J. Biochem. 120, 869-880 (1996)

Further Study on Association of 5SrRNA-L5 Protein Complex and Methionyl-tRNA to Methionyl-tRNA Synthetase in the Macromolecular Aminoacyl-tRNA Synthetase Complex

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Received for publication, March 4, 1996

To obtain direct evidence for the attachment of 5SrRNA-ribosomal L5 protein particles (5SRNP) and methionine-tRNA ($tRNA^{met}$) to methionyl-tRNA synthetase (MetRS) in the macromolecular aminoacyl-tRNA synthetase (ARS) complex of rat liver, a MetRS-5SRNP-$tRNA^{met}$ complex was dissociated from the macromolecular ARS complex fraction by n-octyl-β-D-glucoside (Method I) or by ω-aminooctyl agarose (Method II) chromatography. The dissociated MetRS complex fraction was purified by gel filtration followed by tRNA-Sepharose chromatography using partially purified $tRNA^{met}$ in Method I, and by hydrophobic interaction chromatography in Method II. In both methods, final Superdex™200 chromatography showed that MetRS activity was present in the region corresponding to the molecular weight of the MetRS-5SRNP-$tRNA^{met}$ complex (M_r 200,000). One main protein band corresponding to the molecular weight of MetRS was observed on SDS-PAGE of the final product, which was concentrated by lyophilizing after dialysis against water. Using serum albumin as an inhibitor of adhesion of L5 to the microconcentrators which was used to concentrate the final product, a distinct L5 band was detected on SDS-PAGE, the intensity of which was comparable to that of the MetRS band. Northern blot analysis of RNA prepared from the tRNA-Sepharose fraction showed the presence of 5SrRNA. Dot blot analysis using an antibody against ribosomal protein L5 showed that L5 was present in the Superdex™200 fractions prepared by both methods. The MetRS specific activities in MetRS complex fractions incubated without tRNA increased during the purification procedures, indicating that endogeneous $tRNA^{met}$ exists stably in the MetRS complex. 5SRNP and 5SrRNA markedly enhanced the MetRS activity in the MetRS complex, indicating that 5SRNP(A) plays a role as a positive effector of MetRS.

Key words: methionyl-tRNA, methionyl-tRNA synthetase, ribosomal protein L5, 5S ribosomal RNA, 5SrRNA-L5 complex.

In higher eukaryotic cells, nine ARSs are found in the form of a complex with a molecular weight of about 1.1×10^6. Of these nine, only AspRS and ArgRS are found in cell cytosol, not solely as a macromolecular complex, but also in the free form. The seven other ARSs exist exclusively in the form of multi-ARS complex (see review Ref. *1*).

We recently reported that rat liver cytosolic 5SrRNA has the same 5′- and 3′-terminal sequences as ribosomal, nucleolar and nucleoplasmic 5SrRNA (*2*). The time courses of changes in the specific activities of these four species of 5SrRNA of rat liver from 20 min to 16 h after administration of [^{14}C]orotic acid to rats indicated that cytosolic 5SrRNA has a very high metabolic rate comparable to heterogenous nuclear RNA and is thus not the precursor of ribosomal 5SrRNA (*2*).

We showed that a macromolecular ARS complex containing nine ARSs purified from rat liver cytosol contained 5SRNP (*3*). A ThrRS and HisRS complex also contained 5SRNP (*4*). 5SRNP and 5SrRNA definitely enhanced the MetRS and IleRS activities in the macromolecular ARS complex (*5*). From these results, we assumed that 5SRNP interacts with MetRS and $tRNA^{met}$ in the macromolecular ARS complex.

To verify this assumption, we incubated rat liver cytosol with a trace amount of rat liver 5SrRNA highly labeled with cytidine 3′,5′-[5′-^{32}P]bisphosphate and [^{35}S]methionine in the presence of ATP, and then with an antibody against ribosomal protein L5. The incubation mixture was subjected to protein A-Sepharose column chromatography. The fraction containing overlapping ^{32}P- and ^{35}S-radioactivities was crosslinked by UV-irradiation, and analyzed by PAGE. The results indicated that [^{35}S]methionyl-tRNA interacts with ^{32}P-labeled 5SRNP. The fraction containing overlap-

