Isolation of a single polypeptide leucyl-tRNA synthetase from bakers' yeast[1]

Chiu-Shiong Lin[2], Richard Irwin and Jack G.Chirikjian

Department of Biochemistry, Georgetown University Medical Center, Washington, DC 20007, USA

Received 24 May 1979

ABSTRACT

A single polypeptide of leucyl-tRNA synthetase (LRS) has been purified from budding bakers' yeast by a modification of the procedure published earlier. On denaturing polyacrylamide gel electrophoresis LRS was one band corresponding to molecular weight of 120,000±5,000 daltons. Variable amounts of LRS with a similar molecular weight but which dissociated into equal subunits of 58,000 were also isolated. The affinities (K_M) for substrates for this form of the enzyme were similar to those previously reported for the dimeric form of the enzyme.

INTRODUCTION

The important role that aminoacyl tRNA-synthetases play in protein biosynthesis is well documented (1,2). Each enzyme has the unique ability to recognize and esterify amino acid to specific tRNAs to achieve the required fidelity in protein biosynthesis. Since the initial reports on the purification of leucyl-tRNA synthetase (3) there have been several studies that deal with the purification of enzymes from a variety of sources (1,2). The molecular weights of synthetases show variations among organisms as well as variations within the same organism for synthetases of different amino acid specificity. Although in most cases there appears to be a single enzyme per amino acid, there have been reports of more than one active form (4-7). In several cases, different physical and chemical properties have been reported for such enzymes. It now appears that several of the initial subunit structure assignments for aminoacyl-tRNA synthetases were hampered by the presence of protease activities either present in cells

or released during the purification of these enzymes (8-11). In this report, we wish to describe a modified purification procedure that yields a single polypeptide form for LRS.

EXPERIMENTAL PROCEDURES

A. Materials

Transfer RNA (tRNA): Unfractionated tRNA was extracted from budding bakers' yeast (12). More than 60% of the prepared tRNA contained adenosine residues at the 3'-terminal end. Yeast $tRNA^{leu3}$ (purity in excess of 90%) was prepared by a procedure to be described

Reagents: TPCK-treated trypsin and the molecular weight markers were obtained from Worthington Biochemical. Hydroxyapatite (Bio-Gel HTP) and Coomassie Brilliant Blue R-250 were supplied by Bio-Rad. All other reagents used were of the highest quality available.

Buffers: Buffer A contained 50 mM phosphate (K^+) buffer, pH 7.5 + 0.2 mM DTT + 0.2 mM EDTA. Buffer B contained 20 mM phosphate (K^+) buffer, pH 7.5 + 0.2 mM DTT + 0.2 mM EDTA + 10% glycerol. Buffer C contained 100 mM phosphate (K^+) buffer, pH 7.5 + 0.2 mM DTT + 0.2 mM EDTA + 10% glycerol. Buffer D contained 700 mM phosphate (K^+) buffer, pH 7.5 + 0.2 mM DTT + 0.2 mM EDTA + 10% glycerol.

B. Analytical Methods

Protein Determination: Protein content was determined by the method of Lowry *et al.* (13), using crystalline bovine serum albumin as a standard.

Terminal Adenylation of $tRNA^{leu3}$: Purified $tRNA^{leu3}$ contained only 5%-10% of terminal adenosine moiety. Additional adenosine residues were incorporated enzymatically on the 3'-terminus of $tRNA^{leu3}$ using purified yeast nucleotidyl transferase. The incubation procedure employed was essentially as described by Sternbach *et al.* (14).

Polyacrylamide Gel Electrophoresis: Analytical slab polyacrylamide gel electrophoresis was performed according to the methods of Davis (15) without a stacking gel. Constant current of 1.6 mA/cm was applied until the bromophenol blue marker reached the end of the gel. In order to locate enzy-

matic activity on gels, enzyme samples were applied to two adjacent wells and electrophoresis was performed at 4° in running buffer containing 0.2 mM DTT. After electrophoresis one well was cut along the direction of current and stained; while the other was sliced and the enzyme was eluted with Buffer A, containing 10% glycerol, and assayed for activity.

SDS-Polyacrylamide Gel Electrophoresis: SDS-polyacrylamide gel electrophoresis was performed on 10% slab gel according to the method of Laemmli (16) with the appropriate molecular weight markers.

