The Aminoacyl Transfer Ribonucleic Acid Synthetases

II. PROPERTIES OF AN ADENOSINE TRIPHOSPHATE-THREONYL TRANSFER RIBONUCLEIC ACID SYNTHETASE COMPLEX

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SUMMARY

Rat liver threonyl-transfer RNA synthetase forms an ATP-enzyme complex when incubated with ATP and magnesium ions. The formation, stability, and reactivity of the complex have been studied. Enzyme-bound ATP serves as substrate in the over-all reaction of threonyl-tRNA formation at concentration levels at which free ATP and enzyme do not give rise to product. Threonine-specific tRNA has a labilizing effect on the complex.

The initial velocity of the over-all reaction has been studied. The nature of the kinetic patterns obtained shows that the reaction is bi uni uni bi ping pong. The kinetic evidence also indicates that ATP reacts with the threonyl-tRNA synthetase as the first substrate in the reaction sequence shown:

\[
E + ATP + tRNA \rightarrow E \cdot ATP \cdot tRNA \\
E \cdot AMP-Thr + tRNA \rightarrow E + AMP + Thr \cdot tRNA
\]

The isolation of several aminoacyladenylate-enzyme complexes (1-5) upon incubation of ATP and magnesium ion with various amino acids and the appropriate aminoacyl transfer RNA synthetase has lent considerable support to the long accepted thesis that the formation of aminoacyl-tRNA proceeds through the two-step reaction:

\[
\text{Amino acid} + \text{ATP} + \text{enzyme} \rightarrow \text{aminoacyl-AMP-enzyme + PP}_1
\]

\[
\text{Aminoacyl-AMP-enzyme + tRNA} \rightarrow \text{aminoacyl-tRNA + AMP + enzyme}
\]

MATERIALS AND METHODS

Yeast tRNA was purchased from Schwarz BioResearch and rat liver tRNA was prepared by the method of Brunngraber (13). All tRNA was extensively dialyzed against EDTA, 0.025 M and then against water and was finally lyophilized. Before use the tRNA was dissolved in water at a final concentration of 14 mg per ml. Transfer RNA enriched for threonine- or phenylalanine-accepting activity was prepared by chromatography on MAK columns as described by Yamane and Sueoka (14).

Labeled nucleotides were purchased from Schwarz BioResearch.
plex in fractions from column chromatography, the solution was
for 5 min. The incubation mixtures were then diluted with 5 ml
except that the precipitation at pH 4.5 was eliminated and 1 mM
potato, was kindly donated by Dr. Osvaldo Cori of this Univer-
sity. All Tris buffers were adjusted to the required pH with HCl.

Enzyme Preparation—Threonyl-tRNA synthetase was purified
from rat liver according to the procedure of Allende et al. (11)
except that the precipitation at pH 4.5 was eliminated and 1 mM
dithiothreitol replaced glutathione for storage and was also added
at 0.5 mM to buffers used to incubated the DEAE-cellulose, hydroxyapatite,
and Sephadex chromatographic separations in order to stabilize
the enzyme. The enzyme preparation obtained is free of other
aminoacyl-tRNA synthetases and the C_sC_p-a-adding enzyme.
The actual specific activities of the enzyme preparations used
varied between 900 and 1500 units per mg of protein. Sephadex
G 200 gel filtration of this preparation yielded an active enzyme
fraction of estimated molecular weight of 180,000 which migrated
as a single band on disc gel electrophoresis pH 8.6 (15). Before
use, all enzyme solutions were desalted by passing through a
Sephadex G-50 column equilibrated with 10 mM Tris (pH 7.5),
10 mM MgCl_2, and 0.1 mM dithiothreitol.

