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FUNCTIONAL SIGNIFICANCE OF AMINOACYL-tRNA SYNTHETASE COMPLEX IN THE AMINOACYLATION OF $tRNA^{LEU}$ ISOACCEPTORS

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SUMMARY: Aminoacyl-tRNA synthetases, partially purified from rat liver by two different methods, were used in vitro to study aminoacylation profiles of $tRNA^{Leu}$ isoacceptors. On the basis of molecular weights, one preparation was similar to the multienzyme complex of aminoacyl-tRNA synthetases, whereas the other apparently represents a partially disrupted complex. In the aminoacylation assay, the multienzyme complex produced a profile of leucyl-tRNA isoacceptors that was similar to those found in vivo and in liver perfusion experiments. The aminoacylation profile that was obtained with the partially distrupted complex varied with the enzyme and leucine concentration used. Especially one of the $tRNA^{Leu}$ species was poorly aminoacylated at low leucine and enzyme concentration. These experiments point out that attention should be paid to the nature of the aminoacyl-tRNA synthetase preparation in experiments in which isoacceptor profiles are studied in vitro.

The formation of aminoacyl-tRNA, the first reaction in the cascade leading from free amino acids to proteins, is catalyzed by aminoacyl-tRNA synthetases (EC 6.1.1). It has been documented that at least nine mammalian aminoacyl-tRNA synthetases, namely those for Arg, Asp, Gln, Glu, Ile, Leu, Lys, Met, and Pro exist as a high-M_r complex (1,2), whereas several other aminoacyl-tRNA synthetases are generally present in cell-free extracts as free enzymes (3,4). The aminoacyl-tRNA synthetase complex has been shown to also contain non-protein components such as RNA (5), lipids (5), and carbohydrates (6). The role of these non-enzyme components in the structure and function of the aminoacyl-tRNA synthetase complex has not yet been determined.

The aminoacyl-tRNA synthetase complex is relatively unstable and e.g. vigorous homogenization and ultracentrifugation may lead to its distruption (7-9) thus producing multiple forms of aminoacyl-tRNA synthetases which differ in their molecular weights. The functional properties of the complexed and dissociated forms of aminoacyl-tRNA synthetases in aminoacylation of tRNA isoacceptors have, however, not received much attention. In the present study, we have compared chromatographic profiles of $tRNA^{Leu}$ isoacceptors aminoacylated in vitro with preparations of leucyl-tRNA synthetase representing complexed and dissociated forms of the enzyme. We have also studied profiles of leucyl-tRNA isoacceptors derived from perfused rat liver as well as from rat liver in vivo.

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MATERIALS AND METHODS

L-[^{14}C(U)]-Leucine (spec. act. 347 mCi/mmol) and L-[3,4,5,-^{3}H(N)]-leucine (spec. act. 141 Ci/mmol) were purchased from New England Nuclear Chemicals (F.R.G.).

Two methods were used to prepare aminoacyl-tRNA synthetases from rat liver. The first preparation (enzyme A) was obtained by purification of the 105 000xg supernatant from liver homogenate by DEAE-cellulose chromatography (10) except that 1 mM dithiotreitol was used instead of 10 mM mercaptoethanol. The DEAE-cellulose eluate which contained 4 to 6 mg protein/ml was stored in small aliquots at -20°C. The other preparation (enzyme B) was purified by 35 to 50% ammonium sulfate fractionation of postmitochondrial supernatant from liver homogenate followed by gel filtration on Sepharose 4B (Pharmacia) (11). The first peak eluting from Sepharose 4B and containing 1 to 2 mg protein/ml was pooled and used in the aminoacylation studies. Protein was determined by the fluorescamine method (12).

Molecular weights of both enzyme preparations were estimated by gel filtration on Superose 12 (Pharmacia) equilibrated with 5 mM Tris/HCl, 2 mM EDTA, 0.5 mM dithiotreitol, pH 7.5, containing 10% (v/v) glycerol. The void volume of the column was determined by Blue Dextran 2000 (Pharmacia). Thyroglobulin (M_r 669 000) and aldolase (M_r 158 000) were used as molecular weight standards.

Lipids were extracted according to Sihag and Deutscher (13) from the enzyme preparation B as well as from the second peak that eluted from Sepharose 4B during the purification procedure. Two dimensional thin-layer chromatography was carried out in a solvent system consisting of petroleum ether:diethyl ether:water (80:20:2) for neutral lipids and chloroform:methanol:water (75:25:4) for phospholipids.