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Abbreviations: ARS, aminoacyl-tRNA synthetase; ArgRS, arginyl-tRNA synthetase; AspRS, asparyl-tRNA synthetase; GluRS, glutamyl-tRNA synthetase; IleRS, isoleucyl-tRNA synthetase; LeuRS, leucyl-tRNA synthetase; LysRS, lysyl-tRNA synthetase; MetRS, methionyl-tRNA synthetase; ProRS, prolyl-tRNA synthetase; ValRS, valyl-tRNA synthetase; MetRS complex, MetRS fraction containing 5SRNP and $tRNA^{met}$; octylglucoside, n-octyl-β-D-glucoside; PMSF, phenyl sulfonyl fluoride; 5SRNP, 5SrRNA-L5 protein complex.

ping ^{32}P- and ^{35}S-radioactivities described above was further analyzed by Sephadex G-150 column chromatography. The component containing both radioactivities was found to be distributed in the region corresponding to a molecular weight which was the sum of those of MetRS, 5SRNP, and $tRNA^{met}$ (M_r 200,000). Furthermore, this fraction showed definite MetRS activity. From these results, we assumed that 5SRNP and $tRNA^{met}$ associate with MetRS in the macromolecular ARS complex, and may be released during the preparation procedures described above (*6*).

To obtain more direct evidence for this assumption, we decided to try dissociating the MetRS complex from the macromolecular ARS complex in rat liver cytosol. We used two methods for this purpose. In Method I, octylglucoside was used as a non-ionic detergent. In Method II, ω-aminooctyl agarose chromatography was employed as a form of hydrophobic interaction chromatography (*7*). In both methods, the released fraction was further purified by several kinds of column chromatography. Final products were analyzed by gel-filtration using Superdex™200. This report describes these experiments, the results of which indicate that 5SRNP and $tRNA^{met}$ associate with MetRS in the macromolecular ARS complex, confirming our previous results (*6*).

MATERIALS AND METHODS

Materials—L-[^{35}S]Methionine (1,000 Ci/mmol) and [*U*-^{14}C]lysine (282 mCi/mmol) were obtained from Amersham International, [*U*-^{14}C]arginine (147 mCi/mmol), [*U*-^{14}C]methionine (256 mCi/mmol) from New England Nuclear, and [*U*-^{14}C]isoleucine (240 mCi/mmol), [*U*-^{14}C]-glutamic acid (200 mCi/mmol), [*U*-^{14}C]proline (267 mCi/mmol), and [*U*-^{14}C]aspartic acid (160 mCi/mmol) from Moravek Biochemical. Octylglucoside was purchased from Dojin Chemical Company (Tokyo). ω-Aminooctyl agarose was from Sigma and Bio-Beads SM-2 (20-50 mesh) were from Bio-Rad Laboratories. DEAE Sephadex A-50, Sephacryl S-300, and Superdex™200 were purchased from Pharmacia, Gigapite from Seikagaku Kohgyo (Tokyo), and baker's yeast tRNA from Boehringer. Benzoylated DEAE-cellulose was a gift from Dr. T. Ueda (Faculty of Engineering, The University of Tokyo). Other materials used were as described previously (*3, 5*).

Buffers—Medium A: 0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, and 50 mM Tris-HCl (pH 7.6). Buffer A: 10% glycerol, 5 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.6). Buffer P: 10% glycerol, 5 mM $MgCl_2$, 10 mM DTT, 10 mM K-phosphate buffer (pH 7.0).

