YEAST

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1. Introduction

The aminoacyladenylate-synthetase complex plays a central role in all the discussions about the mechanism of aminoacylation of tRNAs [1]. Without any doubt the aminoacylation of most tRNAs can proceed from this complex but this does not prove that the complex is an obligate intermediate in the enzymatic reaction. For a detailed study of the aminoacylation of a tRNA it is therefore useful to have a direct rapid, quantitative test for the formation of the active intermediate in this reaction.

Trying to apply the filter test of Yarus and Berg [2] on the phenylalanyl-tRNA synthetase (PRS) from yeast, we found that the measurable amount of complex varied with the filter batch, as already mentioned by the authors and never it was quantitative. Therefore we tried to vary some conditions of the test in order to get reproducibly quantitative results.

In this paper we present a modified filter test for the phenylalanyladenylate-synthetase complex, investigations to show that it is quantitative, and some experimental applications of the test. We detected a different heat sensitivity of the activation and aminoacylation activity of PRS.

2. Materials and methods

PRS from *Saccharomyces cerevisiae* C 836 was prepared as in [3]. For the standard test a 200-fold purified preparation was used and for the thermal denaturation we used a pure enzyme. Inorganic pyrophosphatase was from Boehringer (Mannheim, West Germany).

The aminoacylation activity of the PRS was tested according to [3]. The pyrophosphate exchange reaction was measured according to the method described in [4,5]. The incubation mixture contained in 0.1 ml: 2.5 μmol Tris–HCl pH 7.5, 1 μmol ATP, 5 nmol L-phenylalanine, 2 μmol $MgCl_2$, 10 nmol [^{32}P] pyrophosphate, 15 μg bovine serum albumin and 20 to 50 μunit PRS. No phenylalanine was added to the blanks. The samples were incubated at 37°C; after 4 min the reaction was stopped by addition of 0.1 ml 5% perchloric acid. (Under these conditions there was still a linear dependence between the extend of pyrophosphate exchange and the time of incubation). The samples were then shaken 30 min with 2 mg norite in 0.1 ml 0.1 M non-radioactive pyrophosphate, 0.02 M sodium acetate pH 4.5. The suspension was then filtered over glass filters and washed with the buffer used for the norite suspension and with water. The radioactivity of the filters was measured in a liquid scintillation counter.

For the assay of the active intermediate bound to enzyme the incubation mixture contained in 0.1 ml : 2.5 μmol Tris–HCl pH 7.5, 0.005 μmol L-phenylalanine, 0.01 to 1 μmol ATP, an excess of 1 μmol $MgCl_2$ over ATP, 2 μg inorganic pyrophosphatase, 15 μg bovine serum albumin and 0.5 to 5 munit [3] PRS. When the test was carried out with radioactive phenylalanine, the blanks were without ATP, when radioactive ATP was used phenylalanine was omitted in the blanks. The samples were mixed at 0°C and incubated for 2 min at 20°C. Then 2 ml of buffer A

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containing 0.6 M ammonium sulphate, 0.2 mM dithiothreitol, 1 mM EDTA, 1 mM Tris–HCl, pH 7.0 were added and the mixture was poured on nitrocellulose filters. The filters had been pretreated in a solution of 1 mg adenosine per ml, saturated potassium phosphate, pH 7, during at least 9 h. The filters were washed once with 5 ml of buffer A just before the filtration of the samples and three times after this filtration. After drying, the radioactivity on the filters was determined in a liquid scintillation counter. The preparative isolation of the phenylalanyladenylate-synthetase complex was in analogy to [6] using a Sephadex G-50 column equilibrated with buffer A when mentioned in the text.

3. Results

3.1. *Elaboration of the test*

With the test of Yarus and Berg [2] we had a method to detect an aminoacyladenylate enzyme complex. The amount of complex determined was proportional to the amount of enzyme added. However this did not prove that all the complex initially formed was also recovered on the filter. To determine the amount of recovery we prepared the radioactive complex according to [6] by filtration over a Sephadex column. Aliquots of the eluate were immediately applied on the nitrocellulose filters as in [2]. A comparison of the radioactivity found according to this method with the radioactivity of the complex measured directly in the eluate of the column, showed a recovery varying from 50 to 70% with different kinds of nitrocellulose filters. Using two filters superposed instead of one we found that no radioactivity was retained on the second filter. Therefore the adsorption of the synthetase on the first filter is probably quantitative. The loss of radioactivity must have resulted from a degradation of the complex during the filtration. Trying to stabilize the enzyme, we found that the addition of ammonium sulphate and EDTA increased the recovery of complex on the filter up to 95% of the radioactivity eluted from the Sephadex column with the protein. The difference to 100% was mainly due to the degradation of the complex during the time between elution of the product and the moment of the test. The stabilizing effect of the ammoniumsulphate and the EDTA was also observed when the phenylalanyladenylate enzyme complex was isolated on a preparative scale by Sephadex filtration. Running the column in buffer A (see methods) increased considerably the yield of complex in comparison to the buffer used before [6]. Fig.1 demonstrates the linearity between the amount of enzyme and the amount of complex found in the filter test. The addition of bovine serum albumin was particularly necessary to get the curve going through the origin of the coordinates.

