Isolation of An Activator Macromolecule for Alanyl-tRNA Synthetase from Rat Liver

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SUMMARY: Hydrophobic chromatography (1,6 hexanediamine Sepharose 4B) of crude extracts of rat liver leads to a 40-50% loss of total alanyl-tRNA synthetase activity applied to the column. Assaying the alanyl-tRNA synthetase with various pooled fractions from the column indicates the presence of an activator macromolecule for this synthetase. The activator is 260,000 to 350,000 daltons in size. In presence of the activator the alanyl-tRNA synthetase behaves as an allosteric enzyme for tRNA binding. Also, in presence of the activator, the apparent k_m values for ATP and Ala binding to alanyl-tRNA synthetase are lower while the maximum velocity remains the same.

INTRODUCTION: Over the years several reports have appeared suggesting that aminoacyl-tRNA synthetases may actually be associated with other macromolecules in mammalian cells. Thus, under gentle conditions of preparation, isolated microsomes from chick embryo were found to contain aminoacyl-tRNA synthetases (1). Hradec and Dusek (2) have reported that cholesterol-14-methyl hexadecanoate appears to be associated with aminoacyl-tRNA synthetases and in presence of this cholesterol ester aminoacyl-tRNA synthetase activities were stimulated. Recently, Dickman and Boll (3) have isolated an activator macromolecule from rat liver. This activator has a molecular weight of 45,000 and increases acceptance of yeast tRNA for amino acids, Ile, Leu, Lys, Ser and Met.

Bandyopadhyay and Deutscher (4) found that all 18 aminoacyl-tRNA synthetases copurified together by gel filtration with a size of about 1.5×10^6 daltons. They were also able to show that this aminoacyl-tRNA synthetase contained almost all of the tRNA found within the cell (4) and a substantial amount of lipid in the form of cholesterol ester (5).

Other workers have also obtained evidence for the existence of some type of aminoacyl-tRNA synthetase complex. These investigators were not able to isolate a complex containing all 18 aminoacyl-tRNA synthetases, but

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isolated partial complexes containing five aminoacyl-tRNA synthetases (6) and six aminoacyl-tRNA synthetases (7,8). Aminoacyl-tRNA synthetases have also been shown to be associated with ribosome subunits, monoribosomes, and polysomes (9-11).

From the studies reported in the literature, it is apparent that the aminoacyl-tRNA synthetases may exist in mammalian cells in some form of intermolecular aggregates. However, it is not clear whether this aggregate contains all eighteen aminoacyl-tRNA synthetases or whether other types of macromolecules are contained in this aggregate. Studies performed with toluene permeabilized liver cells provides evidence that the aminoacyl-tRNA synthetases may be bound within the cell (12-14).

In attempting to isolate the aminoacyl-tRNA synthetase complex by hydrophobic chromatography, I found that alanyl-tRNA synthetase loses about 50% of its activity. In this communication I report on the isolation of a large molecular weight molecule which when added to alanyl-tRNA synthetase stimulates the activity of the synthetase. In presence of the activator the alanyl-tRNA synthetase behaves as an allosteric enzyme for tRNA binding. The apparent k_m values for ATP and Ala binding are lowered but the maximum velocity of the reaction was unchanged.

MATERIALS AND METHODS: Frozen rat livers were purchased from Pel-Freez Biologicals, Inc. Cyanogen bromide activated Sepharose 4B was purchased from Sigma Chemical Company and 1,6-hexanediamine was purchased from Aldrich Chemical Co. All radioisotopes were purchased from Schwarz/Mann.

Aminoacyl-tRNA synthetases assays were performed as described by Bandyopadhyay and Deutscher (4). The activator assay was run as follows: one assay reaction mixture was the same as for the alanyl-tRNA synthetase assay described (4) except after addition of the enzyme the assay was left on ice for 10 min before incubating at 37C. This reaction mixture was the enzyme control and determined the enzyme activity in absence of the activator. A second reaction mixture was identical to the aminoacyl-tRNA synthetase as described (4) except activator was added in place of enzyme and then the assay was left in ice for 10 min before incubating at 37C. This reaction mixture was the activator control and determined the amount of alanyl-tRNA synthetase activity in the activator sample. A third assay mixture contained both enzyme and activator which was also placed in ice for 10 min before incubating at 37C. All reactions were stopped and processed as described (4). To determine the amount of stimulation, the cpm in the activator control was subtracted from the cpm in assay tube containing both enzyme and activator. Per cent stimulation was determined by dividing the picomoles of the amount