Enzyme Assay—The standard assay of the threonyl-tRNA
synthetase activity was a measurement of ^14C-threonyl-tRNA
formed with the following incubation mixture: 30 mM Tris (pH
8.0), 25 mM KCl, 2 mM GSH, 5 mM MgCl_2, 0.5 mM ATP (K^+ salt
neutralized), 0.01 mM ^14C-threonine (activity 20 mCi per
mmole), and 0.15 mg of yeast tRNA in a final volume of 0.1 ml.
The standard assay reported earlier (11) was the same but was
performed in a 0.5-ml final volume. The reaction was initiated
by the addition of enzyme to tubes that had been equilibrated
at 37°C. After 15 min the reaction was stopped by the addition
of 3 ml of cold 5% trichloroacetic acid containing 1 mM threonine.
The precipitate was filtered on a nitrocellulose membrane, washed
with cold trichloroacetic acid and threonine, and then dried. The
dried discs were placed in 5 ml of a toluene, 2,5-diphenyloxazole
(PPO), and p-bis-[2-(5-phenyloxazolyl)]benzene (POPOP) liquid
scintillation spectrometer with an efficiency of 8.6% for 3H.

To measure the presence or stability of ^3H-ATP-enzyme com-
plexes in fractions from column chromatography, the solution was
poured directly on the wet filter and then washed as described
above.

With the particular batch of Sartorius nitrocellulose filters used
throughout this work and with the washing procedure described
above, 30 to 35% of free enzyme, ATP-enzyme complex, or
threonyladenylate-enzyme complex was retained on the filter; the
amount of enzyme that passed through the filter retained ac-
tivity. This was determined by direct enzyme assay and by
measurement of retention of radioactivity of the labeled com-
plexes.

Binding Assay for Detection of Threonyladenylate-Enzyme
Complex: Complex Binding Assay I—To measure formation of thre-
onyladenylate-enzyme complex with column fractions of ATP-
enzyme, 2 mmoles of ^14C-threonine (specific activity 148 mCi
per mmole) were added to the enzyme solutions, incubated at
20°C for 5 min, and filtered through wet nitrocellulose filters and
washed as described for complex binding assay I.

Threonyl-tRNA Formation from ATP-Enzyme Complex: Com-
plex Activity Assay—To measure formation of threonyl-tRNA
with ATP-enzyme complex isolated by gel filtration on Sephadex
G-50, 2 mmoles of ^14C-threonine (specific activity 148 mCi
per mmole) and 0.1 mg of yeast tRNA were added to the complex
solution and incubated at 37°C for 5 min. The final volume of
the incubation mixture was determined by the amount of enzyme-
bound nucleotide present in the column fractions and was gener-
ally between 0.3 and 0.5 ml. ^14C-Threonyl-tRNA was measured
as described in the standard enzyme assay.

Gel Filtration of ATP-Enzyme Complex on Sephadex G-50—
The complex was prepared by incubating 0.001 m ~1H-ATP
(specific activity 4000 or 5820 mCi per mmole), 10 mM Tris
(pH 7.5), 10 mM MgCl_2, 0.1 mM dithiothreitol, and 50 units of
threonyl-tRNA synthetase unless otherwise indicated. Incuba-
tion was at 20°C for 5 min. The reaction mixture was poured
onto a Sephadex G-50 (coarse) column, 1.8 x 30 cm, that had
been equilibrated previously at 4°C with 10 mM Tris (pH 7.5)
and 10 mM MgCl_2. Elution was carried out with the same buffer at
4°C and fractions of 1 ml were collected. Aliquots were assayd
for total radioactivity on nitrocellulose filters, which were dried
and counted as described for the enzyme assay. Activity assays
were as described. On occasion, smaller Sephadex columns were
used (0.4 x 20 cm) and correspondingly less enzyme. In such
cases, aliquots of 0.1 ml were collected for measurement of
radioactivity.

