Methionyl-tRNA Synthetase from *Phaseolus aureus*: Purification and Properties

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ABSTRACT

L-Methionyl-tRNA synthetase (EC 6.1.1.10) from seeds of *Phaseolus aureus* has been purified approximately 290-fold. Optimum assay conditions were determined by using the ATP-pyrophosphate exchange assay and the aminosylation assay. The enzyme catalyzes both selenomethionine- and selenoethionin-dependent ATP-pyrophosphate exchange in addition to catalyzing the formation of selenomethionyl-tRNA at a rate comparable to the rate of formation of methionyl-tRNA. Competition experiments were conducted to investigate further the substrate specificity of the purified enzyme. Two peaks of methionyl-tRNA synthetase were detected by using Sephadex G-200 gel filtration; the molecular weights of the two enzymes as determined by Sephadex G-200 column chromatography were 340,000 and 85,000. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis suggests that the enzyme is a tetramer consisting of four identical monomers with molecular weights of 85,000.

EXPERIMENTAL PROCEDURE

MATERIALS

L-[2(3H)Methionine (7.8 Ci/mmol) and L-[35Se]selenomethionine (260 Ci/mol) was obtained from the Radiochemical Centre, Amersham, Buckinghamshire, United Kingdom. 32P, in dilute HCl, was obtained from the Australian Atomic Energy Commission, Lucas Heights, New South Wales, Australia, and was converted to [32P]PPi by pyrolysis (19). [32P]PPi was diluted with unlabeled PPi to adjust the specific radioactivity to approximately 0.25 Ci/mol. t-RNA was isolated and purified from mung bean seedlings according to the method of Moustafa (21). Inorganic pyrophosphatase was purified from spinach-leaf tissue (26). Homocysteine was prepared by the method of Flavin (12).

EXTRACTION AND PURIFICATION OF METHIONYL-tRNA SYNTHETASE

All steps were carried out at 0 to 4°C and, following preparation of crude extracts in 100 mM Tris-HCl (pH 8.0), 20 mM MgCl2, 10% (w/v) glycerol and 25 mM β-mercaptoethanol, all proceeding steps were performed in buffer containing 20 mM Tris-HCl (pH 8.0), 10% (w/v) glycerol, 5 mM MgCl2, and 25 mM β-mercaptoethanol unless stated otherwise; the β-mercaptoethanol was added to the buffer solution immediately prior to use.

CRUDE EXTRACT

*Phaseolus aureus* seeds, milled to a powder in a Waring Blender, were homogenized for 2 min (30 s, four times) in a Waring Blender; a tissue buffer ratio of 1 g/7 ml was used. The homogenate was filtered through a double layer of cheesecloth and centrifuged at 25,000g for 60 min. The precipitate was discarded and the supernatant was fractionated with ammonium sulfate.

AMMONIUM SULFATE FRACTIONATION

Solid ammonium sulfate was slowly added to the crude extract, with constant stirring, to 33% saturation. After 15 min, the suspension was centrifuged for 30 min at 25,000g, and the precipitate was discarded. The supernatant was brought to 45% saturation with ammonium sulfate and, after 15 min stirring, was centrifuged at 25,000g for 30 min. The precipitate was discarded, and the supernatant was adjusted to 65% saturation with ammonium sulfate. After stirring for 15 min, the suspension was centrifuged at 25,000g for 30 min. The supernatant was discarded and the precipitate was dissolved in a minimal volume of buffer. The solution was dialyzed against buffer for 24 h.

SEPHADEX G-200 GEL FILTRATION

The dialyzed 45 to 65% ammonium sulfate fraction was applied to a column of Sephadex G-200 (3.5 × 80 cm), equilibrated with buffer and eluted at a flow rate of 0.40 ml/min. Fractions of 5 ml

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were collected and assayed for methionyl- and cysteinyl-tRNA synthetase activities. Fractions containing only methionyl-tRNA synthetase were pooled.

