Biochimica et Biophysica Acta, 452 (1976) 271—283

BBA 67953

MULTIPLE MOLECULAR FORMS OF CYSTEINYL-tRNA SYNTHETASE FROM RAT LIVER: PURIFICATION AND SUBUNIT STRUCTURE

F. PAN, H.H. LEE, S.H. PAI, T.C. YU, J.Y. GUOO and G.M. DUH

Department of Biochemistry, National Defense Medical Center and Medical Research Laboratory, Veterans General Hospital, Taipei (Taiwan)

(Received April 22nd, 1976)

Summary

Cysteinyl-tRNA synthetase (L-cysteine:tRNA^{Cys} ligase (AMP-forming), EC 6.1.1.16) has been purified from rat liver in 23% overall yield. The enzyme was resolved by hydroxyapatite chromatography into three active forms (Fractions CRS-1, CRS-2 and CRS-3). The total activity ratio was about 0.7 : 2 : 1. The fractions CRS-2 and CRS-3 contained no other detectable aminoacyl-tRNA synthetase activity. CRS-2 was homogeneous by polyacrylamide gel electrophoresis, CRS-3 gave two active bands with mobilities corresponding to those of CRS-1 and CRS-2. The molecular weight of CRS-2 was about 240 000 by electrophoretic mobilities on the gels of various porosity, and 115 000—140 000 by sucrose gradient centrifugation. By gel-filtration, CRS-1, CRS-2 and CRS-3 exhibited apparent molecular weights of 122 000, 235 000 and 300 000, respectively. By sodium dodecyl sulfate gel electrophoresis, both CRS-2 and CRS-3 gave a single major band of 120 000 daltons. Stoichiometric study of cysteinyl adenylate formation indicated that CRS-2 has two active sites per molecule. These results are consistent with a dimeric structure of the type α_2 for the major form of rat liver cysteinyl-tRNA synthetase, composed of two probably identical subunits of about 120 000 daltons. Available evidence also suggests that CRS-1 and CRS-3 are α and α_3 (or α_4), respectively.

Introduction

The aminoacyl-tRNA synthetase (EC 6.1.1.-) are some of the most complex enzymes known. They are capable of recognizing three specific substrates in two successive reactions:

$$E + ATP + \text{amino acid} \rightleftharpoons E \cdot \text{aminoacyl adenylate} + PP_i$$
$$E \cdot \text{aminoacyl adenylate} + tRNA \rightleftharpoons E + \text{aminoacyl-tRNA} + AMP$$

Abbreviations: CRS-1, CRS-2 and CRS-3: hydroxyapatite chromatographic cysteinyl-tRNA synthetase fractions 1, 2 and 3 respectively; SDS: sodium dodecyl sulfate; R_M: relative mobility.

A large number of recent reviews [1—9] stress the important role played by these enzymes in cell metabolism, and so considerable effort has been devoted to the purification of individual synthetases for catalytic and structural studies. However, most investigations reported were concerned with the enzymes from microorganisms [8—11]. Therefore, we have initiated a study of the mammalian enzymes with rat liver methionyl-tRNA synthetase [12—15]. The major purpose of the present study was to make available a highly purified cysteinyl-tRNA synthetase (L-cysteine:$tRNA^{Cys}$ ligase (AMP-forming), EC 6.1.1.16) from rat liver for specificity and kinetic studies. In this communication, we wish to present the purification and subunit structure of this enzyme. The purified enzyme appears to be homogeneous according to polyacrylamide gel disc electrophoresis. The multiple oligomeric forms of the enzyme, consisting of subunits of about 120 000 daltons, were resolved by the column chromatography on hydroxyapatite in presence of cysteine. A preliminary report of a part of the present work has appeared [16]. The studies on the specificity and kinetic properties will be reported in a succeeding paper. A partial purification and characterization of cysteinyl-tRNA synthetase from yeast has been published [17].

Materials and Methods

Materials. Livers of adult Sprague-Dawley rats were used as enzyme source. Distilled water was used after being filtered through a research grade ion-exchange demineralizer. Radioactive chemicals were obtained from the Radiochemical Centre. Ammonium sulfate and sucrose were of enzyme grade and from Schwarz/Mann. Chemicals for gel electrophoresis and hydroxyapatite (Bio-gel HTP) were purchased from Bio-Rad; DEAE-cellulose (type DE 52) from Reeve Angel Co.; Sephadex G-200 and Sepharose 6B from Pharmacia; glycerol from E. Merck; and ATP, $PhMeSO_2F$, dithioerythritol and protein standards from Sigma. All other chemicals used were of reagent grade and purchased either from Sigma or E. Merck Co.

Buffers. Tris/sucrose buffer contained 10 mM Tris · HCl (pH 7.5 at 25°C), 0.25 M sucrose, 0.1% Brij 58, 5 mM $MgAc_2$, 0.2 mM EDTA, 0.2 mM $PhMeSO_2F$ and 0.5 mM dithioerythritol. Phosphate buffers were designated as KP-15-7, KP-50-7.5, etc., containing 15 mM potassium phosphate (pH 7.0), 50 mM potassium phosphate (pH 7.5), etc. In addition, all phosphate buffers contained 0.2 mM $PhMeSO_2F$, 0.5 mM dithioerythritol, 3 mM L-cysteine and 20% glycerol.