Partial Purification of Yeast $tRNA^{met}$—Baker's yeast tRNA (0.25 g/30 ml) was subjected to DEAE-Sephadex A-50 chromatography (2.6×90 cm) by the method of Takahashi *et al.* (*9*). The tRNA fraction containing $tRNA^{met}$ was further purified by benzoylated DEAE-cellulose column chromatography (1.5×28 cm) by the method of Takahashi *et al.* (*8*). The activity of the tRNA fraction for the acceptance of amino acids was assayed in an incubation mixture (total volume, 20 μl) containing 25 mM Tris-HCl (pH 7.6), 10 mM KCl, 1 mM $MgCl_2$, 2 mM ATP, 0.02 μCi [^{14}C]amino acid, and 1 μl rat liver cytosol (*3*). Incubation was carried out at 37°C for 10 min. The radioactivity of the trichloroacetic acid-insoluble fraction was measured by the filter disk method of Cherayil *et al.* with some modifications (*10*). The relative activities of the partially purified tRNA for accepting various kinds of [^{14}C]amino acids were as follows: (Figures in parenthesis are the activities of the original yeast tRNA.) [^{14}C]methionine 100(100), [^{14}C]-arginine 27(19), [^{14}C]lysine 20(10), [^{14}C]glutamic acid 10(13), [^{14}C]aspartic acid 10(12), [^{14}C]leucine 30(112), [^{14}C]isoleucine 44(187), [^{14}C]proline 18(175). The tRNA-Sepharose was prepared with partially purified tRNA or unfractionated rabbit liver tRNA by the method of Remy *et al.* (*11*) as described previously (*3*).

Preparation of MetRS Complex—Method I, using octylglucoside: Rat liver cytosol was prepared as described previously (*3*). Usually PMSF, leupeptin, and DTT were added to Medium A at final concentrations of 0.5 mM, 4 μg per ml and 10 mM, respectively. A 12-ml aliquot was applied to a Sephacryl S-300 column (2.6×90 cm) pre-swollen with Medium A. The material was eluted with Medium A at a flow rate of 36 ml/h and 8-ml fractions were collected. Fraction I showing MetRS activity at the void volume (see Fig. 1 in Ref. *3*) was collected (about 30 ml). After concentration on Diaflow ultrafiltration membranes (Amicon Corporation) to 8 ml, 10% octylglucoside in Medium A was added to give a final concentration of 1%, followed by 2.5 M KCl (final concentration 0.2 M). The mixture was left standing at 0°C for 10 min, then Bio-Beads SM-2 were added at 0.25 g per ml and the whole was stirred gently for 90 min at 4°C. It was then applied to an ultramini column (Seikagaku Kohgyo) to remove the Bio-Beads. The material was then applied to a Sephacryl S-300 column (2.6×40 cm) and eluted with KCl-minus Medium A containing 10 mM DTT at a flow rate of 11 ml/h. Fractions of 4 ml were collected and assayed for MetRS activity and A_{280} [Fig. 1(1)]. Fractions containing high MetRS activity and distributed in the region corresponding to a molecular weight from 140,000 (lactate dehydrogenase) to 250,000 (catalase) were collected and the material was applied to a tRNA-Sepharose column prepared with partially purified $tRNA^{met}$. The column was washed with Buffer A, and the material was eluted with 0.4 M NaCl containing Buffer A at a flow rate of 36 ml/h because 0.27-0.35 M KCl eluted 5SRNP-containing macromolecular ARS complex from tRNA-Sepharose (*3*). Fractions of 2 ml were collected and assayed for MetRS activity and for protein with Coomassie assay reagent (Pierce) [Fig. 1(2)]. Fractions showing high specific activities were applied to a Superdex™200 column (2.6×40 cm). The material was eluted with Medium A containing 10 mM DTT at a rate of 48 ml/h. Fractions of 4 ml were collected and assayed for MetRS activity and for protein with Coomassie assay reagent [Fig. 1(3)].

Method II, using ω-aminooctyl agarose chromatography: Fifteen milliliters of rat liver cytosol was subjected to Sephadex G-200 column chromatography and Fraction I was prepared (*3*). Fraction I was concentrated to about 20 ml (15-18 mg of protein) and applied to an ω-aminooctyl agarose column (1.5×7 cm) pre-swollen and washed with 25 mM KCl and 10 mM DTT containing Buffer A. The material was then eluted with 0.3 M KCl and 10 mM DTT containing Buffer A at a flow rate of about 30 ml/h. Fractions of 4 ml were collected and assayed for protein and MetRS activity as described above [Fig. 2(1)]. Fractions with high levels of MetRS specific activity were pooled, and then 1 M K-phosphate buffer (pH 7.0) was added to the mixture at a final concentration of 10 mM. The material

