A NEW ACTIVE INTERMEDIATE IN THE AMINOACYLATION OF tRNA

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1. Introduction

During the past six years the discussion about the mechanism of the enzymatic aminoacylation of tRNAs has been in controversy. Some investigators gave evidence supporting a two step mechanism with an enzyme-bound aminoacyl adenylate as an intermediate [1–7], while others gave evidence for a concerted mechanism in which the tRNA, the ATP, and the amino acid have to be bound to the enzyme and the aminoacylation proceeds without the adenylate as intermediate [8–14].

The filter test described in the preceeding paper is a tool to investigate the properties of the active phenylalanine intermediate bound to the phenylalanyl-tRNA synthetase (PRS) from yeast. The stoichiometry of the enzyme complex in the absence of tRNA was found to be one Phe-AMP per enzyme molecule. In the presence of a periodate treated $tRNA^{Phe}$ ($tRNA^{Phe}_{ox}$), the stoichiometry was different: One Phe-AMP and one Phe were found per molecule of PRS. Both phenylalanines could be transferred onto $tRNA^{Phe}$. Therefore there must exist a reactive Phe on the enzyme that is not bound to AMP. This intermediate and the role of the tRNA in its formation are the clues to the mechanism of the aminoacylation of tRNAs. Probably the transfer of the amino acid on the tRNA via this intermediate is a general mechanism.

2. Materials and methods

Pure PRS from *Saccharomyces cerevisiae* C 836, prepared as in [15] was a gift from R. Hirsch and U. Pachmann in this laboratory. Inorganic pyrophosphatase and hexokinase were from Boehringer (Mannheim). Adenyldiphosphonate-α,β-methylene (Apcpp) was purchased from Serva (Heidelberg).

For the analytical determination of the active intermediate bound to enzyme the standard filter test and for the preparative isolation of this complex the Sephadex filtration presented in the preceeding paper were used. The transfer reaction from this complex was carried out in 0.3 M ammoniumsulphate, 0.1 mM dithiothreitol, 0.05 mM EDTA, 25 mM Tris pH 7.5, 10 mM $MgCl_2$ with 2 pmol of PRS complex and 0.5 nmol of $tRNA^{Phe}$ in 0.1 ml at 20°C for one min.

3. Results

The filter test method presented in the preceeding paper served to determine the stoichiometric composition of the active intermediate enzyme complex. Comparing the radioactivities from [^{14}C]Phe, [^{14}C]ATP and [^{32}P]ATP in the complex formed in the absence of tRNA, it was shown (see table 1) that the complex contains Phe and AMP in the molar ratio of 1:1 and no additional ATP or pyrophosphate. The calculation of the ratio, moles Phe-AMP per mole PRS was based on the amount of protein determined according to Lowry, assuming that the enzyme was 90% pure, as indicated by the acrylamide electrophoresis, the mol. wt. of PRS being 250 000. A ratio near 1:1 was found. When Apcpp was used instead of ATP no complex could be found by the filter test, neither in the absence nor in the presence of $tRNA^{Phe}$.

The absence of $tRNA^{Phe}$ in the assay is an artificial situation. As an analog of $tRNA^{Phe}$ that cannot be

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Table 1
Stoichiometry of the complex between the activated Phe intermediates and PRS

System without $tRNA^{Phe}_{ox}$	(moles)	System with $tRNA^{Phe}_{ox}$	(moles)
PRS	1		1
Phe[a]	0.95		1.9
AMP[b]	0.9		0.9
ATP or pyrophosphate[bc]	0.03		

The values presented here are averaged from three separate experiments, under standard assay conditions (with pyrophosphatase).
[a]Blanks without ATP (0.05 mol) were substracted
[b]Blanks without Phe (0.1 mol) were substracted
[c]In this assay pyrophosphatase was omitted.

aminoacylated, $tRNA^{Phe}_{ox}$ was used to simulate the activation process on the enzyme in the complete system. Two Phe and one AMP were found to be bound under these conditions. The omission of inorganic pyrophosphatase had only an effect in the absence of $tRNA^{Phe}_{ox}$. The amount of Phe-AMP bound to the enzyme was about 20% lower in this case. The blanks indicate that ATP is necessary for the binding of Phe and that Phe is necessary for the binding of ATP. The higher blank 'without Phe' might be due to a contamination of Phe in the assay mixture.

The finding of two Phe bound to one molecule of enzyme raises the question whether both are reactive in the transfer onto $tRNA^{Phe}$. To resolve this problem two different experiments were performed.

(A) Intermediate complex was formed in the presence of $tRNA^{Phe}_{ox}$ and isolated by filtration over Sephadex. The stoichiometry of the complex recovered from the column was verified in an experiment using [^{3}H]ATP and [^{14}C]Phe. After addition of excess $tRNA^{Phe}$, more than 80% of the [^{14}C]Phe was transferred from the complex onto $tRNA^{Phe}$. The conclusion is that both Phe are reactive.