The activity of leucyl-tRNA synthetases was assessed in 0.05 ml reaction mixtures containig 50 mM potassium phosphate buffer, pH 7.4, 5 mM $MgCl_2$, 1 mM spermidine, 2 mM DTT, 10 mM ATP, 10 mM CTP, 8 mg/ml yeast or calf liver tRNA and an appropriate amount of the enzyme. Either ^{3}H- or ^{14}C-labeled leucine was used at concentrations indicated in the text. After incubation at 37°C for 1 to 20 min, the reaction was terminated with 10% (w/v) trichloroacetic acid and the leucyl-tRNA precipitates were collected on glass fiber filters (GF/A). After rinsing and drying, the radioactivity was counted in a toluene-based scintillation fluid.

Transfer RNA was isolated from livers of fed rats by phenol extraction as described previously (14). Purified rat liver tRNA or calf liver tRNA from Boehringer Mannheim GmbH (F.R.G.) was aminoacylated in a volume of 1 ml for 20 min at 37°C. The aminoacylation mixture contained 50 mM potassium phosphate buffer, pH 7.4, 5 mM $MgCl_2$, 1 mM spermidine, 2 mM DTT, 10 mM ATP, 10 mM CTP, and 17.2-25.0 A_{260} units of tRNA. Concentration of leucine was either 20 µM or 800 µM as indicated in the figure legends. After aminoacylation, leucyl-tRNA was separated from free leucine on DEAE-cellulose (10,15).

Isoacceptors of leucyl-tRNA were separated on Sepharose 4B using a linear descending ammonium sulfate gradient from 1.6 M to 1.0 M (16). The column was equilibrated with 10 mM sodium acetate buffer, pH 4.5, containing 1.6 M ammonium sulfate, 10 mM $MgCl_2$, 1 mM EDTA, and 6 mM mercaptoethanol. Albumin was added to each fraction and leucyl-tRNA was precipitated with an equal volume of 10% trichloroacetic acid. The precipitates were collected on glass fiber filters, rinsed, dried, and counted as above.

Leucyl-tRNA was labeled in liver perfusions as follows. Livers from fed rats were first perfused in a single-pass mode for 40 min with a medium containing no amino acids followed by a cyclic perfusion for 3 min with a medium containing ^{14}C-leucine (spec. act. 347 mCi/mmol; 0.8 µmol/l medium). In the second set of perfusions, the medium during the single-pass phase contained 4 times normal plasma concentration of 20 amino acids and during the cyclic phase (2.5 min) 4 times plasma concentration of 20 amino acids with ^{3}H-labeled leucine (spec. act. 5.0 mCi/mmol; 0.8 mmol/l medium). The perfusion method and the composition of the perfusate were in detail as in Pösö et al. (17). While the perfusion was still going on, the livers were frozen with aluminium tongs precooled in liquid nitrogen. Transfer RNA was extracted and the leucyl-tRNA isoacceptors were fractionated as described above.

Leucyl-tRNA was also labeled in vivo by intravenous infusions on ^{3}H-leucine (10 µCi/rat) to v. femoralis of anaesthetized rats. The livers were freeze-clamped 5 min later. Extraction of tRNA and separation of the leucyl-tRNA isoacceptors were as above.

RESULTS

In gel filtration, enzyme A eluted in one major and two minor peaks of activity (not shown). The major peak had an apparent M_r of 400 000 and the minor peaks eluted after the void volume and at the 100 000 M_r-region. For the aminoacylation assays with

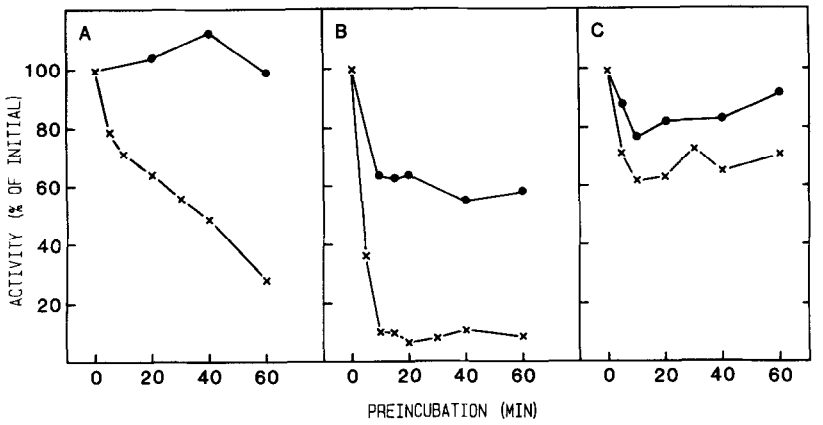

Fig. 1. Thermal stability of leucyl-tRNA synthetases. The enzymes were preincubated for the times indicated at 37°C (A) or 45°C (B and C). The enzyme activity was assayed at 37°C after preincubation. The leucine concentration in the assay was either 20 μM (A and B) or 200 μM (C). Key: x-x, enzyme A; ●–●, enzyme B.

enzyme A, a mixture of all three peaks was used. With enzyme B, the major peak of activity in gel filtration had a M_r of over 10^6 and a second minor peak was seen at the 400 000 M_r-region (not shown). For the aminoacylation assays with enzyme B, only the high-molecular weight fraction was used.