RESULTS
Complex Formation—The incubation of purified threonyl-
tRNA synthetase with magnesium ions and ATP labeled with
^1H and with ^32P in the β- and γ-phosphate moieties in the absence
of threonine gives rise to an ATP-enzyme complex in which both
isotopes were eluted with the enzyme fractions after gel filtra-
tion on Sephadex G-50. The ratios of ^32P/^1H in the protein
fraction obtained in three such experiments were 0.89, 0.92, and
0.83, the original ratio of radioactivity being 1. These results
show that the entire ATP molecule remains with the enzyme upon
gel filtration. Fig. 1 illustrates the separation of such a com-
plex and assay of the resultant fractions. Total enzyme activity
was determined by the standard enzyme assay, that is, with
ATP added, whereas complex activity refers to the formation of
threonyl-tRNA with the column fractions as source of ATP
and enzyme (complex activity assay). It is important to point
out that the concentration of ATP in the aliquots of column frac-
In order to demonstrate the nature of the bound nucleotide, the enzyme fractions which contained radioactivity were precipitated with cold 5% trichloroacetic acid and treated with Norite A. Of both isotopes, 85 to 90% of the radioactivity was adsorbed on the Norite, indicating that there was little or no hydrolysis of the phosphate moieties during the separation process. Only \(^{32}P\)-labeled material (presumably inorganic phosphate) was released upon hydrolysis with 2 N HCl for 10 min at 100°. Only \(^{3}H\)-labeled material was released upon treatment of the acid-treated Norite with 40% acetone-ammonia at pH 8.0. Electrophoresis at pH 4.5 showed the bound nucleotide to be ATP (between 5 and 10% AMP was usually detected and this was most likely a consequence of nucleotide breakdown occurring during electrophoresis (16)).

The extent of recovery of this complex is a function of the stability of this complex as well as that of the ATP-enzyme complex. That more \(^{3}H\) did not remain bound to the enzyme after apyrase treatment was not attempted. If a similar amount of \(^{3}H\)-ATP-enzyme isolated by Sephadex gel filtration is incubated with \(^{3}C\)-threonine and reapplied to a Sephadex column, then the recovery of tritium as \(^{3}C\)-threonyl-\(^{3}H\)-AMP-enzyme is 30 to 40%. The extent of recovery of this complex is a function of the stability of this complex as well as that of the ATP-enzyme complex.

The isolation of ATP-enzyme complex by gel filtration on Sephadex G-50 is a relatively rapid process. The filtration can be completed within 30 min and fractions can be assayed within minutes of being eluted. These studies were greatly facilitated, however, by the fact that many aminoacyl-tRNA synthetases are adsorbed on the Norite, indicating that there was little or no hydrolysis of the phosphate moieties during the separation process. Only \(^{32}P\)-labeled material (presumably inorganic phosphate) was released upon hydrolysis with 2 N HCl for 10 min at 100°. Only \(^{3}H\)-labeled material was released upon treatment of the acid-treated Norite with 40% acetone-ammonia at pH 8.0. Electrophoresis at pH 4.5 showed the bound nucleotide to be ATP (between 5 and 10% AMP was usually detected and this was most likely a consequence of nucleotide breakdown occurring during electrophoresis (16)).

The enzyme-bound nucleotide was shown to be sensitive to an organic pyrophosphatase, potato apyrase (17), and the product obtained by apyrase treatment did not remain bound to the enzyme. The fractions described in Fig. 1 containing \(^{3}H\) and \(^{32}P\) isotopes together with enzyme were pooled, treated with purified apyrase at 20° for 5 min, and reapplied to a Sephadex G-50 column. The total radioactivity remaining in the protein fraction was 12 and 2.5% of the \(^{32}P\) and \(^{3}H\), respectively. Because of the low level of radioactivity bound to the protein fraction, characterization of the product after apyrase treatment was not attempted. If a similar amount of \(^{3}H\)-ATP-enzyme isolated by Sephadex gel filtration is incubated with \(^{3}C\)-threonine and reapplied to a Sephadex column, then the recovery of tritium as \(^{3}C\)-threonyl-\(^{3}H\)-AMP-enzyme is 30 to 40%. The extent of recovery of this complex is a function of the stability of this complex as well as that of the ATP-enzyme complex.

The ATP-enzyme complex is formed very rapidly at 37° and the rate of formation was measured at 0° (Fig. 3). The yield of ATP-enzyme complex, however, is essentially the same at all temperatures between 0° and 37° under the conditions used.

