Tryptophanyl Transfer RNA Synthetase from Lymphocytes of Human Chronic Lymphocytic Leukemia

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Summary

Tryptophanyl-tRNA synthetase has been purified from a lymphocyte-rich fraction of blood cells of patients with chronic lymphocytic leukemia. Normal lymphocytes, other blood cells, and skin also contain the enzyme. After a 240-fold purification by column chromatography, a further purification was obtained by isoelectric focusing, which revealed an isoelectric point of 5.2 to 5.3. The enzyme is stabilized by 2-mercaptoethanol and inactivated by 5, 5'-dithiobis(2-nitrobenzoic acid). The enzyme is stable in, but inhibited by, phosphate buffer. Yeast tRNA\textsubscript{TRP} is a good substrate, with a \( K_m \) of \( 1.1 \times 10^{-7} \) M. ATP and tryptophan have \( K_m \) values of \( 1.6 \times 10^{-4} \) and \( 2.9 \times 10^{-6} \) M, respectively. In sucrose density gradients, the enzyme sediments as a protein with a molecular weight near 90,000.

Introduction

Although comparative studies of mammalian tRNA's from both normal and neoplastic tissues have been made in a number of laboratories, relatively little has been done with tRNA's isolated from man. The human placenta has provided tRNA's and aminoacyl-tRNA synthetases (1, 14). Other human sources for tRNA's are the lymphocytes and leukemic spleen (2, 4, 6). In none of the studies of human tRNA's have highly purified aminoacyl-tRNA synthetases been used. We now report the partial purification and characterization of a tryptophanyl-tRNA synthetase from human lymphocytes.

Materials and Methods

Materials. Brewers' yeast tRNA and \textit{Escherichia coli} tRNA were products of Schwarz BioResearch, Inc., Orangeburg, N. Y. The brewers' yeast tRNA could bond 30 pmole of tryptophan per A\textsubscript{260} unit.\(^6\) Human lymphocyte tRNA was prepared by methods previously described (19); the yield was 6 A\textsubscript{260} units of tRNA per g of packed, wet blood cells prepared as described below. \textit{E. coli} alkaline phosphatase was from Worthington Biochemical Corp., Freehold, N. J. Crystalline human placental alkaline phosphatase (9) was a gift from Dr. Donald Harkness. Isoleucyl-tRNA synthetase was prepared as previously described (18). Human lymphoblastoid cells grown in pure suspension culture as described by Moore \textit{et al.} (15) were generously provided by Mr. Paul Puce and Dr. Adel Yunis. L-Tryptophan\textsuperscript{14}C was purchased from New England Nuclear, Boston, Mass., and purified by column chromatography with spectrophotometric determination of tryptophan concentrations (16). Polyethylene glycol was purchased as Amberlite CG-50 from Mallinckrodt Chemical Works, St. Louis, Mo. Sucrose was from Schwarz. Ampholyte solutions for isoelectric focusing were purchased from LKB Instruments, Inc., Rockville, Md. All other materials were from sources previously named (16, 17).

Methods. Except where specifically noted, Trp-tRNA synthetase and tRNA\textsubscript{TRP} were assayed by the formation of Trp-tRNA as previously described (16, 18) but with 20 mM MgCl\textsubscript{2}, with no bovine serum albumin, with 20 A\textsubscript{260} units of brewers' yeast tRNA (30 pmole tRNA\textsubscript{TRP}/A\textsubscript{260} unit) and with 0.10 mM L-tryptophan\textsuperscript{14}C (9 cpm/pmole) in each 0.5-ml reaction mixture at 30\(^\circ\). Under these conditions with limiting amounts (0.05 to 0.5 unit) of enzyme, reaction rate was nearly constant for 20 min. One unit of enzyme formed 1.0 nmole of Trp-tRNA in 10 min at 30\(^\circ\). Protein was determined by the method of Lowry \textit{et al.} (12). The cells used for the enzyme purification were donated by a single patient with CLL.\(^5\) The patient had a white blood count of 570,000/cu mm with a differential count of 99% lymphocytes and 1% neutrophiles. His hematocrit was 21%, hemoglobin 6.7 g/100 ml, reticulocyte level 11%, and platelet count 100,000/cm mm. The peripheral blood of this patient was enriched for lymphocytes by differential centrifugation at 40 \(\times\) g under sterile conditions. The leukocyte fraction consisted of lymphocytes with some contaminating red blood cells. The cells were stored under liquid nitrogen, in which there was no loss of activity of various aminoacyl-tRNA synthetases for periods up to 2 years.