**DEAE-CELLULOSE (DE52) COLUMN CHROMATOGRAPHY**

The pooled active Sephadex G-200 fractions were applied to a column of DEAE-cellulose (DE52, 3.5 x 15 cm). The enzyme was eluted with a 500-ml linear gradient of KCl, from 0 to 0.3 M KCl, at a flow rate of 0.50 ml/min. Fractions of 4 ml were collected and assayed for methionyl-tRNA synthetase activity, and those fractions containing high specific activity of methionyl-tRNA synthetase were pooled and dialyzed against 20 mM phosphate buffer (pH 7.8) containing 10% (w/v) glycerol, 5 mM MgCl₂, and 25 mM β-mercaptoethanol.

**HYDROXYAPATITE COLUMN CHROMATOGRAPHY**

The dialyzed DEAE-cellulose fractions were applied to a column of hydroxyapatite (10 x 1.5 cm) equilibrated with buffer. The enzyme was eluted with a 250-ml linear phosphate gradient from 0 to 0.3 M at a flow rate of 0.35 ml/min. Those fractions containing the highest specific activity of methionyl-tRNA synthetase were pooled and dialyzed against 20 mM phosphate buffer (pH 7.8) containing 10% (w/v) glycerol and 25 mM β-mercaptoethanol. Following dialysis the enzyme was stored at -20°C.

**ENZYME ASSAY**

**ATP-PPi Exchange.** Methionyl-tRNA synthetase was assayed by the ATP-PPi exchange reaction described previously (7) by using 1.0-ml reaction mixtures containing 200 μmol Tris-HCl (pH 8.0), 10 μmol MgCl₂, 4 μmol ATP, 2 μmol [γ³²P]PPi (0.25 Ci/mmol), 10 μmol l-methionine, and to 70 μg enzyme preparations. Reactions were carried out at 37°C for 10 min. Activity is expressed as nmol methionine-dependent ATP-PPi exchange/min.

**Aminocyclation of tRNA.** The method of Moustafa (21) was used for the measurement of methionine attachment to tRNA. Reaction mixtures contained 100 μmol Tris (pH 8.0), 10 μmol MgCl₂, 0.1 μmol [³⁴Cl]methionine (10 Ci/mmol), 4 μmol ATP, 5 μmol DTT, 5 units of purified inorganic pyrophosphatase from mung bean, 60 μg mung-bean tRNA, and a limiting amount of purified methionyl-tRNA synthetase in a final volume of 1 ml. Radioactivity labeled aminocyclation of tRNA was assayed by precipitation with trichloroacetic acid onto glass fiber (Whatman GF/C) filters, washing with cold trichloroacetic acid, and, after drying, measuring the radioactivity by scintillation counting using a toluene-based scintillator.

**OTHER METHODS**

The protein concentration was determined by the method of Lowry et al. (20) after removal of β-mercaptoethanol by heating (28). SDS-polyacrylamide gel electrophoresis was performed according to Weber and Osborn (29).

**RESULTS**

Purification. The purification procedure yielded an enzyme preparation that had been purified approximately 290-fold with a total enzyme recovery of 9% with respect to the crude enzyme extract (Table 1). Addition of (NH₄)₂SO₄ to 45% saturation in one step resulted in irreproducible fractionation and contamination of the 45 to 65% (NH₄)₂SO₄ saturation fraction with inorganic pyrophosphatase. On Sephadex G-200 two peaks of methionine-dependent ATP-PPi exchange was detected. Peak I consistently represented approximately 20% of the total methionine-dependent ATP-PPi exchange activity. The ratio of methionine-dependent ATP-PPi exchange and the formation of methionyl-tRNA was constant for both Peaks I and II during the purification sequence, provided that fluoride (10 mM) was added to the ATP-PPi exchange assays of the crude extract and ammonium sulfate fractions to inhibit inorganic pyrophosphatase activity. The purification of methionyl-tRNA synthetase peak II by DEAE-cellulose and hydroxyapatite column chromatography is shown in Figures 1 and 2, respectively. Column chromatography of peak I on DEAE-cellulose and hydroxyapatite produced a greater percentage of peak II and less peak I enzyme than obtained during Sephadex G-200 column chromatography. Table I summarizes the purification of Peak II only.

Peak II retained 93% of its activity at -20°C after 3 months. Unlike the methionyl-tRNA synthetases from Escherichia coli and other bacteria, Mg²⁺ ions were required throughout the purification procedure to preserve enzyme stability. In buffer lacking Mg²⁺ ions purified methionyl-tRNA synthetase Peak II lost 90% of its activity when stored at -15°C for 5 days.