(B) Second experiment: (i) Formation of the complex, (ii) addition of glucose and hexokinase to convert the excess of ATP into ADP. (iii) addition of $tRNA^{Phe}$ in excess and precipitation 30 sec after the addition of $tRNA^{Phe}$. When $tRNA^{Phe}_{ox}$ was present during the formation of the complex, about twice the amount of [^{14}C]Phe was incorporated into $tRNA^{Phe}$ as in the absence of $tRNA^{Phe}_{ox}$. This amount corresponded to 110% of the radioactivity in the complex. Only about 80% of transfer of Phe onto $tRNA^{Phe}$ could be expected. As there was still a low concentration of ATP remaining under the conditions of the incubation with glucose and hexokinase, these higher values were due to further aminoacylation mediated by this ATP. However, this experiment is conclusive as the amount of Phe incorporated into the $tRNA^{Phe}$ was proportional to the amount of Phe in the complex.

Both Phe that are bound to the PRS in the presence of $tRNA^{Phe}_{ox}$ are reactive in the transfer reaction.

4. Discussion

In the absence of $tRNA^{Phe}$ only one ATP and one Phe react with an enzyme-bound Phe-AMP. In the presence of $tRNA^{Phe}_{ox}$ one Phe-AMP and one additional reactive Phe are bound to the enzyme. It is plausible that the aminoacylation proceeds only via this reactive Phe. As the reactive Phe has its energy from ATP, one must conclude that the presence of $tRNA^{Phe}$ induces a second strong binding site for ATP and Phe on the enzyme. $tRNA^{Phe}$ may act as an allosteric effector, because the $tRNA^{Phe}_{ox}$ that cannot react produces the same induction. The reactive enzyme-bound Phe is probably formed from Phe-AMP (see the conclusions drawn from the reactivity of the Phe-AMP PRS complex). The conversion of Phe-AMP to enzyme-bound Phe under the release of AMP might also be induced by $tRNA^{Phe}$ or by the occupants of the second binding site for Phe and ATP. As the Phe bound to the enzyme conserves the energy necessary for the ester linkage with the tRNA it is difficult to imagine a

bond with the enzyme other than a covalent one. Murayama et al. [16] already suggested the existence of such a reactive intermediate resulting from the attack of aminoacyladenylate by some nucleophilic group of the protein, upon binding of tRNA.

The aminoacyl adenylate enzyme complex obtained in the absence of tRNA is an artificial product. However, it reacts on addition of tRNA. One can assume that an enzyme catalyzed a reaction only according to one mechanism. Therefore the amino acid can only be transferred directly from one of the intermediates onto the tRNA. Therefore, it is plausible that aminoacyl-AMP is the first intermediate and the amino acid that is bound to the enzyme is formed from this adenylate and reacts directly with the tRNA. The experiment of Lövgren et al. [14] with isoleucyl-tRNA synthetase from *E. coli* shows, that the reaction of the aminoacyl-AMP enzyme complex with tRNA is slow, compared to the reaction in the complete system. This experiment was repeated with phenylalanyl-tRNA synthetase from yeast in the presence of 10 mM Mg^{++} at pH 7.5 and qualitatively the same results were obtained. The slow reaction with the aminoacyl adenylate enzyme complex might be due to a low rate of conversion of the adenylate to the reactive amino acid. There are two possibilities for the rate limiting process in this reaction. One may be the binding of tRNA to the enzyme aminoacyl-AMP complex, if this binding is much slower than the binding to the free enzyme. Then the tRNA induces also directly the conversion of the adenylate to the reactive amino acid. On the other hand, it may be that the conversion itself is rate limiting when the amino acid and ATP are missing in the second binding site.

From the differences in the aminoacylation velocities of complexes formed in the presence and in the absence of tRNA it is evident that at least with most aminoacyl tRNA synthetases the initiation of the reaction proceeds by binding first the tRNA to the enzyme and second the ATP and amino acid followed by formation of aminoacyl-AMP with release of pyrophosphate. This statement is not contradictory to results obtained by steady state kinetic experiments, wherein the pyrophosphate release was found to proceed before the addition of the tRNA to the enzyme [1–7]. The data of the steady state kinetics were obtained from a repeatedly run cyclic process, whereas the conclusions reported here are related to the only once occurring initiation of this cyclic mechanism.

The results of the present investigation help to explain the data interpreted in favour of a two step mechanism with aminoacyl-AMP as an intermediate [1–7] as well as the data in favour of a concerted mechanism with the tRNA in the role of a trigger [8–14]. The results presented here are also in agreement with the data of Dutler [17], who found two reactive Phe per PRS in the presence of $tRNA^{Phe}_{ox}$ but without any further characterisation of these products.

The discrepancy between the data of Fasiolo et al. [18] who found significantly more than one Phe-AMP per molecule of enzyme even in the absence of $tRNA^{Phe}_{ox}$ and the one Phe-AMP per enzyme presented here, are probably due to the methods, that are not equally suitable for the detection of ligands bound with a lower stability.

Note added in proof

A catalytic model involving the attack of aminoacyl adenylate by a nucleophilic group of the protein should allow an ATP–AMP exchange [16]. The general failure to observe such an exchange could be explained by a conformational change of the enzyme upon binding of tRNA, which would be required for the formation of the second active complex. Indeed, Remy et al. [19] observed that only a very faint ATP–AMP exchange can be observed in the absence of tRNA, but that $tRNA^{Phe}$ 3′ deoxy A strongly stimulates this exchange although this modified tRNA is no longer acylable.

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