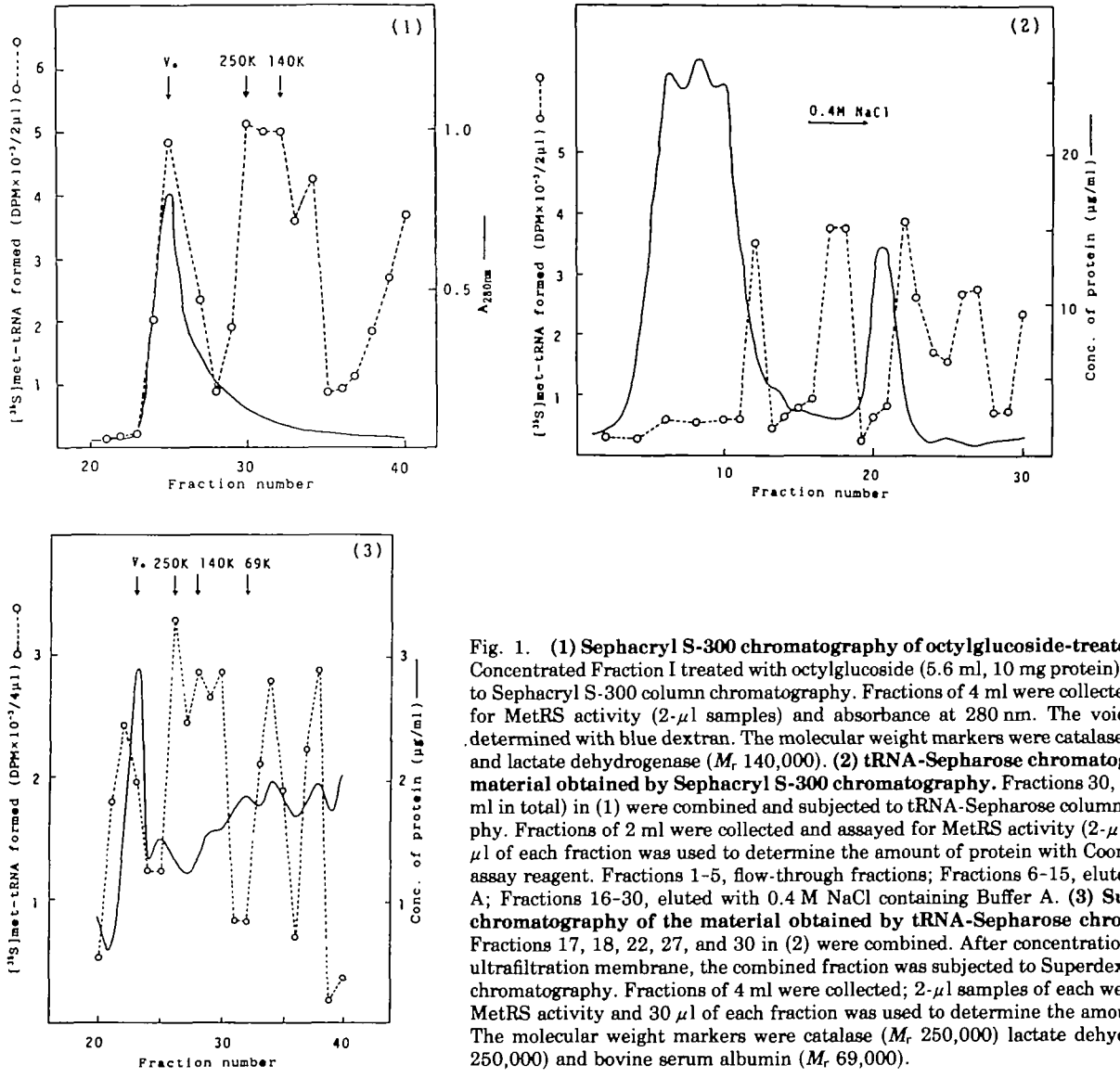

Fig. 1. **(1) Sephacryl S-300 chromatography of octylglucoside-treated Fraction I.** Concentrated Fraction I treated with octylglucoside (5.6 ml, 10 mg protein) was subjected to Sephacryl S-300 column chromatography. Fractions of 4 ml were collected and assayed for MetRS activity (2-μl samples) and absorbance at 280 nm. The void volume was determined with blue dextran. The molecular weight markers were catalase (M_r 250,000) and lactate dehydrogenase (M_r 140,000). **(2) tRNA-Sepharose chromatography of the material obtained by Sephacryl S-300 chromatography.** Fractions 30, 31, and 32 (12 ml in total) in (1) were combined and subjected to tRNA-Sepharose column chromatography. Fractions of 2 ml were collected and assayed for MetRS activity (2-μl samples); 30 μl of each fraction was used to determine the amount of protein with Coomassie protein assay reagent. Fractions 1-5, flow-through fractions; Fractions 6-15, eluted with Buffer A; Fractions 16-30, eluted with 0.4 M NaCl containing Buffer A. **(3) Superdex™200 chromatography of the material obtained by tRNA-Sepharose chromatography.** Fractions 17, 18, 22, 27, and 30 in (2) were combined. After concentration on a Diaflow ultrafiltration membrane, the combined fraction was subjected to Superdex™200 column chromatography. Fractions of 4 ml were collected; 2-μl samples of each were assayed for MetRS activity and 30 μl of each fraction was used to determine the amount of protein. The molecular weight markers were catalase (M_r 250,000) lactate dehydrogenase (M_r 250,000) and bovine serum albumin (M_r 69,000).

was applied to a Gigapite column (1.5×6 cm) pre-swollen and washed with Buffer P. The material was eluted with 0.2 M potassium phosphate containing Buffer P at a flow rate of 20 ml/h. Fractions of 3 ml were collected and assayed for MetRS activity and protein [Fig. 2(2)]. Fractions showing high levels of MetRS specific activity were collected and the collected material was subjected to Superdex™200 chromatography as described above [Fig. 2(3)].