Enzyme assay. Activity of the enzyme was routinely measured by cysteine-dependent exchange of $[^{32}P]PP_i$ into ATP. The final 0.5-ml reaction mixture contained 100 mM Tris-HCl (pH 7.5 at 37°), 3 mM ATP. Na_2 (neutralized to pH 7.5), 10 mM $MgAc_2$, 10 mM L-cysteine, 2 mM $^{32}PP_i$ (pH 7.5, 5—20 · 10^4 cpm), 0.5 mM dithioerythritol, 50 mM KF (for Fractions 1—4 enzymes only), 0.25 mg of dialyzed gelatin (for Fractions 5—6 enzymes), and limiting amount of enzyme. The reaction was continued at 37°C for 15 min and stopped by the addition of 0.2 ml of 7% (w/v) $HClO_4$, followed by 2 ml of water, 0.3 ml of an aqueous suspension of Norit A (50 mg/ml) and 1 drop of 1% Triton X-100. The

mixture was thoroughly mixed and allowed to stand for 10 min. It was then filtered through a Whatman 3MM paper disc (23 mm) and washed with water. The disc was dried and counted in 4 ml of a toluene-based scintillation medium containing 0.49% PPO and 0.01% POPOP. The assay was linear with time (up to 20 min) and enzyme concentration (0.2 to 3 units), until about 5% of the radioactivity from PP_i was exchanged into ATP. One unit of enzyme catalyzes the incorporation of 1 nmol of $^{32}PP_i$ into ATP in 1 min at 37° C. Other amino-acyl-tRNA synthetases were assayed by the same procedure using other amino acids in place of cysteine. Cysteinyl-tRNA formation assay was performed according to the method of Waterson et al. [18].

Protein determination. The concentration of protein at different stages in purification of the enzyme was determined by the Lowry method [19] after precipitation with 10% trichloroacetic acid and solubilized in 0.5 ml of 0.2 M NaOH, since various components in our buffer systems more or less interfered with a direct determination by all the usual methods. Crystalline bovine serum albumin was used as standard.

Polyacrylamide gel disc electrophoresis. The examination of purity of the purified enzyme on polyacrylamide gels was performed in a Tris/glycine system [20] in a Buchler polyanalyst apparatus with the standard 7.5% acrylamide separating gel. Samples (50—200 µl) containing 0.2—50 µg of protein with 20% glycerol were layered over a 2.5% stacking gel. A current of 2 mA/gel was applied until the Bromophenol Blue marker reached the bottom of the gels. The gels were cooled by circulating ice-water during the run, which was performed in a coldroom. After electrophoresis the gels were stained with 0.02% Coomassie Blue R-250 in 50% trichloroacetic acid and diffusion-destained by soaking in 7% acetic acid. A band of 0.2—0.4 µg of protein was visible by this method.

For the identification of cysteinyl-tRNA synthetase activity, an unstained gel was sliced into about 25 segments after electrophoresis and extracted by grinding the segments in Buffer KP-50-7. The supernatant liquid after centrifugation was assayed by the ATP-PP_i exchange reaction as described above.

The same procedure was followed for the estimation of mol. wt. of the enzyme from electrophoretic mobilities on polyacrylamide gels of various porosities. Separating gels containing 5—10% acrylamide were used. Beef liver catalase (M_r 240 000), yeast alcohol dehydrogenase (M_r 151 000), conalbumin (M_r 77 000) and ovalbumin (M_r 43 000) were used as standards. The results were analyzed according to the procedure of Hedrick and Smith [21]. A relationship was found between the mol. wt. of a protein and the slope of a log R_M vs. gel concentration plot (Ferguson plot, ref. 22).

SDS polyacrylamide gel electrophoresis was carried out according to the method of Weber et al. [23]. Ovalbumin, serum albumin, catalase, human transferrin, bovine hemoglobin and rabbit muscle phosphorylase a were used as mol. wt. markers. Denaturation of samples and standards was performed by heating in a solution of 1% mercaptoethanol and 1% SDS at 100° C for 3 min. Staining and destaining of the gels were done as described above. In some experiments any sulfhydryl groups present in the enzyme were blocked after the reduction by the addition of iodoacetamide to a final concentration of 0.12 M followed by incubation in the dark for 30 min at room temperature.

Determination of the apparent molecular weight by gel-filtration. A column of Sephadex G-200 (1.5 × 85 cm) was equilibrated and eluted with 20 mM Tris · HCl buffer (pH 7.5 at 25° C) containing 20% glycerol, 0.5 mM $PhMeSO_2F$ and 3 mM cysteine. The column was calibrated with Dextran Blue 2000, catalase (apparent M_r 195 000, adjusted [24] from 240 000), alcohol dehydrogenase (M_r 151 000), serum albumin (M_r 67 000), hemoglobin (M_r 64 000), chymotrypsinogen A (M_r 25 000), and horse cytochrome *c* (M_r 12 400). The samples were applied in a total volume of 1 ml. Fractions of 1 ml were collected at a rate of about 5 ml/h. The elution positions of the proteins and Dextran Blue were determined either by assaying enzyme activity (cysteinyl-tRNA synthetase, catalase and alcohol dehydrogenase) or by measuring absorbance (albumin and chymotrypsinogen at 280 nm, cytochrome *c* at 415 nm, and Dextran Blue at 620 nm). Dextran Blue emerged at 50 ml. The mol. wt. of cysteinyl-tRNA synthetase was obtained by interpolation according to the method of Andrews [24].

