Interactions of Tyrosyl Transfer Ribonucleic Acid Synthetase from Escherichia coli with Its Substrates

INHIBITION BY TRANSFER RIBONUCLEIC ACID*

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SUMMARY

Transfer RNA inhibited the tyrosine-dependent ATP-pyrophosphate exchange catalyzed by tyrosyl-tRNA synthetase from Escherichia coli. The inhibition was specific for tRNA<sup>TyR</sup>; it was competitive with tyrosine and noncompetitive with ATP. tRNA modified by periodate oxidation or by limited digestion with phosphodiesterase proved to be a stronger inhibitor of the enzyme than native tRNA; the inhibition by these tRNA's, however, was no longer purely competitive with tyrosine. Measurements of the binding of tRNA<sup>TyR</sup> to the enzyme by means of nitrocellulose filters revealed 1 mole of tRNA<sup>TyR</sup> bound per mole of enzyme.

The process by which an amino acid is programmed into its correct position in a polypeptide chain starts with its attachment to a specific transfer RNA molecule. The enzymes that catalyze this step are responsible for the first stage in the translation of the genetic code. The extreme specificity of these enzymes in recognizing only the proper tRNA molecule has been well documented, although the mechanism of recognition is completely unknown. A number of investigations are now focusing on the interaction of the amino acyl-tRNA synthetases with their cognate tRNA's and on the effect of this interaction on enzymatic activity (1–5).

The reaction catalyzed by amino acyl-tRNA synthetases is currently written as two steps.

1. Enzyme + amino acid + ATP $\rightarrow$ enzyme-aminocacyl-AMP + PP<sub>i</sub>
2. Enzyme-aminocacyl-AMP + tRNA $\rightarrow$ aminocacyl-tRNA + AMP + enzyme

Experimental Procedure

Materials—E. coli K12 (W3747) was grown in minimal medium (12) in a 100-liter fermentor and harvested by centrifugation in the late log phase. E. coli B tRNA (stripped) was purchased from General Biochemicals (Chagrin Falls, Ohio). Partially purified tRNA<sup>TyR</sup> was obtained following the chromatographic procedure on DEAE-Sephadex described by Nishimura et al. (13). <sup>32</sup>PP<sub>i</sub> was synthesized from H<sub>3</sub>P<sub>32</sub>O<sub>4</sub> (Schwarz-Mann, Inc.) according to Kornberg and Pricer (14). L-[3, 5-<sup>3</sup>H]Tyrosine, L-[U-<sup>14</sup>C]Tyrosine, and [G-<sup>3</sup>H]ATP were obtained from New England Nuclear. Phosphodiesterase from Crotalus adamanteus venom was purchased from Sigma, and p-nitrophenyl thymidine 5'-phosphate from Calbiochem.

Enzyme Purification—We purified the tyrosyl-tRNA synthetase by the procedure of Calendar and Berg (15, 16) except that nucleic acid autolysis was replaced with protease and the alumina Cr gel step was omitted. We used the enzyme fraction obtained after hydroxylapatite chromatography for most assays. The binding studies were performed with enzyme obtained after DEAE-cellulose chromatography at pH 8. This preparation of enzyme had a specific activity of 80 units per mg of protein and showed one band on acrylamide gel electrophoresis at pH 8.

Assays for Tyrosyl-tRNA Synthetase—The tyrosine-dependent exchange of ATP with <sup>32</sup>PP<sub>i</sub> was assayed as described by Calendar and Berg (15, 16). The volume of the reaction mixture was reduced to 0.5 ml, and 100 mM Tris-HCl buffer, pH 7.1, replaced...
the cacodylate buffer. The standard concentration of tyrosine to measure enzymatic activity was 2 mM. In the experiments measuring inhibition by tRNA, tyrosine was reduced to 0.01 mM unless noted otherwise. One unit of enzyme activity is defined as the amount of enzyme catalyzing the exchange of 1 µmole of \textsuperscript{32P}P\textsubscript{i} with ATP in 1 min at 37°.