Isoelectric Focusing of Protein: Purified enzyme preparations were electrofocused on a minicolumn at 4° by a modified procedure of Godson (17). The 8.5 ml column was run at 350 volts for 3 hours.

Sucrose Gradient Centrifugation: Gradients (4.8 ml) were prepared with 5% and 20% sucrose solutions in Buffer A, containing 0.1 M KCl. The gradients were centrifuged at 35,000 rpm at 2° for 18 hours, using the SW 50.1 rotor. The molecular weight of the enzyme was estimated relative to the migration of protein standards (18).

Determination of Kinetic Constants: The Km values for each substrate were determined as previously described (19).

C. Assay of Aminoacyl-tRNA Synthetase Activities

Aminoacylation Reaction: Leucyl-tRNA synthetase was assayed by measuring the level of aminoacylation of unfractionated tRNA by [^{14}C]-L-leucine (Specific Activity of 25 mCi/mmole) as previously described (3,19).

Pyrophosphate Exchange Reaction: The leucine dependent ATP-^{32}P-PPi exchange activity was determined according to the procedure described by Calendar and Berg (20).

RESULTS

A. Enzyme Purification

The entire procedure was carried out at 4°, unless otherwise indicated. Centrifugations were performed in a Sorvall centrifuge for 30 minutes at 10,000 x g. Solid ammonium sulfate was used to precipitate protein solutions and pH

was maintained between 7.0 and 7.5. All buffers used through the phosphocellulose step contained 1 mM PMSF.

Washing of Yeast Cells: Frozen budding bakers' yeast cells (1 kg) were washed in 2 liters buffer A.

Preparation of Crude Extract: Washed yeast cells were suspended in 50 ml of buffer A and broken by homogenizing with glass beads over a 20 minute period in a Waring Blender. The slurry was diluted with buffer A, glass beads were allowed to settle, and the supernatant was decanted. The beads were washed with the same buffer, and washes were combined with the original extract. Unbroken cells and cell debris were removed by centrifugation. The resulting supernatant will be referred to as the "Crude Extract."

Ammonium Sulfate Fractionation: To the crude extract solid ammonium sulfate was added with continuous stirring to 57% saturation (350 g/l) over a period of 30 minutes. Stirring was continued for an additional 3 hours and the precipitate was collected by centrifugation. The supernatant was brought up to 85% saturation by adding additional ammonium sulfate (210 g/ml); stirring was continued for 10 hours; and the precipitate collected by centrifugation, dissolved in buffer B, and dialyzed overnight against the same buffer.

Chromatography on DEAE-Cellulose Column: Dialyzed enzyme solution was applied onto DEAE-cellulose column, equilibrated with buffer B, and washed with 100 ml of the same buffer. A large protein peak, which contained Leucyl-tRNA synthetase (LRSI) was eluted (Fig. 1). A second well-removed protein peak with higher specific activity referred to as LRS II was eluted from the column with buffer C, containing 0.2 M KCl (Fig. 1). The active fractions from the two peaks were pooled; solid ammonium sulfate (550 g/ml) was added; and the precipitate, collected by centrifugation, was dissolved in a minimum amount of buffer A containing 10% glycerol and dialyzed against the same buffer. Each peak was then treated separately.

As will be shown, the first peak corresponds to the LRS(I) previously reported (3); while the second peak (LRS II) which eluted at higher salt concentration, corresponds to the

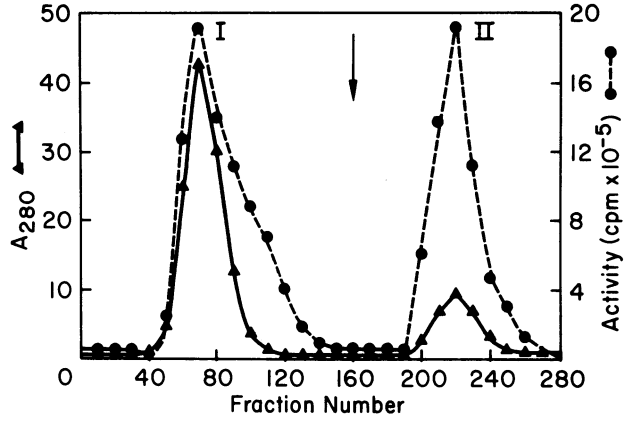

Fig. 1. Chromatography of leucyl-tRNA synthetase on DEAE-cellulose. The elution profile for the two forms of leucyl-tRNA synthetase LRS I (2 subunits) and LRS II (single polypeptide) are shown. Details of the column chromatography were described in the text.

single polypeptide form of the enzyme.