Assay of ARS Activity—The basic incubation mixture (*3, 4*) (total volume, 25 μl) contained 0.125 M sucrose, 50 mM Tris-HCl (pH 7.6), 7.5 mM $MgCl_2$, 160 mM KCl, 8 mM ATP, 1 mM DTT, 17.5 μg of deacylated rabbit liver tRNA, 10 μM [^{14}C]amino acid, and an enzyme preparation. In the case of using [^{35}S]methionine, 0.37 μCi of [^{35}S]-methionine was adjusted to 10 μM with unlabeled methionine. In experiments on the effect of 5SRNP on MetRS activity, the incubation mixture containing yeast tRNA freed from 5SrRNA (*5*) (total volume, 50 μl) was used. Incubation was carried out at 37°C for 10 min. To measure the MetRS activity with endogeneous $tRNA^{met}$ in the MetRS complex, the above incubation mixture was used except that tRNA and carrier methionine were omitted.

The procedures used for washing the RNA and measuring its radioactivity were as described previously (*3, 4*).

PAGE of Protein—Since the amount of the MetRS complex was limited and the loss of material during concentration on a Diaflow ultramembrane (Amicon) was large, firstly we concentrated the material by lyophilization after extensive dialysis against water. The lyophilized material was dissolved in water and subjected to SDS-PAGE as described previously (*3*). It was found, however, that most of L5 was lost. Therefore, later, we used microconcentrators (Centricon™, Amicon) which was treated with bovine serum albumin as recommended by the manufacturer. Since the yield was low when a dilute sample was applied, bovine serum albumin was added to the sample solution to give 10 μg of protein/0.1 ml.

Silver staining of protein on the gel was carried out with a reagent purchased from Daiichi Pure Chemical (Tokyo) using the procedures recommended by the manufacturer.