Results and Discussion

Source of Enzyme. Tryptophanyl-tRNA synthetase is present in erythrocytes, normal lymphocytes, and leukemic leukocytes of chronic myelogenous leukemia as well as in the lymphocytes of CLL. From studies of normal blood fractions freed of monocytes, granulocytes, and platelets on glass bead

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\(^5\) The abbreviation used is: CLL, chronic lymphocytic leukemia.
columns (21), the lymphocyte contains $2 \times 10^{-7}$ unit and the red blood cell contains $5 \times 10^{-9}$ unit, the calculation neglecting the possibility that all of the enzyme present in the red blood cell fraction might reside in the reticulocytes. Lymphoblastoid human cells in suspension culture (15) contain $10^{-7}$ unit/cell, as do the leukemic leukocytes of chronic myelogenous leukemia. The lymphocyte of CLL, too, contains $2 \times 10^{-7}$ unit. Extracts of human skin are another rich source of tryptophanyl-tRNA synthetase.6

The richness of the lymphocyte as a source of Trp-tRNA synthetase should be noted. If mean lymphocyte volume is 260 cu μ (23), the activity is 800 units of tryptophanyl-tRNA synthetase per ml of cells. Under the same arbitrary assay conditions but at 37° instead of 30°, the range of activities of aminocetyl-tRNA synthetases from E. coli in logarithmic growth is 200 to 2800 units/ml (18). On the basis of weight, extracts of the lymphocyte-erythrocyte mixtures provide 200 to 300 units of Trp-tRNA synthetase per g of cells and 4 to 5 units/mg protein, both values at the lower limit of similar calculations from E. coli (18), in which the aminocetyl-tRNA synthetases comprise 10% of the active enzyme mass (24).

### Enzyme Purification

Although the purification which follows is that of an enzyme from a single patient, the tryptophanyl-tRNA synthetase was present at equivalent levels in cells from 2 other patients. All steps in the purification were done at 0—4°. All phosphate buffers were pH 6.9, made by mixing equimolar amounts of KH₂PO₄ and K₂HPO₄. Cells (9.2 g) were thawed in 25 ml of 10 mM Tris-HCL buffer (pH 8.0), 1 mM MgCl₂, and 10% glycerol. The thawed suspension was passed through a French pressure cell at 1200 psi,7 and the lysed cell suspension was centrifuged at 198,000 X g for 90 min. The clear, red supernatant solution was dialyzed against Solution A (20 mM 2-mercaptoethanol-1 mM MgCl₂, 10% glycerol), containing 5 mM phosphate buffer.

The dialyzed extract was placed over a DE-52 column (1.5 x 16 cm) equilibrated with 20 mM phosphate buffer in Solution A. After passage of 230 ml of the equilibration buffer to remove hemoglobin and other unadsorbed proteins, the column was washed with 250 mM phosphate buffer in Solution A to elute the tryptophanyl-tRNA synthetase. The fractions containing activity were dialyzed as above and then placed over a 2nd DE-52 column (0.9 x 18 cm), equilibrated as before. A linear gradient from 20 to 250 mM phosphate buffer

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<table>
<thead>
<tr>
<th>Table 1 Enzyme purification</th>
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<tbody>
<tr>
<td>Fraction</td>
</tr>
<tr>
<td>Extract</td>
</tr>
<tr>
<td>DE-52 batch</td>
</tr>
<tr>
<td>DE-52 gradient</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
</tr>
<tr>
<td>Polymethacrylate</td>
</tr>
</tbody>
</table>

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6 N. S. Penneys and K. H. Muench, unpublished data.
7 All of the activity released by lysis in the French press was released by suspension and thawing of the cells in the initial buffer followed by 8 passes at 1150 rpm in a glass-Teflon homogenizer (Thomas).
recovered in the polymethacrylate fraction had a specific activity of 1300 units/mg.