Fig. 4 illustrates some additional characteristics of the ATP-enzyme complex. The bound \(^{3}H\)-ATP may be separated from free nucleotide by Sephadex G-50 filtration and aliquots assayed for various functions. As shown in Fig. 4A, fractions incubated in the presence of \(^{3}C\)-threonine and tRNA give rise to trichloroacetic acid-precipitable \(^{3}C\)-threonyl-tRNA and this corresponds closely in amount to the \(^{3}H\)-ATP present in the column fractions. Measurement of nitrocellulose membrane-bound ATP-enzyme complex as given in Fig. 4B illustrates that approximately one-third of the total \(^{3}H\)-ATP is retained on filtration of column aliquots through nitrocellulose filters (complex binding assay I). This corresponds to the degree of retention of the enzyme.
en: The effect of ATP and enzyme concentration on ATP-enzyme formation. The effect of increasing amounts of ATP on the amount of complex bound to nitrocellulose filters (complex binding Assay I) was measured with 20 μg of the purified enzyme and incubating in 0.01 M Tris (pH 7.5) and 0.01 M MgCl₂ for 5 min at room temperature in a final volume of 0.1 ml. The effect of increasing amounts of enzyme on the formation of ATP-enzyme was measured at 0.1 μM ATP (specific activity 8520 mCi per mmole) under the same conditions.

enzyme activity by the filtration method as measured independently with these filters.

Aliquots of these same column fractions were incubated with 14C-threonine and subsequently assayed by the filter technique (complex binding Assay II). As seen in Fig. 4C the fractions corresponding to the enzyme and H-ATP were found to give rise to membrane-bound 14C and H in stoichiometric amounts, as would be anticipated from the formation of 14C-threonyl-H-adenylate-enzyme.

The capacity to show equimolar formation of the threonyladenylate-enzyme complex and threonyl-tRNA is dependent upon the rapidity with which the nitrocellulose filter assay is performed. It is also possible, however, to show the formation of the threonyladenylate-enzyme complex by reacting column-isolated ATP-enzyme with free 14C-threonine and recycling through Sephadex, but the yields in this procedure are very low. Despite the low yields, one may measure threonyl-tRNA formation with aliquots from such a recycled chromatographic system upon incubation with tRNA and magnesium ions.

Complex Stability—Table I illustrates some of the conditions in which ATP is released from the protein as measured in the complex binding Assay I. The labeled enzyme-bound nucleotide is released by acid pH, heat treatment, or addition of nonlabeled nucleotide to the reaction mixture. Incubation with

**FIG. 2.** The separation of H-ATP-enzyme complex on Sephadex G-50. The complex was prepared by incubation of H-ATP (specific activity 8520 mCi per mmole) at 0.1 μM with 50 units of threonyl-tRNA synthetase as described under "Materials and Methods" and applied to a Sephadex G-50 column, 1.8 X 30 cm. Aliquots of the eluted fractions were assayed for (A) total H-ATP counts and formation of H-threonyl-tRNA by the complex activity assay, (B) H-ATP-enzyme complex by its retention on nitrocellulose filters with the complex binding Assay I, and (C) H-threonyladenylate-enzyme complex formation by the addition of 14C-threonine to column aliquots and measurement of retention of isotopes on nitrocellulose filters as described in the complex binding Assay II. All results are expressed as micromoles per 1.0 ml, the total fraction volume.
threonine which would permit threonyladenylate-enzyme formation does not alter the amount of tritium retained on the membrane filter. Threonine in combination with tRNA does release all of the membrane-bound labeled material. As is shown below, this release of labeled material may be a result of labilization of the ATP-enzyme complex and not a result of threonyl-tRNA formation.

Fig. 5 illustrates the effect of temperature on the stability of the complex, which was isolated from a Sephadex G-50 column. At 37° the complex has a half-life of about 10 min. In addition, the rate of release of the nucleotide from the Sephadex-isolated complex at 0° is increased by added tRNA. The effect of tRNA on the complex stability was also shown with Sephadex isolation as illustrated in Table II. The addition of tRNA to the incubation mixture containing enzyme and ATP greatly reduces the amount of ATP-enzyme complex that may be isolated by the column separation. Ribonuclease, however, completely reverses this effect; that is, intact tRNA is required for the labilization of the complex. The labilization of the complex by tRNA is apparently related to the tRNA specific for threonine since tRNA preparations enriched for threonine-acceptor activity are effective in preventing the isolation of complex, whereas tRNA enriched for phenylalanine-acceptor activity and free of threonine acceptor activity has no apparent effect on the amount of complex isolated.