Esterification of tyrosine to tRNA was measured in a reaction mixture (0.2 ml) containing 100 mM Tris-HCl buffer, pH 7.1, 5 mM MgCl\textsubscript{2}, 1 mM ATP, 4.8 A\textsubscript{260} units per ml of crude tRNA, 0.1 µg per ml of bovine serum albumin, 1 mM dithiothreitol, 0.01 mM \textsuperscript{3}H\textsubscript{32} tyrosine (specific activity, 10\textsuperscript{6} cpm per µmole), and 0.6 to 3 × 10\textsuperscript{-4} units of enzyme. After 10 min at 37°, the tRNA was precipitated with 1 ml of cold 10% trichloroacetic acid containing 10 mM tyrosine and collected on a Millipore filter (pore size, 0.65 µm). Filters were washed with cold 0.1 N HCl, dried, and counted in 7 ml of a solution of toluene, 2,5-diphenyloxazole (4 g per liter), and 1,4-bis[2-(4-methyl-5-phenylloxazolyl)]benzene (0.1 g per liter) in a Packard model 3320 spectrometer. Measurements of the total amount of tRNA capable of accepting tyrosine were made using a 10-fold excess of enzyme and increasing the time of incubation to 20 min. The accepting capacity of different batches of the unfractionated tRNA varied between 22 and 24 pmoles of [\textsuperscript{3}H]tyrosine per A\textsubscript{260} unit.

Tyrosyl-tRNA Preparation—Crude tRNA (400 to 600 A\textsubscript{260} units) was esterified with 50 to 100 nmoles of [\textsuperscript{3}H]tyrosine and 0.1 unit of enzyme in a total volume of 1 to 2 ml. After 30 min at 37°, the tube was chilled and 1 volume of 20 mM sodium acetate buffer, pH 5.0, was added. The solution was treated with an equal volume of cold phenol that had been previously equilibrated with the same buffer, and the two phases were vigorously shaken. 0.1 volume of 5 mM NaCl and 2 volumes of cold ethanol were added to the aqueous phase. After 1 hour at -20°, the precipitated tRNA was collected by centrifugation, dissolved in 0.5 mM NaCl, and dialyzed against 20 mM potassium phosphate buffer, pH 6.0.

Oxidation of tRNA by Periodate—Typically, 10 mg of crude tRNA were treated with 10 ml of 4 mM KIO\textsubscript{4}. After 60 min at 25° in the dark an excess of ethylene glycol (0.4 mmole) was added to destroy residual periodate. The solution was chilled, 0.1 volume of 5 mM NaCl was added, and the tRNA was precipitated with 2 volumes of cold ethanol. After 1 hour at -20°, the tRNA was collected by centrifugation, redissolved in 0.5 mM NaCl, and reprecipitated as described. The oxidized tRNA showed no acceptor activity under the usual assay conditions.

**RESULTS**

**Inhibition of Tyrosyl-tRNA Synthetase by tRNA**—The activity of tyrosyl-tRNA synthetase is usually measured either by the tyrosine-dependent ATP\textsuperscript{32P} exchange or by the attachment of radioactive tyrosine to tRNA. We observed that tRNA inhibited these reactions at suboptimal concentrations of tyrosine. The following studies were concerned primarily with the exchange reaction because tRNA is neither a substrate nor a requirement for this reaction. Inhibition was affected by both the tyrosine and the tRNA concentration. The addition of tRNA to the exchange reaction should lead to the formation of tyrosyl-tRNA. In the presence of PP\textsubscript{i}, however, acylation proceeded slowly and at the end of the exchange reaction only 20 to 40% of the total tRNA\textsuperscript{32P} was acylated. Furthermore the time course of the exchange reaction with either unacylated or tyrosyl-tRNA was similar, indicating that acylation was not required for inhibition (see Fig. 5 and Table II).

The inhibition by tRNA was noncompetitive with ATP. It made no difference whether the ratio of Mg\textsuperscript{2+} to ATP was held
FIG. 2. Tyrosyl-tRNA synthetase activity as a function of tyrosine concentration in the presence or absence of tRNA. No tRNA (●), tRNA 12 A_260 units per ml (○). The double reciprocal plot of the data is shown in the inset.

FIG. 3. tRNA inhibition of the ATP-PP_i exchange reaction as a function of pH. The tRNA concentration was the same as in Fig. 2. Potassium phosphate buffer (0.1 M) was used over the entire pH range. Tris-HCl (0.1 M) was also used between pH 6.7 and 7.5 with identical results.

constant or was increased. Inhibition by tRNA was also very sensitive to pH; decreasing the pH stimulated the inhibitory activity (Fig. 3). The shape of the curve suggests that this effect of tRNA may be attributed to the dissociation of a single group with a pK around 6.5. The activity of the enzyme and the K_m for tyrosine were essentially constant over the pH range from 5.8 to 8.6 in the absence of tRNA.