Sucrose gradient centrifugation. Determination of the mol. wt. of the enzyme by sucrose density gradient centrifugation was conducted by the method of Martin and Ames [25] with catalase as reference to provide a relation between mol. wt. and distance travelled. Both the sucrose and protein solutions contained 50 mM potassium phosphate (pH 7), 0.5 mM dithioerythritol, 0.2 mM $PhMeSo_2F$ and 3 mM cysteine. Samples (0.3 ml per tube) were layered with Beckman band-forming caps on a 5—20% sucrose linear gradient (5.1 ml) formed with a Beckman gradient former. The tubes were centrifuged at 4° C and 39 000 rev./min for 15 h in a Spinco model L ultracentrifuge with rotor SW50. The polyallomer tubes were then punctured and fractions of about 0.13 ml were collected and assayed for enzyme activities.

Active site titration [26]. Tris · HCl (0.3 M, pH 7.8 at 25° C), [r-^{32}P]ATP (15 μM, 160 000 cpm/nmol), $MgAc_2$ (30 mM), L-cysteine (3 mM), yeast inorganic pyrophosphatase (7.5 U/ml) and dithioerythritol (1.5 mM) were mixed in a total volume of 80 μl at 25° C. Triplicate aliquots of 10 μl (for zero time readings) were separately mixed with 0.1 ml of 7% $HClO_4$ followed by 0.2 ml of a 2% suspension of Norit A. Cysteinyl-tRNA synthetase (0.375 nmol in 0.1 ml) was added to the remaining reaction mixture and aliquots of 10 μl were periodically taken and quenched as above. The quenched samples were filtered through paper discs, washed and counted as described for the assay of cysteinyl-tRNA synthease activity.

Purification of the enzyme. All steps in the purification were performed at 0—5° C, unless otherwise stated.

Postmitochondrial supernatant. Fresh livers (250 g) were homogenized with 3 vols. of the Tris/sucrose buffer in a Waring blendor at full speed for 2 min (with 1-min intermittent cooling). The homogenate was centrifuged at 15 000 × *g* for 30 min, and the supernatant (Fraction 1) was used for subsequent steps.

Heat treatment. Fraction 1 (850 ml) was mixed with 1/39 vol. of 120 mM L-cysteine and 10 mM dithioerythritol, and heated in two batches at 50° C for 25 min. The mixture was cooled quickly to 5° C and centrifuged for 30 min at 15 000 × *g* and the supernatant (Fraction 2) was taken up.

Ammonium sulfate fractionation. Fraction 2 (615 ml) was mixed with 1/79

vol. of 120 mM L-cysteine and 10 mM dithioerythritol. Ammonium sulfate was added slowly with stirring to give 35% saturation. The mixture was allowed to stand for 15 min with stirring and then centrifuged at 15 000 × *g* for 20 min. The supernatant was brought to 58% saturation by the addition of more ammonium sulfate, and the suspension was stirred for 15 min and centrifuged. The precipitate was taken up in 15 ml of KP-15-7 buffer, dialyzed for 3 h against 1 l of the same buffer, and centrifuged (Fraction 3).

Sepharose 6B gel filtration. Fraction 3 (43 ml) was applied to a Sepharose 6B column (5 × 88 cm) equilibrated with KP-15-7 buffer. The enzyme was eluted with the same buffer. The flow rate was 55 ml/h and fractions of 20 ml were collected. The fractions with the highest specific activity were pooled (Fraction 4).

DEAE-cellulose chromatography. Fraction 4 (218 ml) was applied to a DE-52 column (5 × 38 cm) equilibrated with KP-15-7 buffer. The column was washed and the enzyme eluted with a non-linear gradient of potassium phosphate from KP-15-7 to KP-100-7 (total volume, 2.8 l) formed with a mixing system comprising 7 chambers. The chambers 1—3 contained KP-15-7. The chambers 4—7 contained KP-25-7, KP-50-7, KP-75-7 and KP-100-7, resp. The flow rate was 150 ml/h and fractions of 20 ml were collected. The enzyme activity peak appeared at 28 mM phosphate. The fractions containing the highest specific activity were pooled (Fraction 5) for the next step.

Hydroxyapatite chromatography. Fraction 5 (420 ml) was applied to a hydroxyapatite column (2.5 × 6 cm) equilibrated with KP-50-7 buffer. The enzyme was eluted with a linear gradient of potassium phosphate from 50 to 350 mM formed with 160 ml each of KP-50-7.5 and KP-350-7.5 buffers. The flow rate was 20 ml/h. Fractions of 3.5 ml were collected. Three well separated activity peaks appeared at about 105, 180 and 250 mM buffer, respectively. The elution profile is shown in Fig. 1.

Results

Purification of the enzyme

Table I summarizes the purification procedure for the cysteinyl-tRNA synthetase from rat liver. An 880—1680-fold purification was achieved with a 23% overall yield. The enzyme activity was resolved into three well separated fractions at the final stage of purification by hydroxyapatite chromatography in the presence of cysteine. They are designated as fractions CRS-1, CRS-2 and CRS-3, respectively, as shown in Fig. 1. The results were reproducible with only slight variation between different experiments in the total activity ratio. The ratio calculated from the data in Table I was 0.7 : 2 : 1. In the cysteine activation reaction as measured by the ATP-PP_i exchange assay, the calculated turnover number (in mol of $^{32}PP_i$ per mol of enzyme per min) of CRS-2 is about 400, and that of CRS-3 is 700—1000, based on a molecular weight of 240 000. The corresponding value for the yeast cysteinyl-tRNA synthetase was 200 [9] and those for other aminoacyl-tRNA synthetases ranged from 15 to 13 000 [9].