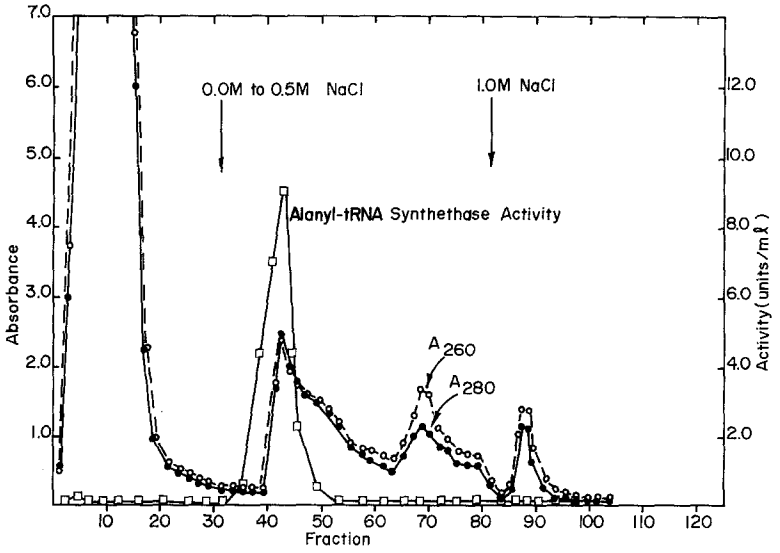

Fig. 1 Chromatography of low speed supernatant fraction on 1,6 hexanediamine CNB-Sepharose 4B.

The low speed supernatant fraction (6.0 ml) was applied to a column (0.9 by 13 cm) of 1,6 hexanediamine CNBr-Sepharose-4B and eluted with Buffer I until the A_{280} was less than 0.10, then the alanyl-tRNA synthetase was eluted by a 0.0 to 0.5 M NaCl in Buffer I gradient (total vol of the gradient was 80 ml). At the end of this gradient 50 ml of 1 M NaCl in Buffer I was applied to the column. Fractions of 1.6 ml were collected at a flow rate of about 30 ml per hour. Alanyl-tRNA synthetase activity □-□-□ ; A_{280} o-o-o, A_{260} o---o---o. Zero time counts were subtracted from each value. The column was run at room temperature. Aliquots of 15 µl were assayed of Ala incorporation.

of stimulation by the picomoles in the enzyme control assay and multiplying by 100.

To isolate alanyl-tRNA synthetase and the activator frozen livers were partially thawed, minced and homogenized in 5-10 gm portions with 2 vol of medium containing 0.02 M KPO_4, pH 7.0, 8 mM $MgCl_2$, 10 mM 2-mercaptoethanol and 10% glycerol (final vol) (Buffer I). Homogenization consisted of six up-to-down strokes on a motor-driven Teflon homogenizer. The homogenate was centrifuged for 15 min at 12,100 x g to remove cellular debris (low speed supernatant fraction).

The hydrophobic affinity column was formed by coupling 1,6 hexanediamine to cyanogen bromide activated Sepharose (CNBr-Sepharose 4B) suspended in water at a concentration of 350 mg of 1,6 hexanediamine per ml of CNBr-Sepharose 4B. The pH was adjusted to 9-10 with HCl. The mixture was stirred for 16 hr at room temperature. Then the uncoupled 1,6 hexanediamine was removed by 10 washings with water and 4 washings with Buffer I.

RESULTS: As shown in Figure 1, alanyl-tRNA synthetase activity was retained on a 1,6 hexanediamine CNBr-Sepharose 4B column under conditions employed in

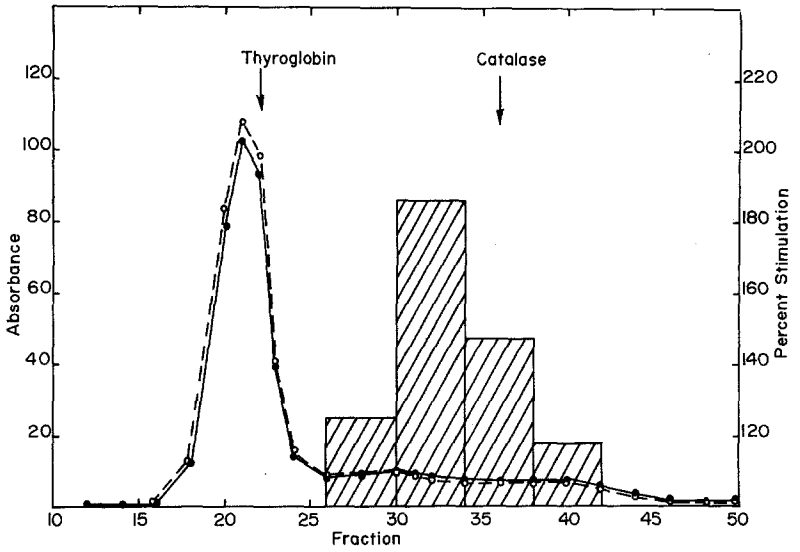

Fig. 2 Chromatography on G-200.