**Isoelectric Focusing.** When 188 µg of the polymethacrylate fraction from the 63 g of cells were subjected to isoelectric focusing in an experiment similar to that in Chart 1, 30% of the activity was recovered in a single peak with a pI of 5.2 to 5.3. The 4 fractions with the highest activity were pooled, dialyzed for 48 hr against 0.2 mM ammonium acetate (pH 6) and 24 hr against water, then frozen, dried, and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (22). After staining with Coomassie brilliant blue, the gel was estimated to contain less than 5 µg protein. Therefore, isoelectric focusing as described (Chart 1) may effect a further enzyme purification of as much as 10-fold. Presence of 17 mM dithiothreitol throughout the pH gradient affects neither the pI nor the recovery of activity.

**Stability.** Human tryptophanyl-tRNA synthetase is stable in 10 mM potassium or sodium phosphate buffers, pH 6.9, in the presence of 10% glycerol and 20 mM 2-mercaptoethanol at 0°C. When the DE-52 gradient fraction of the enzyme was stored under these conditions at a concentration of 12 µg/ml, it retained 50% of its original activity after 17 days. In parallel experiments without 2-mercaptoethanol, the enzyme lost all activity in 17 days; and with 0.5 mM 5,5'-dithiobis(2-nitrobenzoic acid) in place of 2-mercaptoethanol, all activity was lost within 3 days. Although stable in Tris-HCl buffer, pH 7.5, the enzyme is not stable in Tris-HCl buffers of pH 8.2 and 8.6 or in sodium or potassium cacodylate buffers, pH 5.6 to 6.9, in 10% glycerol to which 20 mM 2-mercaptoethanol has been added. The instability in the presence of cacodylate and 2-mercaptoethanol could be related to their reaction, causing elevation of pH (11).

**Assay Conditions.** Although potassium phosphate buffer stabilizes the enzyme during purification and storage, phosphate buffer inhibits the enzymatic activity, and no activity is seen in an assay mixture containing 100 mM potassium phosphate buffer, pH 6.9, in place of 100 mM sodium or potassium cacodylate buffer, pH 6.9. The optimum ATP concentration is 1 to 3 mM. Larger concentrations decrease the rate of tryptophanyl-tRNA formation, and at 20 mM ATP remaining activity is less than 5%. Tryptophanyl-tRNA synthetase of *E. coli*, also, is inhibited by ATP concentrations above 1 mM. That enzyme forms tryptophanyl-ATP (16), and the competition between ATP and tRNA as acceptors of tryptophan may relate to the decrease in rate of tryptophanyl-tRNA formation in the

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### Table 2: Kinetic data

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (M)</th>
<th>Relative $V_{max}$</th>
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<tbody>
<tr>
<td>ATP</td>
<td>$1.6 \times 10^{-4}$</td>
<td>71</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>$2.8 \times 10^{-4}$</td>
<td>69</td>
</tr>
<tr>
<td>tRNA$\text{Trp}$</td>
<td>$1.1 \times 10^{-7}$</td>
<td>77</td>
</tr>
</tbody>
</table>

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**Chart 2.** Kinetic data is plotted by the method of least squares as relative $v$ against relative $v/s$ (5). Each 0.5-ml assay tube was as described in "Methods" except for the relevant substrate, and contained 0.65 µg of the hydroxylapatite fraction of tryptophanyl-tRNA synthetase. Whole brewers' yeast tRNA containing 28 pmol of tRNA$\text{Trp}$ per $A_{260}$ unit was used as substrate.

**Chart 3.** Sucrose density gradient centrifugation. In a total volume of 4.7 ml, 22 units of the polymethacrylate fraction of tryptophanyl-tRNA synthetase (•), 42 units of *E. coli* isoleucyl-tRNA synthetase (○), and 1.5 units (1.0 unit changes $A_{400}$ by 1.0 in 1.0 min at 23°C) of human placental alkaline phosphatase (●) were layered on a linear 5% to 20% sucrose gradient in 4.6 ml of 10 mM potassium phosphate buffer, pH 6.9-20 mM 2-mercaptoethanol and centrifuged for 18 hr at 39,000 rpm in the SW 39L rotor on a Beckman ultracentrifuge (13). Fractions were 0.12 ml. Isoleucyl-tRNA synthetase and alkaline phosphatase were assayed as previously described (7, 18). Tryptophanyl-tRNA synthetase activity in the fractions shown is 30% of the input, and no separate peak was detectable. The top of the gradient is on the right.
presence of high ATP concentrations. With ATP at 1 mM, the optimum Mg\(^{2+}\) concentration is 20 mM. The L-tryptophan concentration is nearly optimal at 0.1 mM, with concentrations up to 0.4 mM giving a slight increase in activity. With sodium cacodylate and Tris-HCl buffers in the range of pH from 6.6 to 8.6, enzyme activity increases with rising pH.