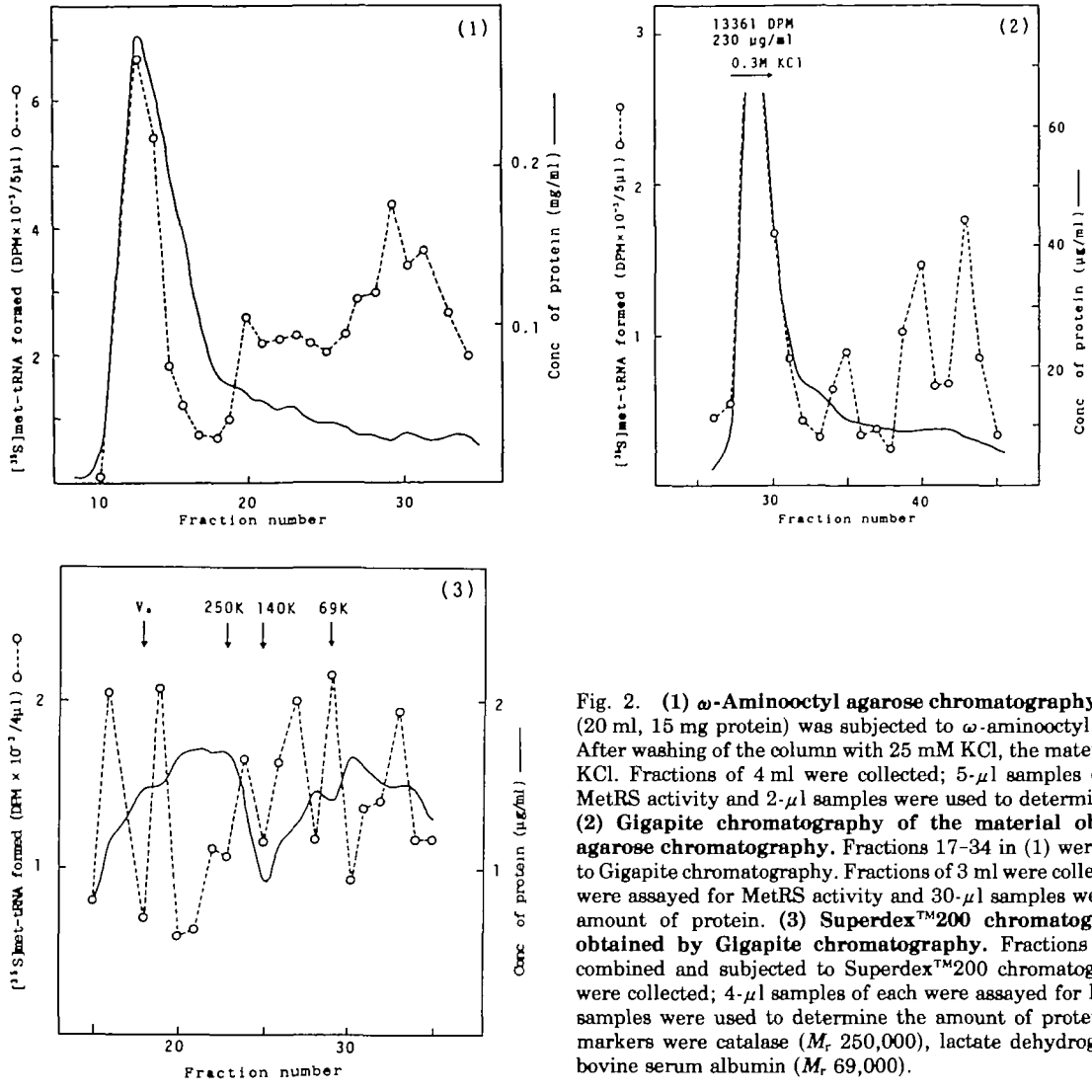

Fig. 2. **(1) ω-Aminooctyl agarose chromatography of Fraction I.** Fraction I (20 ml, 15 mg protein) was subjected to ω-aminooctyl agarose chromatography. After washing of the column with 25 mM KCl, the material was eluted with 0.3 M KCl. Fractions of 4 ml were collected; 5-μl samples of each were assayed for MetRS activity and 2-μl samples were used to determine the amount of protein. **(2) Gigapite chromatography of the material obtained by aminooctyl agarose chromatography.** Fractions 17–34 in (1) were combined and subjected to Gigapite chromatography. Fractions of 3 ml were collected; 5-μl samples of each were assayed for MetRS activity and 30-μl samples were used to determine the amount of protein. **(3) Superdex™200 chromatography of the material obtained by Gigapite chromatography.** Fractions 40 and 43 in (2) were combined and subjected to Superdex™200 chromatography. Fractions of 4 ml were collected; 4-μl samples of each were assayed for MetRS activity and 30-μl samples were used to determine the amount of protein. The molecular weight markers were catalase (M_r 250,000), lactate dehydrogenase (M_r 140,000), and bovine serum albumin (M_r 69,000).