Chromatography on Phosphocellulose Column: LRS I pool was fractionated on a phosphocellulose column. Elution with buffer A washed out a large protein peak that usually was devoid of enzyme activity. In some preparations, a small amount of activity was also found in this peak. A linear gradient of 500 ml buffer A + 10% glycerol and of 500 ml buffer C + 300 mM KCl eluted the second protein peak, which contained high enzymatic activity. The active fractions in this peak were pooled, and concentrated by ammonium sulfate precipitation, and the precipitate was dialyzed against buffer B overnight.

Similarly LRS II was fractionated by phosphocellulose chromatography. Elution with equilibrating buffer resulted in an inert protein peak completely devoid of enzyme activity. A linear gradient, as that described above, eluted a second well-removed protein peak with very high specific activity. The active fractions were pooled, and processed in a similar fashion.

Chromatography on Hydroxyapatite Column: The dialyzed enzyme fractions obtained from the phosphocellulose step were

further fractionated on Bio-Gel HTP. After washing with 100 ml buffer B, the column was eluted with linear gradient of 80 ml buffer B and of 80 ml buffer D. The bulk of the protein eluted early, while the enzyme appeared later in the gradient as a discrete peak. The active fractions were pooled and concentrated and were used for further analysis or stored at -90° after increasing the glycerol concentration to 20%. Under these conditions, the enzyme activity of both forms appeared to be relatively stable for several months.

Second Chromatography on DEAE-Cellulose Column: In some preparations, LRS II enzyme form was still contaminated with other proteins. A second pass through DEAE cellulose and phosphocellulose columns were necessary prior to obtaining the pure leucyl-tRNA synthetase. A summary of the purification is given in Table 1.

B. Characterization of the Multiple Forms of Bakers' Yeast Leucyl-tRNA Synthetase

Assessment of Purity and Determination of Subunit Structure: The polypeptide composition of the two enzyme forms were determined (16) using several standard proteins. Values obtained were of 120,000±5,000 for the LRS II and 58,000 for

TABLE I

Purification of Budding Yeast Leucyl-tRNA Synthetase

Enzyme Fraction	Volume (ml)	Protein (mg/ml)	Specific Activity	Purification (-fold)	Yield (%)
Crude Extract	3100	106.5	0.8	1	100.0
$(NH_4)_2SO_4$	340	69.4	10.6	13	94.7
DEAE-Cellulose (7 x 55 cm)					
a) LRS I	184	45.9	16.1	20	51.5
b) LRS II	81	17.4	21.0	26	11.2
Phosphocellulose (45 x 35 cm)					
a) LRS I	180	4.0	80.0	100	21.8
b) LRS II	38	2.4	170.0	213	5.8
Hydroxyapatite (1 x 30 cm)					
a) LRS I	45	1.6	265.2	331	7.2
b) LRS II	30	1.4	292.1	365	4.6
DEAE-Cellulose (2 x 15 cm)					
a) LRS II	26	0.6	434.0	543	2.6