**Specificity of Complex Formation**—Nonlabeled ADP and AMP reduce the affinity of ATP as shown in preliminary studies with the complex binding Assay I. Similar results have been observed with Sephadex isolation as a means of measuring complex formation. Parallel experiments with 14C-AMP or 14C-ADP and 3H-ATP were performed to measure the relative capacities of these nucleotides to bind to the enzyme. The results are presented in Table III. It may be seen that ADP binds very efficiently to the enzyme. The nucleotide diphosphate, however, was used at 12 times the concentration of the ATP. AMP has less affinity for the enzyme under the conditions tested. Some 3GTP can also bind to the enzyme; however, this may be displaced by cold ATP. CTP and UTP are not bound. Further studies of the specificity of the nucleotide binding are warranted.

**Magnesium Ion Requirement**—Magnesium ions are required for the formation of the ATP-enzyme complex and retention of the complex on nitrocellulose filters is enhanced when the washing solution contains magnesium ions. Incubation of ATP and enzyme at approximately 0.5 mM MgCl₂ gives optimum forma-

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**Table I**

*Characteristics of binding of ATP-enzyme complex on nitrocellulose filters*

<table>
<thead>
<tr>
<th>Conditions</th>
<th>³H bound (μmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>0.87</td>
</tr>
<tr>
<td>Plus threonine, 0.2 mM</td>
<td>0.90</td>
</tr>
<tr>
<td>Plus threonine, 0.2 mM and tRNA, 8 μg</td>
<td>0.05</td>
</tr>
<tr>
<td>Plus ATP, 0.1 mM, unlabeled</td>
<td>0.02</td>
</tr>
<tr>
<td>Plus 5% trichloracetic acid, 100°, 5 min</td>
<td>0.02</td>
</tr>
</tbody>
</table>

**Table II**

*Effect of tRNA on isolation of ATP-enzyme complex by Sephadex G-50 filtration*

<table>
<thead>
<tr>
<th>Conditions</th>
<th>³H-ATP-enzyme isolated (μmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Complete system</td>
<td>1.50</td>
</tr>
<tr>
<td>Plus tRNA, 0.3 mg</td>
<td>0.23</td>
</tr>
<tr>
<td>Plus tRNA, 0.3 mg, and then ribonuclease, 25 μg</td>
<td>1.87</td>
</tr>
<tr>
<td>2. Complete system</td>
<td>1.73</td>
</tr>
<tr>
<td>Plus tRNA, 0.1 mg</td>
<td>0.30</td>
</tr>
<tr>
<td>Plus tRNA, 0.1 mg</td>
<td>0.08</td>
</tr>
</tbody>
</table>

**Table III**

*Nucleotide specificity for formation of labeled enzyme*

<table>
<thead>
<tr>
<th>Radioactive nucleotide</th>
<th>Nucleotide bound (μmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>³H-ATP (1.6)</td>
<td>45</td>
</tr>
<tr>
<td>³H-ADP (14.0)</td>
<td>41</td>
</tr>
<tr>
<td>¹⁴C-AMP (14.4)</td>
<td>6</td>
</tr>
<tr>
<td>¹⁴C-ADP (14.0)</td>
<td>40</td>
</tr>
</tbody>
</table>

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Fig. 6. Reciprocal plots of the velocity of the formation of $^{14}C$-threonyl-tRNA by threonyl-tRNA synthetase. A, measurements were made at saturating concentrations of tRNA (10 μg) and different concentrations of ATP at various threonine levels. The inset shows the slope versus the reciprocal of the threonine concentration. B, measurements were made at saturating concentrations of tRNA (10 μg) with different concentrations of threonine at various levels of ATP. The inset shows the slope plotted against the reciprocal of the ATP concentration. Initial rates were measured by the assay described under "Materials and Methods."

The effect of substrate concentration on initial rate of reaction—Certain kinetic properties of the purified enzyme have been studied with the aim of defining the order of addition of the various substrates in the overall reaction sequence. Fig. 6A shows the intersecting pattern that is obtained with saturating concentrations of tRNA, varying concentrations of ATP, and different levels of threonine. A replotted of the slopes against the reciprocal of threonine concentration is linear (inset). These findings indicate that ATP and threonine are interconnected through one enzyme form.