Inhibition by tRNA_Tyr—The previous results were obtained with crude tRNA, but the inhibition could be attributed solely to tRNA_Tyr; with preparations of tRNA that were approximately 7-fold enriched in tRNA_Tyr, 7-fold less tRNA was required to obtain the same inhibition (Table I). Furthermore, chromatog-

<table>
<thead>
<tr>
<th>RNA</th>
<th>Acceptor activity (μmoles Tyr bound/μmol)</th>
<th>Concentration of tRNA in assay (A_260 units/ml)</th>
<th>Inhibition %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>24</td>
<td>3.5</td>
<td>19</td>
</tr>
<tr>
<td>tRNA_Tyr from DEAE-Sephadex</td>
<td>165</td>
<td>0.5</td>
<td>20</td>
</tr>
<tr>
<td>tRNA_Tyr from DEAE-Sephadex</td>
<td>170</td>
<td>0.5</td>
<td>20</td>
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</tbody>
</table>

TABLE I

Inhibition of tyrosyl-tRNA synthetase by crude tRNA and purified fractions of tRNA_Tyr in exchange assay.

FIG. 4. Inhibition of tyrosyl-tRNA synthetase by tRNA_Tyr. Crude tRNA (about 13,000 A_260 units) was loaded onto a DEAE-Sephadex column (2 X 125 cm) equilibrated with 20 mM Tris-HCl, pH 7.5, 8 mM MgCl_2, 375 mM NaCl (13). The tRNA was eluted with a linear salt gradient from 20 mM Tris-HCl, pH 7.5, 8 mM MgCl_2, 375 mM NaCl to 20 mM Tris-HCl, pH 7.5, 16 mM MgCl_2, 525 mM NaCl with an ISCO Dialagrad model 380. The flow rate was 32 ml per hour and the gradient was run for four days. The fractions showing absorbance at 260 nm were assayed for tyrosine-acceptance capacity as described under "Experimental Procedure." Before assaying for inhibitory activity, a sample from every other tube was dialyzed against distilled water to remove NaCl which inhibited the synthetase activity. Absorbance at 260 nm (●), tyrosine-acceptor activity (○), inhibition of the exchange assay (O).

<table>
<thead>
<tr>
<th>RNA</th>
<th>K_m (μM)</th>
<th>V_max (μmol/min)</th>
<th>K_i (μM)</th>
<th>V_p (μmol/min)</th>
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<tr>
<td>None</td>
<td>12</td>
<td>4.7</td>
<td>4.7</td>
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<tr>
<td>Native</td>
<td>35</td>
<td>0.13</td>
<td>4.7</td>
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</tr>
<tr>
<td>Tyrosyl</td>
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<td>0.11</td>
<td>4.6</td>
<td>4.6</td>
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<tr>
<td>Phosphodiesterase treated</td>
<td>23</td>
<td>0.064</td>
<td>3.6</td>
<td>3.6</td>
</tr>
<tr>
<td>Periodate oxidized</td>
<td>20</td>
<td>0.049</td>
<td>2.4</td>
<td>2.4</td>
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</tbody>
</table>
Inhibition of Tyrosyl-tRNA Synthetase by tRNA\textsuperscript{\text{TyR}}

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Inhibition of Tyrosyl-tRNA Xy~~thetase by tRNATyr

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FIG. 5. Double reciprocal plot of the tyrosyl-tRNA synthetase activity as a function of tyrosine with native or modified tRNA's.

The concentration of tRNA calculated as tRNATyr was 0.32 PM. The values of the kinetic parameters are given in Table II. No tRNA (●), crude tRNA (○), tyrosyl-tRNA (△), phosphodiesterase-treated tRNA (▲), periodate-oxidized tRNA (□).

### TABLE III

Inhibition of ATP-PP\textsubscript{i} exchange by tRNA after digestion with snake venom phosphodiesterase

<table>
<thead>
<tr>
<th>Time of incubation</th>
<th>Inhibition</th>
<th>Hydrolysis</th>
</tr>
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<tbody>
<tr>
<td>hours</td>
<td>%</td>
<td>%</td>
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<tr>
<td>0</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>31</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
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<td>4</td>
<td>8</td>
<td>24</td>
</tr>
<tr>
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<td>1</td>
<td>30</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>33</td>
</tr>
</tbody>
</table>

Inhibition by Modified tRNA's—The activation of tyrosine was also inhibited by tyrosyl-tRNA demonstrating that a free 3'-terminal adenosine was not required for inhibition. The inhibition by tyrosyl-tRNA was also competitive with tyrosine, and only small variations were observed for the kinetic constants determined with free or acylated tRNA (Table II). About 20% of the tyrosyl-tRNA was deacylated during the exchange reaction.