**Molecular Weight.** Peak II was purified to homogeneity as determined by polyacrylamide gel electrophoresis (Fig. 3) by using the procedure summarized in Table I. Peak I was purified by the same procedure as peak II but was always accompanied by some peak II protein as determined by polyacrylamide gel electrophoresis.

The molecular weights of peaks I and II were determined by gel filtration on Sephadex G-200 according to Andrews (3). The elution volumes were compared to those obtained for ferritin, catalase, alkaline phosphatase, inorganic pyrophosphatase, and peroxidase. By using this method, mol wt of 340,000 ± 10,000 and 84,000 ± 5,000 for peaks I and II, respectively, were obtained (Fig. 4). A single band of protein was obtained when both peaks I and II were examined by SDS-polyacrylamide gel electrophoresis. Band mobility, compared to that of BSA, alkaline phosphatase, inorganic pyrophosphatase, and peroxidase under the same conditions indicated a mol wt of 84,000 ± 2,500.

**Kinetic Properties.** The kinetic properties of mung-bean methionyl-tRNA synthetase were determined from ATP-PPi exchange and aminocyclation data using peak II enzyme. Kinetic constants were calculated as described (30). The rates of methionine-dependent ATP-PPi exchange and methionyl-tRNA synthesis were linear with enzyme concentrations in the range 5 to 80 μg protein, and both assays were linear with time for at least 60 min at 27°C. When a series of buffers was used to study the pH optimum of the enzyme, the pH optimum was found to lie between 7.6 and 8.3. The Michaelis constants for ATP and methionine were 1.3 mM and 32 μM, respectively, as determined by the ATP-PPi exchange assay. Purified methionyl-tRNA synthetase had an absolute requirement for Mg²⁺ ions; the optimum Mg/ATP ratio was 2.5:1. The enzyme was also strongly inhibited by sulfhydryl-group reagents.

**Substrate Specificity.** Only l-methionine of the 20 amino acids commonly found in proteins acted as a substrate for ATP-PPi exchange, indicating that the purified methionyl-tRNA synthetase was not contaminated with other aminocyclation-tRNA synthetases. Furthermore, the enzyme was specific for the l isomer of methionine. However, a relatively large number of nonprotein amino acids acted as alternative substrates (Table II). Unlike the cysteinyl-tRNA synthetase from mung bean seeds, the methionyl-tRNA synthetase catalyzed ATP-PPi exchange in the presence of the methylated derivative of the true substrate. The Km for l-methionine was 1 μM, which is significantly higher than the Km for methionine. l-Selenomethionine, which is a good substrate for rat liver (8), Sacrina lutea (14), Paracoccus denitrificans (7) and E. coli (22) methionyl-tRNA synthetases, was also a good substrate for the mung bean seed enzyme. In contrast to other methionyl-tRNA synthetases which have been studied, the Vmax (selenomethionine) (24.04 nmol ATP-PPi exchange per min)  was...
Table I. Procedure for Purification of Methionyl-tRNA Synthetase from Ph. aureus

<table>
<thead>
<tr>
<th>Purification Procedure</th>
<th>Protein</th>
<th>Specific Activity</th>
<th>Total Activity</th>
<th>Recovery</th>
<th>Purification</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>mg</td>
<td>nmol mg(^{-1}) min(^{-1})</td>
<td>units</td>
<td>%</td>
<td>-fold</td>
</tr>
<tr>
<td>Crude extract after centrifugation</td>
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<td>0.55</td>
<td>6358</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulfate fractionation (45–65%)</td>
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<td>3.68</td>
<td>2570</td>
<td>42</td>
<td>7</td>
</tr>
<tr>
<td>Sephadex G-200 filtration</td>
<td>255</td>
<td>8.22</td>
<td>2098</td>
<td>33</td>
<td>15.0</td>
</tr>
<tr>
<td>DEAE-cellulose (pH 8.0) elution between 0 and 0.3 M KCl</td>
<td>46</td>
<td>31.79</td>
<td>1462</td>
<td>23</td>
<td>57.8</td>
</tr>
<tr>
<td>Hydroxyapatite (pH 8.0) elution between 0 and 0.3 M KCl</td>
<td>3.6</td>
<td>161.15</td>
<td>580</td>
<td>9</td>
<td>293</td>
</tr>
</tbody>
</table>

FIG. 1. Column chromatography of methionyl-tRNA synthetase peak II on DEAE-cellulose. (●—●), methionyl-tRNA synthetase activity; (■—■), protein (mg/ml); (-----), KCl gradient.