Active activator fractions from the 1,6 hexanediamine CNBr-Sepharose-4B (tubes 3-16) were pooled and concentrated by ultrafiltration in an apparatus obtained from the Amicon Corporation using XM-300 membranes at a nitrogen pressure of 20 lb/in^2. Concentration of 2 to 3 fold was accomplished by this procedure with greater than 80% recovery of the activator molecule. The XM-300 concentrate (5.8 ml) was applied to a column (2.5 cm by 80 cm) of Sephadex G-200 and eluted with Buffer I. Fractions of 5 ml were collected at a flow rate of about 8.0 ml per hr. Standards on the column were thyroglobulin (peaked at fraction 22), catalase (peaked at fraction 36), and bovine serum albumin (peaked at fraction 51). Activator activity ( ) was determined by pooling the fractions from the G-200 column (5 fractions per pool) and using the activator assay described in the text. A_{280} o-o-o and A_{260} o---o---o. Zero time counts were subtracted from each value. About 114% of the activator activity applied to the column was recovered. The column was run at 4C.

this experiment. Essentially, all of the alanyl-tRNA synthetase activity eluted off the column at about 0.1 M NaCl with a recovery of 40-50% of the total activity applied to the column. Fractions 39 to 44 were pooled and stored at -18C in 20% glycerol (alanyl-tRNA synthetase fraction).

Pooling the various fractions from the column and mixing these pooled fractions with the alanyl-tRNA synthetase fraction showed that an activator molecule for alanyl-tRNA synthetase was in fraction numbers 3-20 (data not shown). In presence of the activator essentially 100% of the alanyl-tRNA synthetase activity applied to the column was recovered.

The activator isolated on the hydrophobic column could be purified further by chromatography on a column of Sephadex G-200 (Fig. 2). This procedure served to separate the activator, which was included in the column, from higher molecular weight contaminants. The greatest amount of stimulation occurred with the pool containing fractions 30-34. The molecular size of this pool was estimated to be between 260,000 and 350,000 daltons. Fractions 30-34 were concentrated by ultrafiltration using an Amicon PM-30 membrane under nitrogen. The samples were concentrated 3 to 5 fold by this method with greater than 90% recovery of the activator molecule. This PM-30 concentrated sample was used as the activator molecule in the remaining experiments in this paper.

There were no alanyl-, arginyl-, lysyl-, threonyl-, or methionyl-tRNA synthetase activities in this activator sample (data not shown). This suggests that the activator is not an aggregate of other aminoacyl-tRNA synthetases which simply bind to alanyl-tRNA synthetase and alter its activity.

In all experiments performed in this communication the activator was used at saturating concentrations (10 µl). The amount of enzyme used was rate limiting (2 µl) because the initial rate of the reaction was increased in the presence of the activator but the maximum velocity remained the same (data not shown).

The data in Fig. 3 shows that in presence of the activator and increasing concentrations of tRNA, alanyl-tRNA synthetase behaves as an allosteric enzyme. In absence of the activator the synthetase activity shuts off at relatively low concentration of tRNA (100 µg of tRNA per assay) as shown in Fig. 3. This rapid shut off of alanyl-tRNA synthetase activity was not due to some component in the assay becoming rate limiting because if the enzyme concentration used in the assay was doubled, the activity of the synthetase doubles and the synthetase activity was inhibited at 200 µg instead of 100 µg of tRNA per assay (data not shown).