The present sequence of procedure enable us to complete the purification without intermediate concentration and prolonged dialysis step. Heating of the crude extract (Step 2) was effective in reducing the high level of endogenous

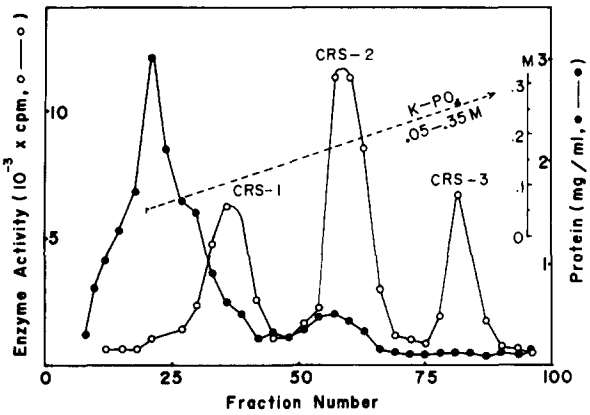

Fig. 1. Elution diagram of rat liver cysteinyl-tRNA synthetase from a hydroxyapatite column (2.5 × 6 cm).

synthetase activity as measured by the ATP-PP_i exchange without any added amino acid. At an early stage of our investigation, we tried to purify the enzyme without the addition of $PhMeSO_4F$ and cysteine in the buffers. The overall recovery of enzyme activity was low and variable, ranging from 3 to 10%.

Purity of the enzyme

The purified enzyme (Fractions CRS-2 and CRS-3) was tested for the presence of other aminoacyl-tRNA synthetases by the ATP-PP_i exchange assay. We found no detectable activity for 19 other coded amino acids. Homogeneity of the purified enzyme was checked by polyacrylamide gel disc electrophoresis. Fig. 2 compares the electrophoresis patterns of the three active fractions

TABLE I

PURIFICATION OF THE CYSTEINYL-tRNA SYNTHETASE FROM RAT LIVER

Starting material was 250 g rat liver.

Step	Volume (ml)	Total protein (mg)	ATP-PP_i exchange		Yield (%)
			Total activity (units)	Specific activity (units/mg)	
1 Postmitochondrial supernatant	850	32 800	121 000	3.7	100
2 Heating with cysteine (50° C, 25 min)	615	12 300	84 600	6.9	70
3 Ammonium sulfate 35—58% saturation	43	4 250	49 500	11.6	41
4 Sepharose 6B	218	1 350	58 200	43	48
5 DEAE-cellulose	420	117	41 600	356	34
6 Hydroxyapatite					
Peak I (CRS-1)	25	4.6	5 400	1200	
Peak II (CRS-2)	32	4.5	14 800	3290	33
Peak III (CRS-3)	32	1.2	7 500	6250	

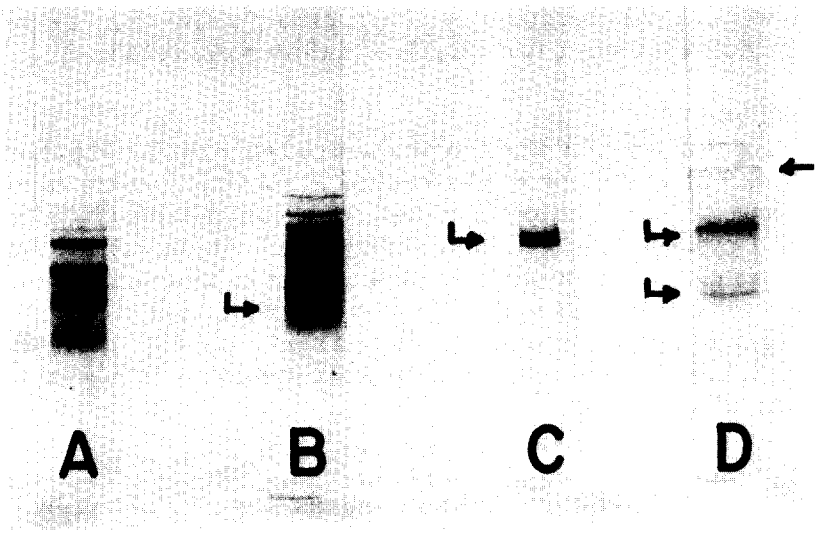

Fig. 2. Polyacrylamide gel electrophoresis of various fractions of rat liver cysteinyl-tRNA synthetase. (A) DEAE-cellulose fraction, (B) CRS-1, (C) CRS-2, (D) CRS-3. The bands with the enzyme activity are marked by the arrows ↳. The origin of migration is indicated by ←.

obtained from the hydroxyapatite column. The major form (CRS-2) migrated as a single protein band. An assay for cysteinyl-tRNA synthetase activity across an unstained gel gave one peak of activity at the position of the protein band. CRS-3 gave two bands, both being enzymatically active. The mobilities of these two bands were similar to those of the active bands of CRS-1 and CRS-2. On the other hand, CRS-1 gave four major bands, a pattern similar to that obtained with the DEAE-cellulose fraction. Among the bands from CRS-1, only the one