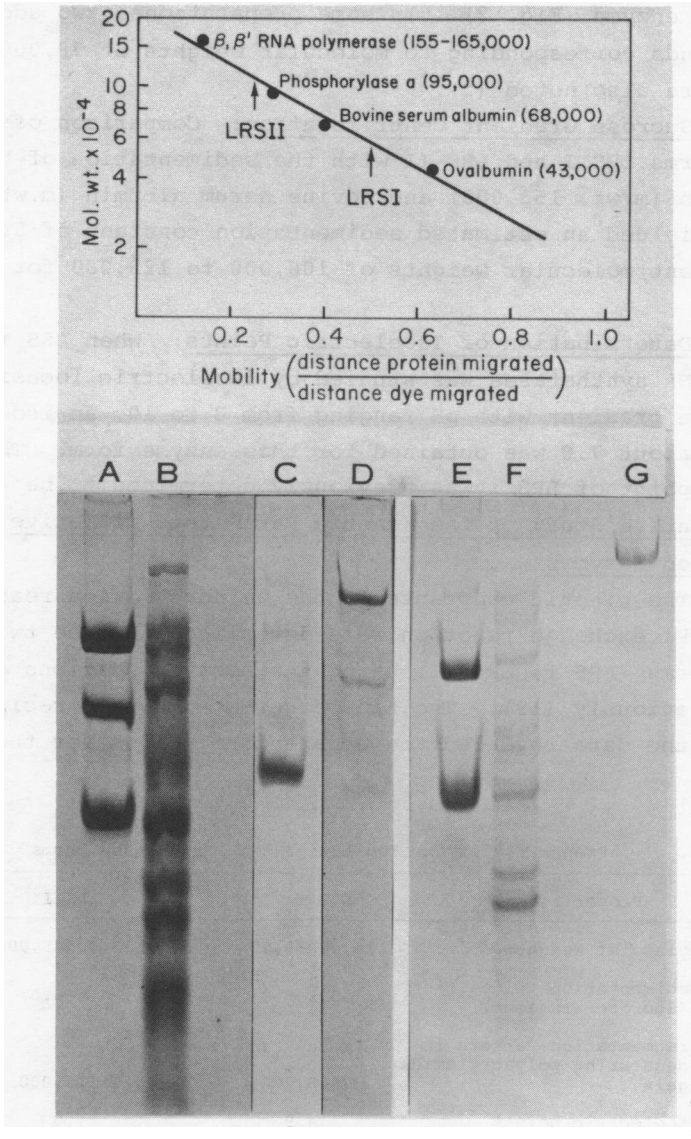

Fig. 2. Subunit determination for LRS I and LRS II by denaturing SDS-polyacrylamide analysis: Top figure shows the mobility for LRS II (120,000) and LRS I (58,000) relative to protein standards. Phosphorylase a, BSA and ovalbumin are shown in Lanes A and E , while the E. coli RNA polymerase subunits are represented by the top protein bands in the bacterial crude extract (Lanes B and F). Purified LRS I and LRS II are shown in lanes C and G respectively. Lane D represents aged LRS II which upon storage generated two nonidentical subunits (3).

the LRS I enzyme (Fig. 2). In some preparations, two additional bands corresponding to molecular weights of 78,000 and 38,000 were also noted (3).

Sucrose Gradient Centrifugation: Comparison of the enzyme forms LRS I and LRS II with the sedimentation of human γ-globulin (m.wt. 153,000) and bovine serum albumin (m.wt. 68,000) yielded an estimated sedimentation constant of 5.5-6.0S and apparent molecular weights of 100,000 to 125,000 for both enzymes.

Determination of Isoelectric Points: When LRS II leucyl-tRNA synthetases was studied by isoelectric focusing in sucrose gradient with pH ranging from 3 to 10, an isoelectric point of about 7.0 was obtained for this enzyme form. The isoelectric point of LRS I was previously determined to be 4.9 (3).

C. Comparative Study of the Kinetic Parameters of Native and Modified Enzymes

The kinetic constants of the aminoacylation reaction and ATP-PPi exchange reaction were determined for the two enzyme forms (LRS I, LRS II) using the assay conditions described previously (19). The Lineweaver-Burke double reciprocal plots of the data revealed the apparent Km values for the two

TABLE II

Properties of the Two Leucyl-tRNA Synthetase Forms

Properties	LRS I	LRS II
Molecular Weight	116,000±5,000	115,000±5,000
Sedimentation Value (Sucrose Gradient)	5.5-6.0	5.5-6.0
Fragmentation Pattern in denaturing polyacrylamide gels	2x58,000	1x115,000
Isoelectric points	4.9	7.0
K_m Values (xM) obtained by Aminoacylation reaction:		
ATP	1.1×10^{-4}	0.4×10^{-4}
L-leucine	2.0×10^{-5}	1.0×10^{-5}
$tRNA^{leu3}$	1.0×10^{-7}	1.3×10^{-7}
ATP-PP_i Exchange:		
ATP	3.3×10^{-4}	2.5×10^{-4}
L-leucine	2.5×10^{-5}	2.5×10^{-5}

leucyl-tRNA synthetase forms for the substrates, ATP, L-leucine and $tRNA^{leu3}$ in the aminoacylation and ATP-PPi exchange reac-reactions. Table II summarizes Km values as well as other properties described above for the two enzyme forms. As shown, both forms of the enzyme exhibit very similar Km values for their substrates.