Further modification of tRNA altered but did not destroy its inhibitory activity. tRNA was subjected to periodate oxidation or to partial digestion with snake venom phosphodiesterase; the latter treatment removed two or three nucleotides from the 3'-terminus (21). These tRNA fractions were unable to accept tyrosine, but they were more effective inhibitors of tyrosyl-tRNA synthetase than the native tRNA (Fig. 5). Moreover the inhibition was mixed with variations not only in the apparent K\text{m} for tyrosine, but also in the apparent maximum velocity of the reaction (Table II).

When tRNA was treated with snake venom phosphodiesterase at 37\({}^\circ\)C in the absence of magnesium ions, more extensive degradation was obtained. Hydrolysis and the loss of inhibitory activity paralleled each other with increased time of exposure to the exonuclease. After about 6 hours of digestion, the remaining tRNA showed no inhibitory activity. In this time about 30% of the A\text{260} material had become acid soluble (Table III).

Detection of Enzyme-tRNA Complex with Membrane Filter Assay—tRNA is both a substrate and an inhibitor of tyrosyl-tRNA synthetase. We asked whether the inhibitor-binding site for tRNA was identical with the substrate-binding site by determining the number of tRNA-binding sites on the enzyme molecule. For this study we utilized the membrane filter assay of Yarus and Berg (1, 18). Fig. 6 shows the effect of varying pH, Mg\textsuperscript{2+}, and phosphate on the efficiency of the assay, defined as the fraction of tRNA\textsuperscript{\text{TyR}} which can be bound to the filter with an excess of enzyme. Mg\textsuperscript{2+} appeared to be required for complex formation. As with the isoleucyl-tRNA synthetase, pH was an important variable. At pH 4.7 with either [\text{3H}]tyrosine-tRNA\textsuperscript{\text{TyR}} or [\text{3H}]tyrosine-tRNA\textsuperscript{\text{TyR}} we obtained an efficiency of 70 to 83%. The binding of [\text{3H}]tyrosine-tRNA\textsuperscript{\text{TyR}} to purified tyrosyl-tRNA synthetase is shown in Fig. 7a. These data were corrected for efficiency (70%) and were plotted according to the equation described by Yarus and Berg (Fig. 7b). We calculated that 1.0 mole of tRNA was bound per mole of tyrosyl-tRNA synthetase based on a molecular weight for the enzyme of 96,000 (18) and our unpublished results). The same experiment with [\text{3H}]tyrosine-tRNA\textsuperscript{\text{TyR}} gave 0.8 binding site per mole of enzyme.
In these binding studies the tRNA<sup>Tyr</sup> concentration was varied from 0.2 μM to 0.75 μM. These amounts were comparable to the concentration of 0.3 μM tRNA<sup>Tyr</sup> that we routinely added to the ATP-PP<sub>1</sub> exchange assay to measure tRNA inhibition. In these experiments, where tRNA interacted with the free enzyme, we obtained no evidence for more than one binding site. The binding constants obtained were $1.8 \times 10^8$ liters per mole and $1.3 \times 10^8$ liters per mole for tyrosine-tRNA<sup>Tyr</sup> and tyrosine-tRNA<sup>Ale</sup>, respectively. These numbers are similar to those previously reported for isoleucyl-tRNA synthetase (1) and valyl-tRNA synthetase (3).

**DISCUSSION**

Our studies demonstrated that the interaction between tyrosyl-tRNA synthetase and tRNA inhibited the rate of tyrosine-dependent ATP-PP<sub>1</sub> exchange. This inhibitory effect of tRNA was complete only for those fractions containing tRNA<sup>Tyr</sup>; tRNA fractions deficient in tRNA<sup>Tyr</sup> lacked this property. Only one binding site for tRNA was found per mole of tyrosyl-tRNA synthetase suggesting that the binding site for inhibition is the same as the substrate-binding site. Further support for the identity of the sites comes from the observation that the $K_a$ for tyrosyl-tRNA measured at pH 5.5 was lowered to $2 \times 10^{-4}$ M, a value very close to the binding constant for tRNA<sup>Tyr</sup> of 1 to $2 \times 10^8$ liters per mole. Our results, however, cannot exclude the existence of a second binding site, which could become accessible only in the presence of substrate(s). In one case it has been shown that the presence of one substrate affected drastically the binding of tRNA to the enzyme, Knowles et al. were unable to detect the complex between seryl-tRNA synthetase and tRNA<sup>Ser</sup> by density gradient centrifugation when ATP was present (22).