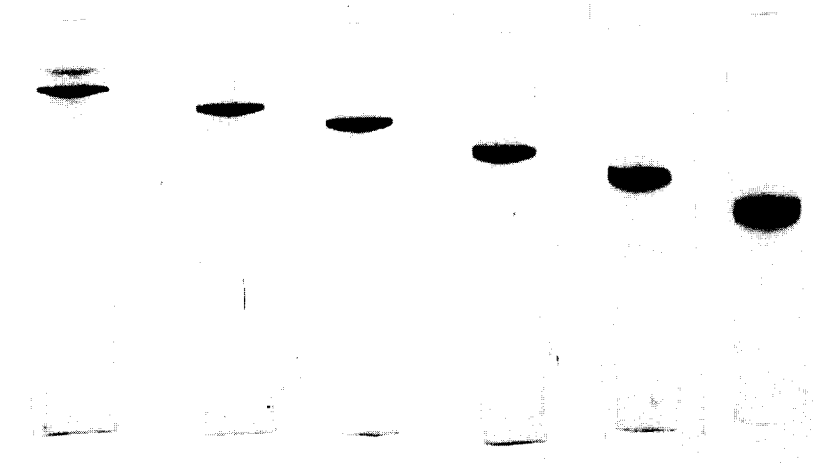

Fig. 3. Electrophoretic mobilities of rat liver cysteinyl-tRNA synthetase (CRS-2) on polyacrylamide gels of various porosities. Protein load: 22 μg. Gel concentrations: 5—10% (from right to left).

with the highest mobility was found to be enzymatically active (Fig. 2). In addition, CRS-2 migrated as a single band in gels of various porosity (Fig. 3).

Stability of the enzyme

Storage of the purified cysteinyl-tRNA synthetase (50 μg/ml in 0.1 M potassium phosphate, pH 7.5) at 4°C for 6 days resulted in about 70% decrease in the ATP-PP_i exchange activity. The loss in activity was greatly retarded by 50% glycerol or 3 mM L-cysteine plus 0.5 mM dithioerythritol. About 90% of the original activity remained in the presence of these protecting agents. However, glycerol was much less effective than cysteine plus dithioerythritol in protecting the enzyme against heat inactivation (50°C, 30 min). ATP-Mg^{2+} provided little stabilization. Glycerol did not stabilize the yeast cysteinyl-tRNA synthetase [17] during storage.

Specificity of the enzyme

The specificity studies showed that the substrate binding sites of this liver enzyme are highly specific. None of the various analogues tested could replace L-cysteine or ATP as substrate in the ATP-PP_i exchange reaction. Cysteamine and *S*-methyl-L-cysteine were competitive inhibitors with respect to cysteine. 2'-Deoxy-ATP, α,β-CH_2-ATP and sodium tripolyphosphate were competitive inhibitors of ATP. Other compounds examined were inactive or acted as weak, noncompetitive inhibitors. The details will be reported in a succeeding paper.

Molecular weight and subunit structure

The mol. wt. of the major form (CRS-2) of the purified enzyme was studied by three different methods. Studies on the behavior during gel-filtration on Sephadex G-200 obtained a value of 230 000. By sucrose gradient centrifugation, a sedimentation coefficient of 7.4 S was found for the enzymes, corresponding to a mol. wt. of 127 000 (between 115 000 and 140 000 in other experiments), taking catalase (s = 11.3 S, mol. wt. = 240 000) as reference. On the basis of electrophoretic mobilities on the polyacrylamide gels of various

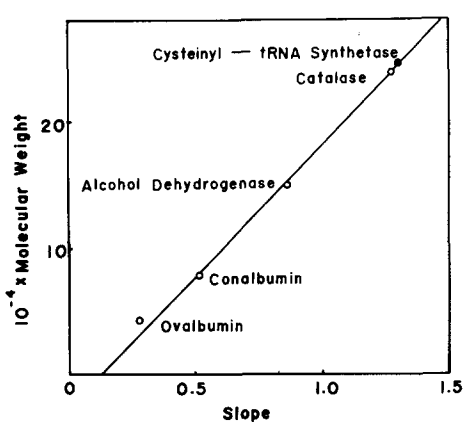

Fig. 4. Determination of molecular weight of rat liver cysteinyl-tRNA synthetase (CRS-2) by polyacrylamide gel electrophoresis with varying concentrations of acrylamide. The slopes for different proteins were obtained from the plots of log R_M vs. gel concentration.

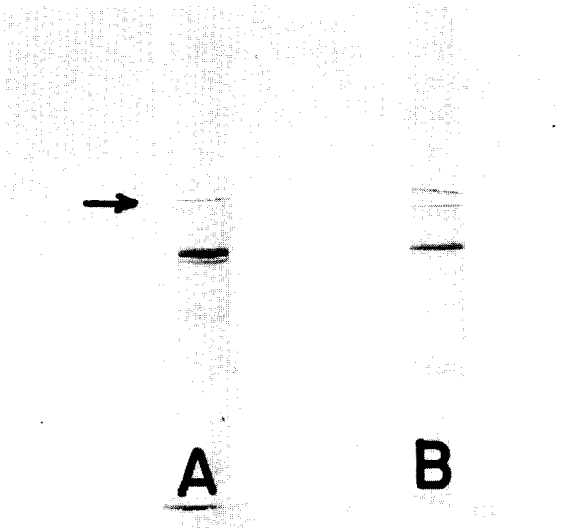

Fig. 5. SDS polyacrylamide gel electrophoresis patterns of rat liver cysteinyl-tRNA synthetase: (A) CRS-3, (B) CRS-2. The origin of migration is marked by the arrow.

porosities, an estimate of 240 000 for the mol. wt. of CRS-2 could be derived from Fig. 4.