**Kinetics.** Kinetic studies are summarized in Chart 2 and Table 2. The K_m's for all 3 substrates are typical of values found for a wide variety of aminoacyl-tRNA synthetases from bacteria and other sources. For example, the tryptophanyl-tRNA synthetase of *E. coli* has K_m values for tryptophan, ATP, and tRNA\(^{\text{TPP}}\) of 3 \times 10^{-5}, 5 \times 10^{-4}, and 3 \times 10^{-3} M, respectively (10, 16). Interestingly, the K_m of the human enzyme for yeast tRNA is equivalent to that of the *E. coli* enzyme for *E. coli* tRNA and the K_m for tryptophan is an order of magnitude lower for the human enzyme than for the *E. coli* enzyme. Not enough human tRNA was available to compare it with yeast tRNA in kinetic studies. For whole-yeast tRNA, the K_m was equivalent to 4 A\(_{260}\) units of tRNA per ml of reaction mixture.

**Specificity.** The enzyme forms tryptophanyl-tRNA, 14 pmol/A\(_{260}\) unit, from tRNA prepared from lymphocyte-rich blood of patients with CLL. Although yeast tRNA is a good substrate, as shown by the kinetic studies, the enzyme forms tryptophanyl-tRNA from *E. coli* tRNA at less than 3% the rate achieved with yeast tRNA, and *E. coli* tRNA does not inhibit the enzyme in forming yeast tryptophanyl-tRNA. Thus a sample of the polymethacrylate fraction contained 680 units/ml when assayed with 27 A\(_{260}\) units of yeast tRNA alone, 640 units/ml when assayed with 13 A\(_{260}\) units of yeast tRNA, and 13 A\(_{260}\) units of *E. coli* tRNA, and no detectable activity (fewer than 17 units/ml) when assayed with 25 units of *E. coli* tRNA alone.

This species specificity for tRNA is the same for the enzyme from human skin. Moreover, both enzymes charge yeast tRNA to the same extent, 30 pmol/A\(_{260}\) unit, and a mixture of the 2 enzymes, each present in excess, does not increase that level. Therefore, the enzymes from both human sources appear to charge the same isoacceptors in yeast tRNA.

Chloroquine, which increases *E. coli* tryptophanyl-tRNA formation by activating *E. coli* tRNA\(^{\text{TPP}}\) (16, 17) does not convert *E. coli* tRNA\(^{\text{TPP}}\) into a suitable substrate for the human enzyme and at 2.5 mM decreases by 40% the rate of tryptophanyl-tRNA formation from yeast tRNA by the human enzyme.

**Sedimentation.** As shown in Chart 3 the human tryptophanyl-tRNA synthetase sediments as a single, symmetrical peak in sucrose density gradient centrifugation, and by this criterion has an apparent molecular weight of 90,000 when compared to *E. coli* alkaline phosphatase (M.W. 90,000) (8) and human placental alkaline phosphatase (M.W. 116,000 to 125,000) (8, 9), and *E. coli* isoleucyl-tRNA synthetase (M. W. 112,000) (3) as standards. In this respect, the human enzyme is typical of most aminoacyl-tRNA synthetases from *E. coli* (20). In similar studies, the *E. coli* tryptophanyl-tRNA synthetase sediments as a protein with a molecular weight of 75,000; it has probably identical subunits with a molecular weight of 37,000 (10). The elucidation of the possible subunit structure of the human enzyme must await its complete purification.

**REFERENCES**

Human Trp-tRNA Synthetase

Discussion

Dr. Gallo: Dr. Muench, one question on your presentation, are you thinking of systematically comparing some of the synthetases of the lymphocytic leukemia cells to normal cells or just beginning to see the actual mechanisms of how they are working and characterizing their K_m's.

Dr. Muench: The comparison with normal lymphocytes is a long-term goal. However, obtaining normal lymphocytes in sufficient quantities to do the types of comparisons we want is a formidable task. Moreover, we have no evidence so far for any difference between the normal lymphocyte enzyme and the leukemic enzyme.

Dr. Adamson: May I ask Dr. Muench if there is any evidence for subunits in the human?

Dr. Muench: We haven't been able to do the required studies on the human enzyme, because we haven't had it pure.
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