The pH dependence of inhibition may reflect an increase in the binding of tRNA<sup>Tyr</sup> to the synthetase with decreasing pH. This proposal is supported by our observations that more complex was detected on membrane filters as the pH was lowered. A corresponding effect of pH has been reported for other synthetases using diverse methods for measuring interactions between the enzyme and its nucleic acid. Mitra, Chakraburtty, and Mehler studied the ability of tRNA<sup>Arg</sup> to protect arginyl-tRNA synthetase from heat denaturation and the $K_a$ for tRNA<sup>Arg</sup> in the ATP-PP<sub>1</sub> exchange as a function of pH (4). They also reported an analogous decrease in the $K_a$ for tRNA in the tRNA esterification reaction at pH below 7 for the glutamine, lysine, and proline enzymes. Measurements of the binding of tRNA<sup>VaI</sup> to valyl-tRNA synthetase with the techniques of sucrose gradient centrifugation, membrane binding, and fluorescence quenching all confirmed that association increased as the pH was lowered (3, 5). The similarity of these findings suggests that some of the effects on the protein or the nucleic acid, or both, responsible for binding may be comparable in many tRNA synthetases and tRNA's.

We found that an intact 3'-adenosine terminus of tRNA<sup>Tyr</sup> was not required for inhibition of tyrosine activation. With tRNA<sup>Tyr</sup> oxidised by periodate, however, we observed greatly lower inhibition than with the unaltered tRNA. Furthermore, inhibition by modified tRNA was mixed; both the apparent $K_a$ for tyrosine and the apparent maximum velocity of exchange were affected. The contribution of the 3'-terminus of a tRNA molecule to its interaction with an amino acyl-tRNA synthetase is still poorly understood. At least in one case, that of tRNA<sup>VaI</sup> from yeast, Lagerkvist, Rymo, and Waldenström found that after periodate oxidation tRNA<sup>VaI</sup> no longer was bound by valyl-tRNA synthetase (23). Conversely, Yarus and Berg, comparing the association constants of the isoleucyl-tRNA synthetase for unaltered and phosphodiesterase-treated fractions of tRNA<sup>1le</sup>, observed that the terminal cytidylyl and adenylyl residues contributed very slightly to the strength of the binding of tRNA<sup>1le</sup> to the enzyme (2). By means of circular dichroism, Ohta, Shimada, and Imahori detected a conformational change in tyrosyl-tRNA synthetase from yeast with intact but not with periodate-treated tRNA<sup>Tyr</sup> (24). They did not determine, however, if periodate-treated tRNA was capable of binding to the enzyme. In studies with arginyl-tRNA synthetase, Mitra et al. demonstrated that the function of tRNA<sup>Arg</sup> as an activator was distinct from its ability to bind the enzyme (4). Treatment of this tRNA with periodate or with phosphodiesterase did not destroy binding but did prevent activation of the enzyme. Periodate-treated tRNA also no longer activated glutamyl-tRNA synthetase from rat liver even though it was still bound by the enzyme (7). Further analyses of the binding properties of native and modified tRNA's and of the effect of substrates on binding may explain why changes in the 3'-terminus alter the inhibitory or activation patterns of the tRNA.

We do not know if binding per se of tRNA to tyrosyl-tRNA synthetase is sufficient to cause inhibition. Extensive degradation with phosphodiesterase eliminated the inhibitory activity, but we have not yet determined whether the tRNA remaining is still capable of binding to the enzyme. If there is a direct correlation between the binding of tRNA and its ability to inhibit the exchange reaction, it may be possible to utilize this property in identifying that part of the tRNA molecule required for recognition by tyrosyl-tRNA synthetase.

Inhibition of an amino acyl-tRNA synthetase by its cognate
tRNA may be a general phenomenon and could serve an important physiological function in the bacterial cell. Amino acyl-tRNA synthetases are involved in regulating the levels of at least some of the amino acid biosynthetic enzymes, but it is not known if they function directly as repressors, or if they have a more indirect role, for example, catalyzing the formation of a corepressor such as charged tRNA (25). In a few cases derepression of the biosynthetic enzymes has been correlated with a decrease in amino-acylated tRNA. The level of acylated tRNA which drops at low intracellular concentrations of the amino acid would be reduced even further when amino acid activation is inhibited by tRNA. The net result would be a greater derepression of the amino acid biosynthetic enzymes which could provide more of the amino acid to the cell.

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REFERENCES