FIG. 2. Column chromatography of methionyl-tRNA synthetase peak II on hydroxyapatite. (●—●), methionyl-tRNA synthetase activity; (■—■), protein (mg/ml); (-----), phosphate gradient.

higher than the \(V_{\text{max}}\) (methionine) (21.27 nmol ATP-PPi exchange mg\(^{-1}\) protein min\(^{-1}\)); the \(K_m\) (selenomethionine) was 38 \(\mu\)M, only slightly higher than the \(K_m\) (methionine).

Those amino acid analogs which did not support ATP-PPi exchange, which included \(\alpha\)-methionine, \(\beta\)-ethlycysteine, \(\lambda\)-methionine sulfoxide, and \(\lambda\)-methionine sulfoximine, were tested for inhibition of methionine-dependent ATP-PPi exchange. None of them inhibited the methionine-dependent ATP-PPi exchange.

Formation of \(^{3}H\)Methionyl-tRNA. In addition to the purified mung-bean methionyl-tRNA synthetase catalyzing the synthesis of \(^{3}H\)methionyl-tRNA, the enzyme also catalyzed the formation of \(^{75}\)Se-selenomethionyl-tRNA. The \(K_m\) (selenomethionine) as

calculated from aminoacylation assays was almost the same as the \(K_m\) value for methionine. However, the \(V_{\text{max}}\) (selenomethionine) was slightly less than the \(V_{\text{max}}\) (methionine). This is in direct contrast to the results of ATP-PPi exchange data, which gave a \(V_{\text{max}}\) (selenomethionine) significantly higher than the \(V_{\text{max}}\) (methionine).

Inhibition of Formation of Methionyl-tRNA by Analogs. Selenomethionine was tested for its ability to inhibit the extent of formation of methionyl-tRNA (Fig. 5). The results of reciprocal experiments show the inhibition of selenomethionyl-tRNA formation by methionine (Fig. 6). Both selenomethionine and methionine inhibited the formation of methionyl-tRNA and selenomethionyl-tRNA, respectively. Figure 5 shows that, at a molar ratio of 1:1 (methionine to selenomethionine), the inhibition of methionyl-tRNA formation by selenomethionine is approximately 50%. Figure 6 shows a 50% inhibition of selenomethionyl-tRNA formation by methionine at a similar molar ratio.

The other methionine analogs which supported ATP-PPi ex-
change were tested for their ability to inhibit the formation of methionyl-tRNA in assays containing a fixed concentration of \(^{3}H\)methionine (0.1 mM) and increasing concentration of analogs (Table III). Of the other methionine analogs tested, selenomethionine and ethionine were the most efficient inhibitors of methionyl-tRNA formation; both these amino acid analogs inhibited methionyl-tRNA formation by more than 30% at an analog to amino acid molar ratio of 100:1. Homocysteine and norleucine were less efficient inhibitors.

**DISCUSSION**

The aminoacylation of tRNA involves three steps: (a) the binding of the amino acid and ATP to the enzyme (1); (b) the formation of the aminoacyl adenylate complex and the release of PPi (2); and (c) the binding of the amino acid with its cognate tRNA.

The ATP-PPi exchange assay tests only the recognition of amino acid analogs in the first two steps. The only true means of determining the specificity of amino acid-activating enzyme is to measure the formation of the aminoacyl-tRNA complex. However, this cannot always be done due to the unavailability of radioactively labeled amino acids. Notwithstanding this limitation a measure of the specificity of an aminoacyl-tRNA synthetase can be obtained by assaying the abilities of amino acid analogs to inhibit the extent of aminoacylation of tRNA with the true substrate. This method may be misleading, inasmuch as some analogs, such as amino acid esters and \(\delta\)-amino acids, have been found to bind to aminoacyl-tRNA synthetases but unable to form an aminoacyl-adenylate complex (4, 11, 24). Failure to form an aminoacyl-adenylate complex also results in the failure of these compounds to support ATP-PPi exchange. Therefore, provided an amino acid analog is capable of supporting ATP-PPi exchange, the ability of an analog to inhibit the extent of aminoacylation of tRNA by the true substrate can be used as a measure of determin-
Table III. Effect of Analogs on Formation of Methionyl-tRNA in a P. aeuros System

Results are expressed as percentages of the methionyl-tRNA formed in the absence of analog. The assays were performed as described under "Experimental Procedures," except that increasing concentrations of analogs were included in the assays as indicated in the table. The concentration of [3H]methionine in the assays was maintained at 0.1 mm.