Preincubation of enzyme A at 37°C linearly decreased its activity when assayed in the presence of 20 μM leucine, whereas enzyme B was stable under similar conditions (Fig. 1A). At 45°C, enzyme A completely lost its activity within 10 min, while enzyme B, after losing about 40% of its activity within 10 min, maintained the remaining activity for at least 30 min (Fig. 1B). After preincubation at 45°C, enzyme B behaved almost identically in the presence of 20 μM and 200 μM leucine in the assay, whereas enzyme A showed higher activity remaining after preincubation when assayed at 200 μM leucine concentration (Fig. 1C).

When leucine concentration in the assay was varied from 1 to 400 μM, both enzymes showed linearity in the Eadie-Hofstee plot over the whole range of leucine concentration (not shown). The K_m values were 33.9 μM and 8.7 μM for enzyme A and enzyme B, respectively, and the corresponding V_{max} values were 294 and 217 pmol leucine/min · mg protein.

Separation of lipids from enzyme B preparation by thin layer chromatography revealed nine spots of neutral and polar lipids (results not shown). Lipid extraction was also performed from the second peak eluting at the M_r-region of 400 000, but only one spot of neutral lipids was observed.

Rat liver $tRNA^{Leu}$ isoacceptors acylated with either enzyme A or enzyme B eluted from Sepharose 4B in three peaks (Fig. 2). Both ^3H- or ^{14}C-labeled leucine gave identical results. When the amount of enzyme A in the acylation reaction was gradually increased to a twofold excess, the relative area of the second leucyl-tRNA peak increased significantly (Fig. 2, panels I-III). No change in the relative area of the third peak was observed. Once the size of the second peak had reached that of the first peak, further addition of the enzyme did not change the profile. The concentration of leucine used (20 μM) is above the reported K_m for leucyl-tRNA synthetase (8.5 μM for the complex and 9.1 μM for the free enzyme) (18). Rechromatography of peaks I and II on benzoylated DEAE-cellulose (not shown) revealed that, while peak I elutes in three