The mol. wt. of the subunits of the enzyme was determined by polyacrylamide gel electrophoresis after reduction and denaturation in the presence of SDS and mercaptoethanol. From freshly prepared, homogeneous enzyme (CRS-2), a major band of protein was obtained after electrophoresis in either 7.5 or 10% gel (Fig. 5). No alteration in the mobility of the band could be detected after reduction and carboxymethylation of the protein. Comparisons of the mobility of the enzyme with those of several proteins of known size (Fig. 6) yielded an estimate of 120 000 for the mol. wt. of the subunits of the enzyme. These results would suggests that CRS-2 is a dimer composed of probably identical subunits of about 120 000 daltons.

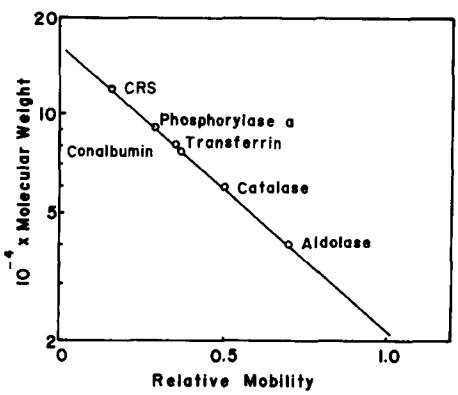

Fig. 6. Molecular weight determination of the subunit of rat liver cysteinyl-tRNA synthetase (CRS) by SDS polyacrylamide gel electrophoresis.

The multiple forms of the enzyme

As mentioned in the Purification section, rat liver cysteinyl-tRNA synthetase activity was resolved into three well separated peaks by hydroxyapatite chromatography in the presence of cysteine. All the three fractions (CRS-1, CRS-2 and CRS-3 in Fig. 1) were active both in the ATP-PP_i exchange and cysteinyl-tRNA formation reactions. They were all derived from the DEAE-cellulose fraction. The DEAE-cellulose fraction as well as CRS-1 and CRS-2 exhibited an apparent mol. wt. of 230 000—240 000 by Sephadex G-200 gel-filtration, when the column was equilibrated and eluted with KP-10-7 buffer. When CRS-1 was dialyzed against KP-350-7.5 buffer and then subjected to gel filtration with the same buffer, it gave an elution volume corresponding to a mol. wt. of 122 000. On the other hand, CRS-3 showed an apparent mol. wt. of about 300 000 and 270 000 with buffers KP-10-7 and KP-350-7.5, respectively. By sucrose gradient centrifugation, CRS-3, as well as CRS-2, showed a mol. wt. of 110 000—140 000 in different experiments. By SDS polyacrylamide gel electrophoresis, freshly prepared CRS-3 gave a major band of protein with a mobility apparently identical with that obtained from CRS-2 under the same conditions (Fig. 5). Furthermore, as mentioned in the Purity section, CRS-3 gave two active bands with mobilities corresponding to those of the active bands of CRS-1 and CRS-2 after gel electrophoresis without SDS.

These results indicate that the multiple forms of rat liver cysteinyl-tRNA synthetase observed in vitro are oligomeric isomers. CRS-1 and CRS-2 may be represented as α and α_2, respectively, while CRS-3 could be α_3 or α_4 which dissociates readily during the molecular weight determination by various methods.

Besides the chromatographic differences between CRS-1, CRS-2 and CRS-3, we could not find any significant difference in the K_m values for ATP and cysteine. The specific activity (Table I) of CRS-3 was almost 100% higher than that of CRS-2. Although the specific activity of CRS-1 as isolated was lower than that of CRS-2, the turnover number of CRS-1 could be at least comparable to that of CRS-2, if the purity of CRS-1 (Fig. 2) is taken into consideration.

Formation of cysteinyl adenylate

The usefulness of equilibrium dialysis was limited in our case by the instabil-

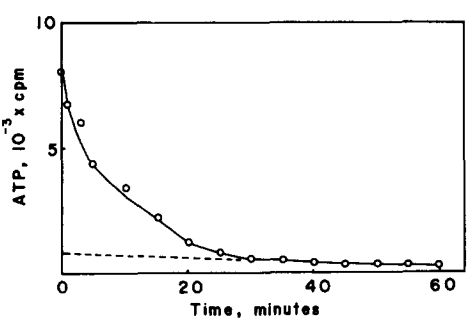

Fig. 7. Formation of cysteinyl adenylate from L-cysteine (1 mM) [r-^{32}P]ATP (5 μM) and rat liver cysteinyl-tRNA synthetase (CRS-2, 2.5 μM) at 25°C, pH 7.8, in the presence of inorganic pyrophosphatase (2.5 units/ml), dithioerythritol (0.5 mM) and $MgAc_2$ (10 mM).

ity of the enzyme under study. Partial denaturation led to inaccurate and non-reproducible quantitative results. Therefore we determined the stoichiometry of cysteinyl-adenylate formation and binding and calculated the number of catalytically competent active site according to a recently published method of "active site titration" for aminoacyl-tRNA synthetases [26]. From the results presented in Fig. 7, an n value of 1.9 was obtained, indicating that the dimeric cysteinyl-tRNA synthetase forms two cysteinyl adenylate per molecule. Thus it may be reasonable to assume that each subunit of the rat liver cysteinyl-tRNA synthetase has one active site.