It is possible that the activator might bind to some inhibitor in

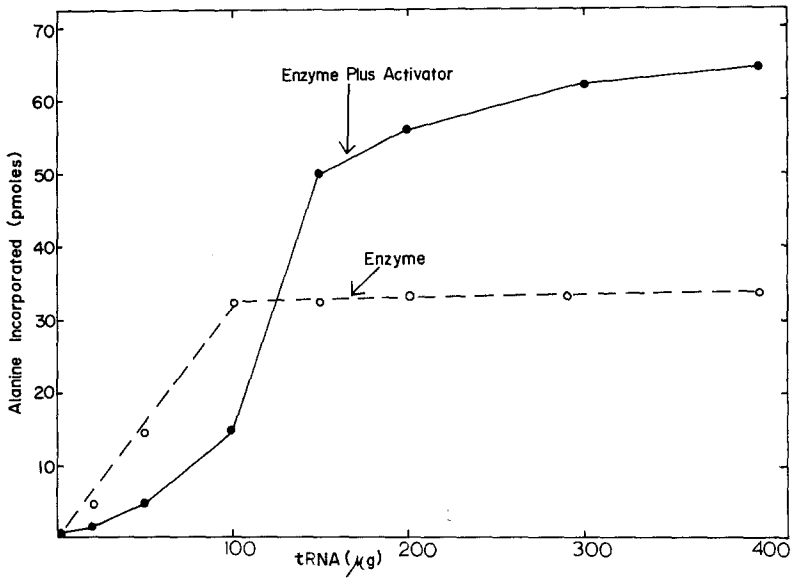

Fig. 3 Effect of Varying tRNA Concentrations on Alanyl-tRNA Synthetase Activity.

Alanyl-tRNA synthetase assays and activator assays are described in the text. All assays contained 2 µl of alanyl-tRNA synthetase fraction and when added the amount of activator used was 10 µl. All assays were incubated for 5 min at 37C before stopping as described in the text. Zero time counts were subtracted from each value and activator control counts were subtracted from the appropriate values.

the tRNA preparation; consequently, the activator might merely be reversing some artifactual inhibition. To check this possibility, I tested the effect of the activator on alanyl-tRNA synthetase using the PP_i - ATP exchange assay in absence of added tRNA. The procedure used was that of Deutscher (15). If the assay was performed without tRNA the activator increased alanyl-tRNA synthetase PP_i - ATP exchange by 2-fold. Also in presence of the activator there was a decrease in the apparent k_m's for ATP and Ala (data not shown). This suggests that there was not an inhibitor for alanyl-tRNA synthetase presence in the tRNA preparation.

Figure 4 shows that in presence of the activator the apparent k_m for ATP binding to alanyl-tRNA synthetase was lower than when the activator was not present. However, the maximum velocity was essentially the same in presence or absence of the activator.