It has to be mentioned that under our test conditions Phe-tRNA^{Phe} from yeast is quantitatively retained by the filter, several other aminoacylated tRNAs are also adsorbed but not quantitatively. The filter test can be used as well with radioactive Phe as with radioactive ATP however the blanks (without Phe) are higher when radioactive ATP is used. This might be due partly to the presence of other synthetases and to the contamination with amino acids when crude enzyme fractions are used. The main cause of these high blanks is the adsorption of ATP on the nitrocellulose filters. This adsorption of ATP can be reduced considerably by pretreating the filters with phosphate and adenosine (see Materials and methods). However, when less than 1 pmol of complex are to be filtered, it would be advantageous to use smaller filters and lower amounts of ATP.

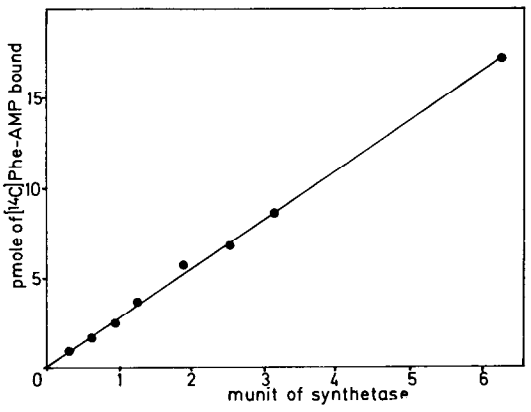

Fig.1. Dependence of the amount of active phenylalanine enzyme complex from the amount of synthetase as measured by the filter test. The samples were incubated with variable amounts of PRS and the test was carried out as described in Materials and methods.

3.2. *Applications of the test*

Repeating the test, we found within one batch of enzyme a reproducible quotient of aminoacylation units per nmole of active complex. However, this relation was different from one enzyme preparation to another and decreased with the time of storage of the enzyme. One could interpret these observations as due to a different sensitivity to denaturation of the aminoacylation activity and of the complex forming activity of the PRS. The evidence that the aminoacylation activity of PRS decreases faster than the amino acid activation activity of the PRS during thermal denaturation is demonstrated by fig.2. The use of a highly purified enzyme (4 units per A_{280} unit of protein) and the increase of the rate of inactivation with the temperature are evidences for a thermal effect rather than a proteolytic one. This figure also shows that the change in the amino acid activation activity measured by three different methods: formation of the enzyme complex with the radioactive label in the Phe, with the label in the adenosine of the ATP, or pyrophosphate exchange, coincided very well. This coincidence is a further evidence for the validity of the filter test. The diagram in fig.2 is characteristical for most enzyme preparations. With one batch of PRS we occasionally found a near to 100% increase in the aminoacylation activity during the first three minutes of heating, at 43°C, then this activity decreased as usual. This observation shows, that the PRS can also be denatured reversibly, but we do not know the special conditions of such a denaturation. The complex formation activity of this special enzyme batch did not show any increase during the first few minutes of heating, the slight decrease as in fig.2 was also observed in this case.

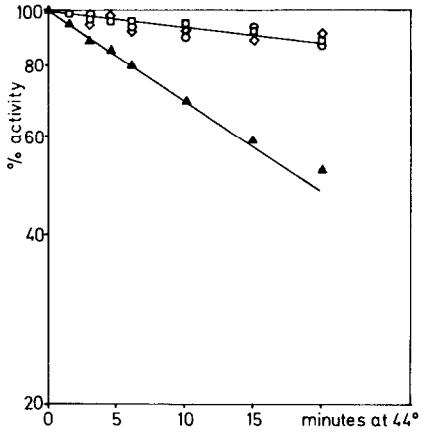

Fig.2. Thermal inactivation of PRS represented in a semi logarithmic plot. PRS was incubated at 44°C in a buffer containing 1 mM glutathione, 0.5 mM EDTA, 10% glycerol, 0.1 M potassium phosphate pH 7.3 and 1 mg/ml bovine serum albumine. Aliquots were withdrawn at the times indicated in the figure and assayed in the different tests. (▲—▲) Aminoacylation activity according to [3]. (○—○) Pyrophosphate exchange. (□—□) Yield of PRS active intermediate complex with [^{14}C] phenylalanine as the marker. (◇—◇) Yield of PRS–active intermediate complex with [^{14}C] ATP as the marker.

4. Discussion

Once the number of phenylalanyladenylate binding sites per molecule of PRS is established unequivocally, the quantitative test for the complex permits to determine the absolute purity of the PRS, whereas the usual kinetic tests based on pyrophosphate exchange or on aminoacylation of the tRNA can indicate only a relative purification of the enzyme. It was already known from other publications [7–9] that the aminoacylation activity of an aminoacyl-tRNA synthetase can be destroyed in preference to the amino acid activation using very special conditions. Here we showed that thermal denaturation has a similar effect and we have evidence that even during the purification of the PRS the aminoacylation activity may change rather independently of the amino acid activation capacity. Therefore it is controversible to measure a synthetase activity by pyrophosphate exchange, because also enzyme molecules inactive in aminoacylation are included.

The quotient of the aminoacylation units per μmole of adenylate complex represents a criterion for the quality of the enzyme. In our investigation we found a variance between 0.2 and 1.2 unit per nmole of complex with the different treatments during the purification of the enzyme.

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