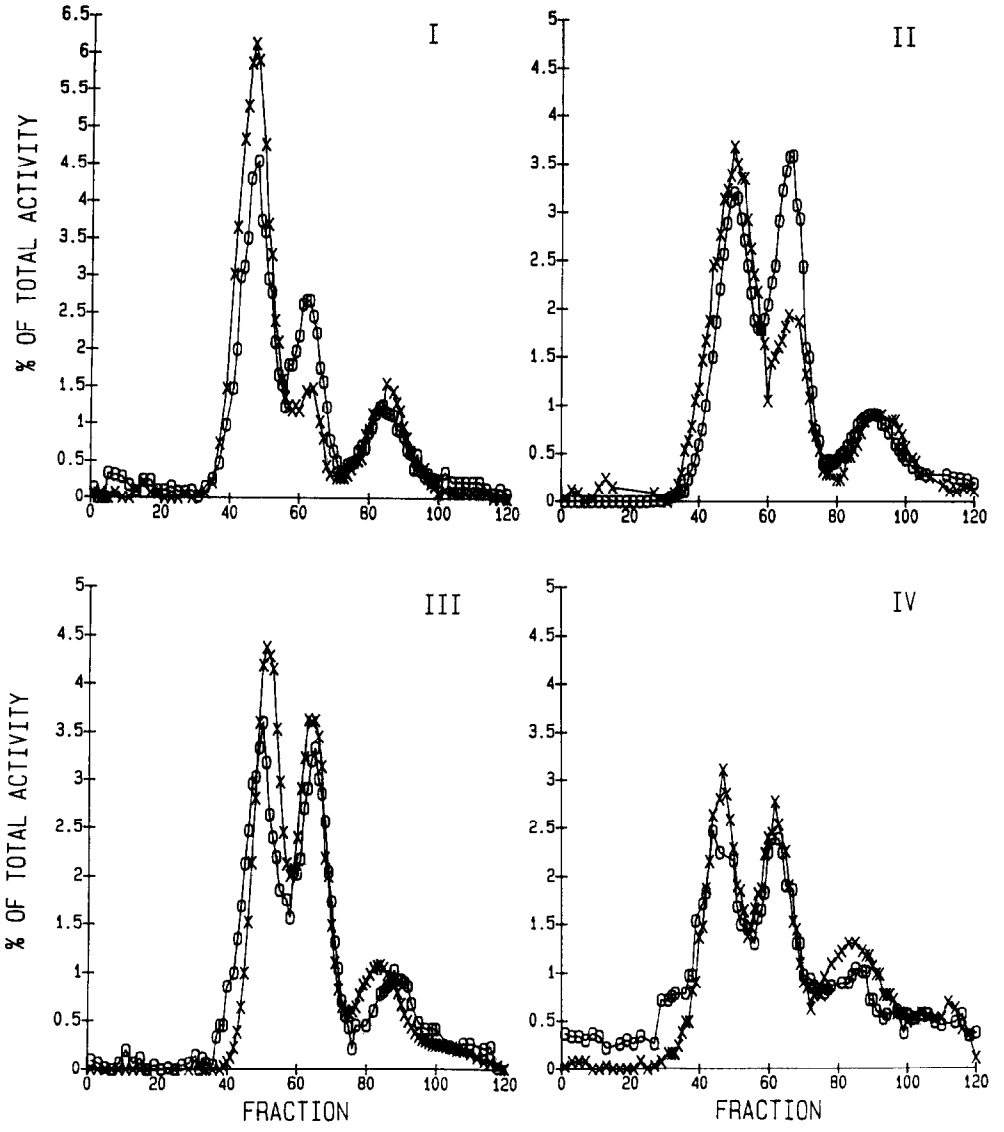

Fig. 2. Effect of leucyl-tRNA synthetase concentration on the aminoacylation of rat liver $tRNA^{Leu}$ isoacceptors in the presence of various concentrations of leucine. Rat liver tRNA (29-40 μM) was acylated with ^{3}H- or ^{14}C-leucine (x-x, 20 μM, o-o, 800 μM) for 20 min and the leucyl-tRNAs were separated on Sepharose 4B as described in Materials and Methods. The protein concentration in the enzyme preparation was 6.04 mg/ml (enzyme A) and following amounts were used; panel I, 0.24 mg; panel II, 0.36 mg; panel III, 0.48 mg ; with enzyme B, the amount of protein was 22 μg (panel IV).

separate peaks, peak II remains as a single peak suggesting that peak II contains only one leucyl-tRNA isoacceptor species (19).

At high leucine (800 μM) and low enzyme concentration in the acylation reaction, the relative area of peak II was considerably larger than at low leucine concentration (Fig 2, panel I). Also in this case, no further increase in the aminoacylation of peak II was observed once it had reached the aminoacylation level of peak I (not shown).

The leucyl-tRNA isoacceptor profile from acylation with enzyme B was similar to that seen when high concentration of enzyme A was used in the acylation reaction (Fig.2, panel IV). Changing the concentration of enzyme B over a 8-fold range did not alter the