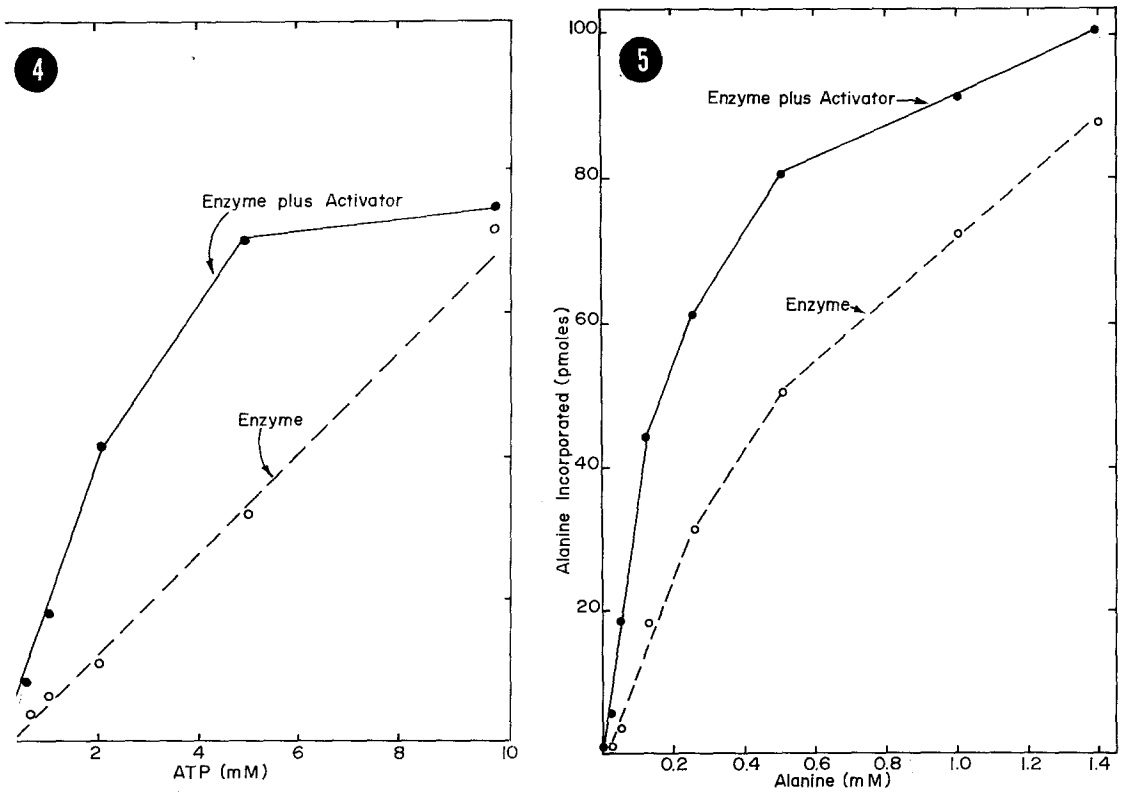

Fig. 4 Effect of Varying ATP Concentrations on Alanyl-tRNA Synthetase Activity.

Assay conditions are the same as described in Figure 3 except at each ATP concentration equal molar amounts of $MgCl_2$ are added.

Fig. 5 Effect of Varying Alanine Concentration on Alanyl-tRNA Synthetase Activity.

Assay conditions are the same as described in Figure 3 except the concentration of [^{14}C]-labeled alanine is varied.

In presence of the activator the apparent k_m for Ala binding to alanyl-tRNA synthetase was lower than when the activator was absent (Fig. 5). As in the case of ATP binding the maximum velocity for alanyl-tRNA synthetase activity was the same in presence or absence of the activator.

Since Hradec and Dusek (2) reported that cholesterol-14-methyl-hexadecanoate stimulated aminoacyl-tRNA synthetases, it was necessary to determine whether the activator isolated in our laboratory was a lipid. Lipids were extracted from the activator by the procedure of Bligh and Dyer (15).

After chloroform:methanol extraction no activator activity was in the organic phase (data not shown). This suggests that the activator was probably not a lipid. However, this does not rule out the possibility that the activator was a lipoprotein.

The activator did not increase the acceptance of rat liver tRNA for the amino acids Ala, Arg, Lys, Thr, Met, Ile, Leu or Ser (data not shown). Thus the activator isolated in this communication was not the same activator isolated by Dickman and Boll (3).

Since there was a stimulation of synthetase activity by PP_i - ATP exchange in absence of tRNA it appears that the activator was not a pyrophosphatase or any other activity that removes the products of aminoacylation. Also, the PP_i - ATP exchange reaction rules out the possibility that the activator was a tRNA-nucleotidyltransferase activity that adds CCA to the termini of tRNAs that lack this sequence. However, using the tRNA nucleotidyltransferase assay described by Deutscher (16), I was not able to obtain a significant incorporation of [^{14}C]-ATP or [^{14}C]-CTP into the tRNA used in experiments outlined in this communication (data not shown). Therefore, the activator was not adding a CCA to the terminus to tRNA lacking this sequence.

DISCUSSION: In this communication, I present evidence for isolation of a large molecular weight activator molecule which stimulates alanyl-tRNA synthetase. In presence of the activator alanyl-tRNA synthetase behaves as an allosteric enzyme for tRNA binding. Also, the apparent k_m values for ATP and Ala binding are lowered but the maximum velocity of the reaction was unchanged.

In attempting to provide evidence that aminoacyl-tRNA synthetases exist in some form of an intermolecular aggregate, we have evidence which suggests that some of the aminoacyl-tRNA synthetases exist in two forms. For example, both arginyl-tRNA synthetase and lysyl-tRNA synthetase exist as a large molecular weight aggregate (1.5×10^6) and as individual enzymes (100,000) (16). However, we have never been able to isolate alanyl-tRNA

synthetase as a large molecular weight aggregate; it always elutes off a gel filtration column at about 100,000 daltons (16). One explanation of why alanyl-tRNA synthetase does not exist as a large molecular weight aggregate is that cellular disruption is so harsh that the interaction between the activator and alanyl-tRNA synthetase is destroyed. If we could determine how alanyl-tRNA synthetase interacts with the activator it is possible that this type of information could aid us in understanding how the intermolecular aggregation of synthetases occur within the cell and how aminoacyl-tRNA synthetases fit into the highly organized protein synthesizing apparatus. Thus, we are in the process of purifying both the activator and alanyl-tRNA synthetase to homogeneity to find answers for these questions.

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