Fig. 6B illustrates that a similar intersecting pattern is obtained with saturating concentrations of tRNA, different threonine concentrations, and three levels of ATP. Again, the replot of the slopes against the reciprocal of ATP concentration is linear (inset), thus confirming that both substrates (ATP and threonine) are interconnected through the path of reaction.

The effect of varying the level of the third substrate, tRNA, was next observed. As shown in Fig. 7 a parallel pattern is obtained at saturating ATP concentration, varying threonine concentration, and three different levels of tRNA. A replot of the intercepts of the lines against the reciprocal of the concentration of tRNA is linear. These data are an indication that...
threonine and tRNA are not interconnected through reversible steps under these conditions. The pattern is also parallel when one performs the converse experiment, that is, studying initial velocities with saturating threonine concentration and varying ATP at different tRNA levels.

Effect of Substrate Concentration on Initial Reaction Rate in Presence of Pyrophosphate—As shown in Fig. 8, A and B, in the presence of pyrophosphate, a first product of the reaction (6), a noncompetitive pattern is obtained at either saturating or non-saturating ATP concentrations when threonine concentration is varied at two different levels of tRNA.

The initial velocity pattern is also noncompetitive in the presence of pyrophosphate when ATP is varied at two different tRNA concentrations and non-saturating concentration of threonine (Fig. 8C).

The initial velocity pattern, however, is again parallel (Fig. 9) in the presence of pyrophosphate, saturating concentrations of threonine, at three different levels of tRNA, and varying ATP.

DISCUSSION

The isolation of intermediary complexes between enzymes and substrates can be very useful in the study of the mechanism of enzyme-catalyzed reactions. The mere detection of binding of a substrate to the enzyme protein, however, does not show that such an intermediary complex is functional in the normal reaction sequence. Small molecules can interact with proteins either nonspecifically or at sites removed from the “active” site of the enzyme (19).

The evidence presented above, however, does indicate that the nucleotide bound is in fact ATP, that the ATP-enzyme complex is a true intermediate, and that its formation represents the first step in the reaction catalyzed by the threonyl-tRNA synthetase.

The β- and γ-phosphate moieties of 3H-ATP-β,γ-32P remained with the enzyme through the Sephadex isolation with constant isotope ratio and the radioactive material was identified as ATP. The possibility, therefore, that the binding of labeled material resulted from a reaction of ATP with enzyme-bound threonine to form a threonyladenylate-enzyme complex was ruled out. Free threonine does not bind to the enzyme in the absence of ATP (1).
The ATP-enzyme complex can react stoichiometrically either with threonine to form threonyladenylate-enzyme complex or with threonine and tRNA to yield threonyl-tRNA. These results support the conclusion that the ATP-enzyme complex is functional. The levels of ATP bound to the enzyme are not sufficient to carry out threonyl-tRNA formation if added as free ATP in the presence of threonine and tRNA. In defining a role for the ATP-enzyme complex we must consider that the conditions for observing the formation of this complex may be considerably modified as compared to those of the "normal" situation, that is, when the enzyme acts in the presence of the full complement of substrates and factors required for the over-all reaction. The isolated ATP-enzyme complex is sufficiently stable to permit isolation but may exist as an enzyme form that has a "wrong" configuration for catalytic decomposition and this configuration may be affected by the presence of one or both substrates and/or products. This idea is supported by the finding that tRNA decreases the stability of the ATP-enzyme complex. This effect appears to be specific for the threonine-specific tRNA which is the normal substrate for this synthetase. Directly related to this effect is the apparent discrepancy between the approximate $K_m$ for ATP in the formation of the ATP-enzyme complex (0.3 $\times$ 10$^{-7}$ M) in the absence of the other substrates and the apparent $K_m$ for ATP in the over-all reaction (i.e., 1 $\times$ 10$^{-7}$ M). The kinetically determined apparent $K_m$ is thus not simply a measure of the affinity of ATP for this enzyme.