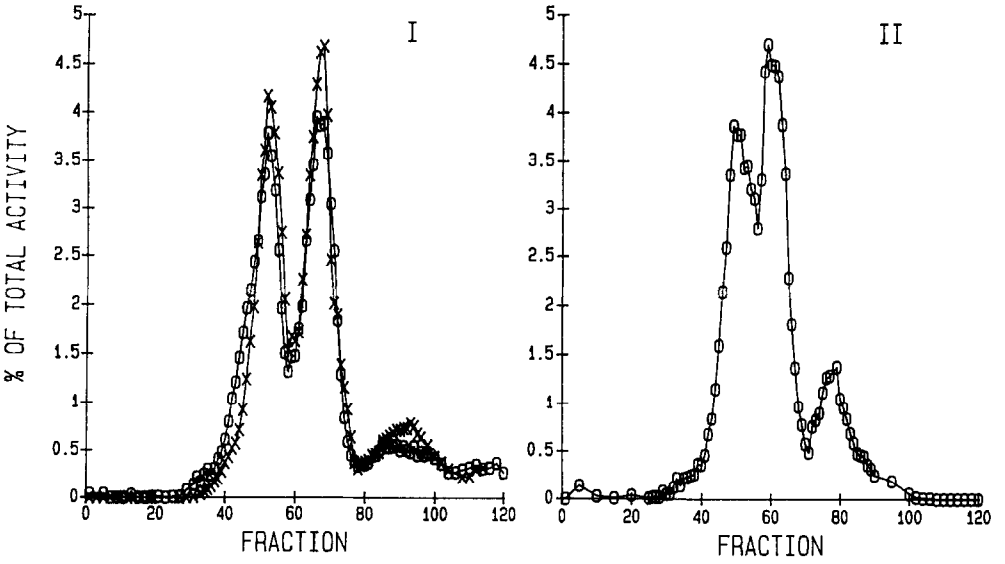

Fig. 3. Leucyl-tRNA isoacceptors in liver perfusion experiments and in vivo. Panel I. Livers were perfused in a single-pass mode for 40 min either without any amino acids (0x aa) or in the presence of 4 times normal concentration of 20 amino acids (4x aa). Leucyl-tRNA was then labeled in a cyclic perfusion for 3 min with ^{14}C-leucine (0x aa, x-x) and for 2.5 min with ^{3}H-leucine (4x aa, o-o). Panel II. ^{3}H-leucine was injected to v. femoralis of anaesthetized rats 5 min before the liver was frozen to label leucyl-tRNA in vivo. ^{3}H-leucyl-tRNAs were isolated and separated as in Panel I.

leucyl-tRNA profile. Further, no changes in the aminoacylation profile were found when enzyme B was used to aminoacylate rat liver tRNA in the presence of 800 μM leucine (Fig. 2, panel IV).

Isoacceptor profiles of leucyl-tRNA from livers perfused with 4 times the plasma concentration of amino acids or without added amino acids are shown in Fig. 3, panel I. The profiles were similar to those seen in experiments with enzyme B or with high concentrations of enzyme A. No effect of varying amino acid concentrations was found.

Leucyl-tRNA isolated from rat liver produced essentially similar isoacceptor profiles as those from liver perfusion experiments and from in vitro acylation with enzyme B (Fig. 3, panel II).

DISCUSSION

The occurrence of aminoacyl-tRNA synthetases as a multienzyme complex, although its significance is not fully understood, together with its relative unstability draws attention to types of enzyme preparations used in studies involving tRNA aminoacylation. In the present study, enzyme B with its high molecular weight seems to correspond to the multienzyme complex, whereas the molecular weight of enzyme A suggests that it represents a partially dissociated complex. The apparent molecular weight of the main fraction of enzyme A (400 000) indicates that even this preparation does not represent truly free leucyl-tRNA synthetase (M_r 129 000) (20). Thus, the methods employed to isolate enzyme A, namely mechanical homogenization, DEAE-cellulose chromatography and ultracentrifugation, appear to cause disassembly of the multienzyme complex. Further, when distrupted, the complex appears to lose its lipid components that have been shown to be mainly glycolipids (5). The methods used

to isolate enzyme A in this study are similar to those generally used for in vitro studies on aminoacylation (for further references, see 19).

When compared with the results from labeling tRNA in vivo or in liver perfusion experiments, the dissociated form of leucyl-tRNA synthetase (enzyme A) aminoacylated poorly one $tRNA^{Leu}$ isoacceptor when both enzyme and leucine concentrations were low. On the other hand, aminoacylation of tRNA with the partially purified multienzyme complex produced a leucyl-tRNA isoacceptor pattern which closely resembled that found both in vivo and in liver perfusion experiments. These studies demonstrate that, in vitro, the aminoacylation profile of leucyl-tRNA isoacceptors may vary considerably depending on the integrity of the aminoacyl-tRNA synthetase complex used.

The kinetic parameters of the two enzyme preparations differed significantly from each other. The complexed leucyl-tRNA synthetase (enzyme B) had greater affinity toward leucine than the dissociated form of the enzyme (enzyme A). The K_m value for enzyme B (8.7 μM) was similar to that for the 24S complex of leucyl-tRNA synthetase (8.5 μM) (18), whereas the K_m value for enzyme A (33.9 μM) is considerably higher than that for the 6S leucyl-tRNA synthetase (9.1 μM) (18). Also, the dissociated form of leucyl-tRNA synthetase was more thermolabile than the multienzyme complex. Similar findings on the thermostability of leucyl-tRNA synthetase and lysyl- and arginyl-tRNA synthetases have been reported previously (21-23). In addition to these differences in the kinetic parameters, enzyme B preparation contained lipids, whereas the 400 000 M_r-fraction from Sepharose 4B was practically devoid of lipids. It has been recently suggested that the lipid components of the multienzyme complex may be essential for a proper function of the complex (24).

In conclusion, these studies show that, when compared with in vivo aminoacylation of $tRNA^{Leu}$ isoacceptors, the aminoacylation profile in vitro depends, in addition to the presence of particular isoacceptors, also on the nature of the enzyme preparation used in the aminoacylation reaction. Further studies are required to clarify why dissociation of the multienzyme complex leads to poor aminoacylation of specific tRNA isoacceptors.

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