Discussion

The present study describes a rather rapid procedure for the purification of cysteinyl-tRNA synthetase from a mammalian source in 23% overall yield. The purified enzyme contained no other detectable aminoacyl-tRNA synthetase activity and appears to be homogeneous by polyacrylamide gel disc electrophoresis (Figs. 2 and 3). Under our experimental conditions, 0.2—0.4 μg of protein could give a clearly visible band, while 20 μg or more of CRS-2 migrated as a single band in gels of various porosity. This would indicate a minimum purity of 98—99%. CRS-3 could also be considered as 'pure' preparation since it gave two protein bands after gel electrophoresis but both bands showed cysteinyl-tRNA synthetase activity. To our knowledge, it is the first cysteinyl-tRNA synthetase ever purified to apparent homogeneity. The only other cysteinyl-tRNA synthetase available in a highly purified state was from bakers' yeast [17] which was reported to be 70—75% pure.

Classical preparative procedures assumed that the majority of aminoacyl-tRNA synthetases are small and cytoplasmically localized as has been found in prokaryotic systems [9]. However, the presence of these synthetases together with some tRNA in multienzyme complexes of high-molecular weight in rat [27—29] and mouse [30] liver has been established. During the present study, we also observed that the cysteinyl-tRNA synthetase activity of our Fraction 3 was eluted from Sephadex G-200 column in the void volume. Therefore, we have avoided a high-speed centrifugation of the liver extract as the first step of enzyme purification and used Sepharose 6B instead of Sephadex G-200 in the gel-filtration step.

The polypeptide chains of aminoacyl-tRNA synthetases from various organisms, with few exceptions, are composed of about either 450 or 900 amino-acid residues per chain. They are usually isolated as monomeric proteins (α) of roughly 100 000 daltons, or as dimers (α_2 or $\alpha\beta$) or tetramers ($\alpha_2\beta_2$) of about 50 000 or 100 000 daltons per subunit [8—10]. The mol. wts. of cysteinyl-tRNA synthetase from *Escherichia coli* [31] and yeast [17] have been reported to be 44 000 and 160 000, respectively. The data of our present study indicate that the major form of the purified cysteinyl-tRNA synthetase from rat liver is a dimer composed of probably identical subunits of about 120 000 daltons, and therefore has a quaternary structure of the α_2 type. This conclusion is further supported by the stoichiometric study of cysteinyl adenylate formation and the behavior of the multiple forms of the enzyme resulted from the chromatography on hydroxyapatite as described in the Results section. However, further

studies are required to establish the identity of the two subunits of identical or similar mass.

Regulatory enzymes are usually allosteric proteins, and the allosteric control mechanism is closely related to the subunit structure of the protein. Therefore, the first step of the procedure required for evaluation of an allosteric enzyme is to purify the protein and determine the numbers and kinds of subunits [32]. A comprehensive list of the subunit structure of over 300 proteins has been recently published [33].

By the so-called "active site titration" method for aminoacyl-tRNA synthetases [26], our data (Fig. 7) suggest two active sites for the dimeric form of rat liver cysteinyl-tRNA synthetase. For other aminoacyl-tRNA synthetases there seems to be no common relation between the subunit composition and the number of substrate-binding sites or active sites. Reports ranged from the simple pattern of one site per subunit to one per tetramer [9,34]. It is of special interest to note that tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* consists of two identical subunits but binds only a single molecule of tyrosine or tyrosyl adenylate per dimer [35].

The present study demonstrates that rat liver cysteinyl-tRNA synthetase can function in multiple molecular forms at least in vitro, although the physiological significance of this phenomenon is not clear. The possible artifactural cause of multiforms can not be ruled out. However, we are not able to attribute the formation of the high-molecular-weight CRS-3 to the heat treatment and presence of substrate involved in the purification procedure, since the enzyme was heated at step 2 and the substrate cysteine was present in buffers used from steps 2 through 5 as well as the final hydroxyapatite step. Yet, the Fraction 5 enzyme (SEAE-cellulose eluate) exhibited a mol. wt. of 240 000 by gel-filtration, as CRS-2 did. Since cysteinyl-tRNA synthetase activity was eluted from Sephadex G-200 column at void volume before DEAE-cellulose step, the enzyme probably exists in vivo in a high-molecular-weight complex. Then it may be irrelevant to argue whether CRS-2 or CRS-3 is the "native" form in vivo. The interpretation that three forms of CRS as isolated from the hydroxyapatite column are oligomeric isomers is consistent with the following observations: (a) CRS-2 consists of two subunits of about 120 000 daltons; (b) after gel electrophoresis in the absence of sodium dodecyl sulfate, CRS-3 gave two enzymatically active bands with mobilities corresponding to those of the active bands of CRS-1 and CRS-2, respectively; (c) The electrophoretic mobilities of CRS-2 and CRS-3 in the presence of sodium dodecyl sulfate were indistinguishable, suggesting that they were composed of the same kind of subunit; and (d) CRS-1 showed an apparent mol. wt. of 122 000 or 230 000 by gel-filtration depending on the ionic strength. The apparent mol. wt. of 270 000—300 000 for CRS-3 could result from a partial dissociation during the gel-filtration. The low molecular weights of CRS-2 and CRS-3 shown by sucrose-gradient centrifugation may also reflect a dissociation into subunits. The dissociation of aminoacyl-tRNA synthetases into subunits was also encountered previously when the rat liver phenylalanyl-tRNA synthetase [36] and *E. coli* prolyl-tRNA synthetase [37] were subjected to ultracentrifugation, while the yeast cysteinyl-tRNA synthetase showed aggregation during sedimentation [17]. The poor reproducibility of the kinetic data sometimes we observed with different preparations of