It would appear that the properties of the aminoaeryl-tRNA synthetases are in general affected by their substrates. The isoleucyl-tRNA synthetase described by Baldwin and Berg (20) is very stable in the presence of both ATP and isoleucine, whereas amino acid or ATP alone is not as effective. This enzyme as well as other synthetases is more resistant to proteolytic digestion or sulphydryl reagents in the presence of substrates (20, 21). The valyladenylate-enzyme complex is very stable but decomposes rapidly in the presence of magnesium ion (3). Certain aminoaeryl-tRNA synthetases show a requirement for tRNA for the exchange of $^{32}$P and ATP, suggesting conformational control of the first step of the reaction (6, 10).

The kinetic data obtained indicate that the mechanism of the threonyl-tRNA synthetase reaction is bi uni bi uni bi ping pong (nomenclature of Cleland (22, 23)). The finding of an intersecting initial velocity pattern for ATP and threonine in the presence of saturating amounts of tRNA indicates that these substrates are interconnected through one enzyme form. The parallel pattern of threonine and tRNA in the presence of saturating amounts of ATP indicates that threonine and tRNA are not interconnected.

The threonyladenylate-enzyme complex from rat liver can be isolated and subsequently reacted with tRNA to form threonyl-tRNA (11). Pyrophosphate then has to emerge as the first product in the reaction sequence prior to the addition of tRNA. Thus, when measuring initial rates of reaction, when the pyrophosphate concentration is effectively zero, there is no reversible connection possible between threonine and tRNA in the absence of added pyrophosphate.

The results obtained when initial rates were measured in the presence of pyrophosphate show that ATP is the first substrate in this reaction. This is indicated by a comparison of Figs. 8 and 9. The addition of ATP in saturating amounts does not break the reversible connection between threonine and tRNA in the presence of added pyrophosphate (Fig. 8, A and B). However, in the presence of pyrophosphate, the reversible connection between ATP and tRNA is broken by a saturating amount of the second substrate, threonine, and a parallel pattern is again observed (Fig. 9).

Our present kinetic data do not allow us to give quantitative assignments to the kinetic constants involved. It must be emphasized that, although it is possible to obtain the order of addition of substrates in this system (or in any other that is complicated by the presence of more than one substrate or product and is reversible), the numerical value of the apparent $K_m$ or apparent $V_{max}$ does not represent a simple mathematical solution, but is the result of a series of terms that include concentration, kinetic constants, and equilibrium constants. The fact, however, that our kinetic studies show that ATP is the first substrate to enter the reaction sequence lends support to the idea that the ATP-enzyme complex is one of the functional complexes in this reaction.

The results, therefore, are consistent with the following reaction mechanism:

$$
\begin{align*}
&\text{ATP} \quad \text{Thr} \quad \text{PP}_i \quad \text{aa-tRNA} \quad \text{AMP (or tRNA)} \\
&\text{↓} \quad \text{↓} \quad \text{↑} \quad \text{↓} \quad \text{↑}
\end{align*}
$$

$$
\begin{align*}
&\text{E} \quad \text{E-ATP} \quad (\quad) \quad \text{E-AMP-Thr} \quad (\quad) \quad (\quad) \quad \text{E}
\end{align*}
$$

We cannot assert, however, that the mechanism described here holds for all synthetases since such different properties are evident among the aminoaeryl-tRNA synthetases even when isolated from the same species (7, 8).

The isolation of ATP-enzyme complexes has not been achieved with Sephadex gel filtration with the lysyl- or seryl-tRNA synthetases studied (3, 5). This, however, does not rule out the existence of ATP-enzyme complexes with other synthetases since it is possible that their affinities or stabilities are too low to allow detection by this method.

Rougct and Chaperville (12) have reported the isolation of an ATP-enzyme complex with leucyl-tRNA synthetase from Escherichia coli. These authors concluded that this complex represents the first intermediate in the reaction sequence. Mehler and his collaborators likewise have kinetic data that show a similar mechanism with the prolyl-tRNA synthetase from E. coli.

This work illustrates that our present knowledge of the reaction catalyzed by amino acid-activating enzymes is as yet very limited because of our lack of information on the presumed controlling effects of substrates or other factors on enzyme activity. We are presently investigating these and other aspects of the aminoaeryl-tRNA synthetase reaction mechanism.

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