<table>
<thead>
<tr>
<th>Analog/ amino acid</th>
<th>Methionyl-tRNA Formation after Addition of Analogs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L-Homocysteine</td>
</tr>
<tr>
<td>ratio</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>97.4</td>
</tr>
<tr>
<td></td>
<td>89.3</td>
</tr>
<tr>
<td></td>
<td>83.3</td>
</tr>
<tr>
<td></td>
<td>79.2</td>
</tr>
</tbody>
</table>

ing the specificity of an aminoacyl-tRNA synthetase.

Of the amino acid analogs examined here, selenomethionine, selenoethionine, ethionine, homocysteine, and norleucine supported ATP-PPI exchange to the greatest extent. The availability of [75Se]methionine allowed examination of the ability of purified methionyl-tRNA synthetase from mung bean seeds to catalyze the formation of [75Se]methionyl-tRNA at a rate similar to the rate of formation of methionyl-tRNA. The similarity of the \( V_{max} \) (selenomethionine) and the \( V_{max} \) (methionine) and the \( K_m \) (selenomethionine) and the \( K_m \) (methionine) in both the ATP-PPI exchange assay and the aminoacyl-tRNA-synthesizing assay indicates that selenomethionine is as good a substrate as methionine for mung-bean methionyl-tRNA synthetase. The fact that the \( V_{max} \) (selenomethionine) is higher than the \( V_{max} \) (methionine) in the ATP-PPI exchange assay but lower in the aminoacyl-tRNA assay may be an indication of the operation of an editing mechanism.

Further supporting evidence for this statement is provided by the competition experiments provided in Figures 5 and 6.

Ethionine, selenoethionine, homocysteine, and norleucine, all of which supported ATP-PPI exchange, also inhibited the formation of methionyl-tRNA. However, this inhibition was significant only at high substrate concentrations, which are considered to be nonphysiological. The similarity of the per cent inhibition of methionyl-tRNA formation by ethionine and its selenium-substituted analog (Table III) provides another example of the acceptance by an aminoacyl-tRNA synthetase of a selenium-substituted analog of a sulfur-containing amino acid.

The \( K_m \) value of 32 \( \mu \)M obtained for methionine is similar to the value reported for the methionyl-tRNA synthetase from E. coli (23), wheat germ (25), and yellow lupin seeds (17) but is 1 order of magnitude lower than the value reported for S. lutea (14) and rat liver (8, 9). The enzyme was similar to other methionyl-tRNA synthetases in having an absolute requirement for Mg\(^{2+}\) ions, having an optimum \( M_g \) ATP ratio and being sensitive to sulphydryl-group reagents.

Two peaks of methionyl-tRNA synthetase activity were recognized by Sephadex G-200 gel filtration. The molecular weight of peak I was twice the value obtained for the methionyl-tRNA synthetase from E. coli (18), wheat germ (10), and lupin seeds (17). The molecular weight of peak II was similar to the molecular weight of the protein band obtained by SDS-polyacrylamide gel electrophoresis of wheat germ methionyl-tRNA synthetase (25). The fact that SDS-polyacrylamide gel electrophoresis of peak I gave a single protein band with a molecular weight the same as peak II indicates that the enzyme may be composed of four identical subunits. This contrasts with the enzyme from E. coli (18), lupin seeds (17), or wheat germ (25), which seems to be a dimeric protein. However, it has been reported that concentrated pure methionyl-tRNA synthetase from lupin seeds formed a number of polymeric forms, including one with a mol wt of 340,000. Therefore, it seems that the active monomorphic subunit of methionyl-tRNA synthetase from mung-bean seeds is similar to that obtained from lupin seeds.

LITERATURE CITED