rat liver cysteinyl-tRNA synthetase or with the same preparation after storage of various period of time may be attributable to the existence of and interchange among the multiple oligomeric states of the enzyme.

Acknowledgment

This investigation was supported in part by the National Science Council, Taiwan (Project No. NSC-63B-0412-06-02).

References

1 Soll, D. and Schimmel, P.R. (1974) in The Enzymes (Boyer, P.D., ed.), Vol. 10, Part A, pp. 489—538, Academic Press, New York
2 Kisselev, L.L. and Favorova, O.O. (1974) Adv. Enzymol. Relat. Subj. Biochem. 40, 141—238
3 Mehler, A.H. (1971) Methods Enzymol. 20, 203—210
4 Mehler, A.H. and Chakraburtty, K. (1971) Adv. Enzymol. Relat. Subj. Biochem. 35, 443—501
5 Mehler, A.H. (1970) Prog. Nucl. Acid Res. Mol. Biol. 10, 1—22
6 Muench, K.H. (1971) Methods Mol. Biol. 1, 215
7 Novelli, G.D. (1967) Annu. Rev. Biochem. 36, 449—484
8 Neidhardt, F.C., Parker, J. and McKeever, W.G. (1975) Annu. Rev. Microbiol. 29, 215—250
9 Loftfield, R.B. (1972) Prog. Nucl. Acid Res. Mol. Biol. 12, 87—128
10 Penneys, N.S. and Muench, K.H. (1974) Biochemistry 13, 560—565
11 Goto, T. and Schweiger, A. (1973) Hoppe-Seyler's Z. Physiol. Chem. 354, 1027—1033
12 Pan, F. and Chang, G.G. (1971) Bull. Sun Yat-Sen Cult. Found. 8, 87—95
13 Chang, G.G. and Pan, F. (1972) J. Chin. Biochem. Soc. 1, 1—12
14 Chang, G.G., Lin, T.Z., Yu, T.C., Guoo, J.Y. and Pan, F. (1975) J. Chin. Biochem. Soc. 4, 10—16
15 Pan, F., Guoo, J.Y. and Yu, T.C. (1975) J. Chin. Biochem. Soc. 4, 17—23
16 Pan, F. and Duh, G.M. (1974) Bull. Sun Yat-Sen Cult Found. 13, 241—258
17 James, H.L. and Bucovaz, E.T. (1969) J. Biol. Chem. 244, 3210—3216
18 Watterson, J.R., Winter, W.P. and Schmickel, R.D. (1974) J. Clin. Invest. 54, 182—187
19 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
20 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
21 Hedrick, J.L. and Smith, A.J. (1968) Arch. Biochem. Biophys. 126, 155—164
22 Ferguson, K.A. (1964) Metab. Clin. Exp. 13, 985—1002
23 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
24 Andrews, P. (1965) Biochem. J. 96, 595—606
25 Martin, R.G. and Ames, B.N. (1961) J. Biol. Chem. 236, 1372—1379
26 Fersht, A.R., Ashford, J.S., Bruton, C.J., Jakes, R., Koch, G.L.E. and Hartley, B.S. (1975) Biochemistry 14, 1—4
27 Tscherne, J.S., Weinstein, I.B., Lanks, K.W., Gersten, N.B. and Cantor, C.R. (1973) Biochemistry 12, 3859—3865
28 Bandyopadhyay, A.K. and Deutscher, M.P. (1971) J. Mol. Biol. 60, 113—122
29 Vennegoor, C. and Bloemendal, H. (1972) Eur. J. Biochem. 26, 462—473
30 Berg, B.H. (1975) Biochim. Biophys. Acta 395, 164—172
31 Fayat, G., Blanquet, S., Dessen, P., Batelier, G. and Waller, J.P. (1974) Biochimie 56, 35—41
32 Koshland, D.E., Jr. (1970) in The Enzymes (Boyer, P.D., ed.), Vol. 1, pp. 341—396, Academic Press, New York
33 Klotz, I.M., Darnall, D.W. and Langerman, N.R. (1975) in The Proteins (Neurath, H. and Hill, R.L., eds.), Vol. 1, pp. 294—411, Academic Press, New York
34 Fasiolo, F. and Ebel, J.P. (1974) Eur. J. Biochem. 49, 257—263
35 Bosshard, H.R., Koch, G.L.E. and Hartley, B.S. (1975) Eur. J. Biochem. 53, 493—498
36 Tscherne, J.S., Lanks, K.W., Salim, P.D., Grunberger, D., Cantor, C.R. and Weinstein, I.B. (1973) J. Biol. Chem. 248, 4052—4059
37 Lee, M.I. and Muench, K.H. (1969) J. Biol. Chem. 244, 223—230