DISCUSSION

Previous studies assigned leucyl-tRNA synthetase to be composed of two equal subunits with 55,000 (3). With the knowledge of the presence of proteases in yeast extracts that modify aminoacyl-tRNA synthetases, we added several protease inhibitors in later studies (21). The presence of extracellular proteases was also considered, and cells were prewashed with buffer containing PMSF. From prewashed cells and upon using protease inhibitors, a new enzyme form (LRS II) with a single polypeptide of $120,000 \pm 5,000$ daltons was isolated, in addition to the previously observed LRS I. The properties for both enzyme forms are summarized in Table II. The relationship between the two forms remains to be determined, although it is likely that the dimeric form is a limited protease digest of the single polypeptide LRS. The LRS from the closely related *Candida utilis* has also been reported to be a single polypeptide with a molecular weight of 128,000 (22).

FOOTNOTES

1. Supported by Grant PCM76-14147 from the National Science Foundation and Grant 74-959 from the American Heart Association.

2. Submitted in partial fulfillment for the Ph.D. degree. Present address: Laboratory of Nutrition and Endocrinology, NIAMDD, NIH, Bethesda, Maryland 20014.

REFERENCES

1. Söll, D., and Schimmel, P. (1974) The Enzymes (Boyer, P., ed.) 10: 489-538, Academic Press, New York.
2. Kisselev, L.L., and Favorova, O.O. (1974) Advances in

Enzymology (Meister, A., ed.) 40: 141-238, John Wiley, New York.
3. Chirikjian, J.G., Kanagalingam, K., Lau, E., and Fresco, J.R. (1973) J. Biol. Chem. 248: 1074-1079.
4. Kern, D., Giege, R., Robre-Saul, S., Boulanger, Y., and Ebel, J.P. (1975) Biochimie 57: 1167-1176.
5. Paradies, H.H. (1975) Biochem. Biophys. Res. Commun. 64: 1253-1262.
6. Surguchov, A.P., and Surguchova, I.G. (1975) Eur. J. Biochem. 54: 175-184.
7. Lemaire, G., Gros, C., Epely, S., Kaminski, M., and Labouesse, B. (1975) Eur. J. Biochem. 51: 237-252.
8. Bruton, C.J., and Hartley, B.S. (1970) Biochem. J. 117: 18P-19P.
9. Schmidt, J., Wang, R., Stanfield, S., and Reid, B. (1971) Biochemistry 10: 3264-3268.
10. Gros, C., Lemaire, G., Rapenbusch, R. van, and Labouesse, B. (1972) J. Biol. Chem. 247: 2931-2943.
11. Kucan, Z., and Chambers, R.W. (1973) J. Biochem. (Tokyo) 73: 811-819.
12. Lindahl, T., and Fresco, J.R. (1967) Methods Enzym. 12: 601.
13. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193: 265-275.
14. Sternbach, H., Haar, F.V.D., Schlimme, E., Gaertner, E., and Cramer, F. (1971) Eur. J. Biochem. 22: 166-172.
15. Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121: 404-427.
16. Laemmli, U.K. (1970) Nature 227: 680-685.
17. Godson, G.N. (1970) Anal. Biochem. 35: 66-76.
18. Martin, R.G., and Ames, B.N. (1961) J. Biol. Chem. 236: 1372-1379.
19. Lin, C.S., Irwin, R., and Chirikjian, J.G. (1975) J. Biol. Chem. 250: 9299-9303.
20. Calendar, R., and Berg, P. (1966) Biochemistry 5: 1681-1690.
21. Lin, C.S. (1976) Ph.D. Thesis, Georgetown University.
22. Murasugi, A., and Hayashi, H. (1975) Eur. J. Biochem. 57: 169-175.