Northern Blotting and Immune Dot Blot Analysis—Several tRNA-Sepharose fractions pooled in liquid nitrogen were collected and the material containing 540 μg protein in 113 ml was lyophilized after extensive dialysis against water. RNA was purified and subjected to Northern blotting as described previously. Immune dot blot analysis was carried out using an ECL Western blotting system (Amersham) as described previously (*3*).

Analytical Procedures—Protein concentrations required for calculating the specific activity of MetRS were determined by using Coomassie protein assay reagent as described previously (*4*).

RESULTS

Release of the MetRS Complex from the Macromolecular ARS Complex—It was essential to find suitable conditions for the release of the MetRS complex from the macromolecular ARS complex without loss of activity. As the macromolecular ARS complex fraction, we used Fraction I obtained by Sephadex G-200 chromatography of the cytosol, which showed MetRs activity exclusively at the void volume (*3*).

Deutscher reported that rat liver ARS complex was dissociated by a process of freezing and thawing (*12*). We found, however, that freezing (−80°C) and thawing (+20°C) Fraction I several times released only slight MetRS activity (data not shown).

Recently, a macromolecular ARS complex containing nine ARSs was reported to be stable toward high-ionic-strength or non-ionic detergents (*13*). Partial dissociation was observed only after simultaneous treatment with 1 M NaCl and a non-ionic detergent, Triton X-100 (*14, 15*). Since we had previously observed that a high concentration of KCl dissociated 5SRNP from the macromolecular ARS complex (*3*), it was thought that the foregoing treatment might not be suitable to our purpose. Instead, we used an octylglucoside, and found that 1 or 2% octylglucoside in 0.2 M KCl released the MetRS complex from Fraction I, as shown by following gel filtration on Sephadex G-200. The bound octylglucoside, however, could not be removed by gel filtration. Overnight dialysis of the material after the treatment caused a loss of MetRS activity. Dialysis for one to 2 h only partially removed the detergent and caused

partial loss of MetRS activity. Octylglucoside was firmly bound to macromolecular ARS complex or its components, and inactivated MetRS. Therefore, we used Bio-Beads SM-2 to remove the detergent from the material completely. The results are summarized in Table I. Treatment with octylglucoside at 1% in the presence of 0.2 M KCl followed by Bio-Beads treatment dissociated the MetRS complex partially without loss of activity. However, the efficiency of release was usually rather low (about 20%). Therefore, we had to use diluted solution in the following purification, which led to the difficulties described below.

Purification of the MetRS Complex with Octylglucoside (Method I)—Most of the difficulties in this experiment concerned the inactivation of MetRS during the purification procedures following gel filtration of detergent-treated Fraction I, especially in a diluted solution. Addition of DTT (10 mM) partially prevented this inactivation. To maintain the MetRS activity, we shortened the purification period to 2 days after the octylglucoside treatment by using Sephacryl S-300 and Superdex™200 for gel-filtration.

Figure 1(1) shows the elution pattern of Fraction I treated with 1% octylglucoside and Bio-Beads on Sephacryl S-300 column chromatography. Since the molecular weight of the MetRS-5SRNP-tRNA^{met} complex is about 200,000 (*6*), we collected the fractions in the region between the markers catalase (M_r 250,000) and lactate dehydrogenase (M_r 140,000) [Fractions 30-32 in Fig. 1(1)]. The collected fraction was immediately subjected to chromatography on tRNA-Sepharose, in which partially purified yeast tRNA^{met} was used. Since 0.4 M KCl eluted the 5SRNP containing macromolecular ARS from the tRNA-Sepharose column (*3*), we usually used 0.4 M NaCl for the elution of MetRS complex. Figure 1(2) shows the elution pattern on tRNA-Sepharose chromatography. Fractions showing high MetRS specific activity were combined, because only a limited volume could be subjected to gel filtration. The combined fraction was analyzed by Superdex™200 column chromatography; Figure 1(3) shows the elution pattern. MetRS activity is present in the region corresponding to the molecular weight of the MetRS-5SRNP-tRNA^{met} complex (M_r 200,000), and this was stored in liquid nitrogen for further analysis. The activity is also present in the void volume, and in the region corresponding to the marker serum albumin (M_r 69,000), indicating that aggregation, dissociation and degradation of the MetRS complex occurred during the preparation procedures owing to the instability of the complex. Dissociation and degradation of the MetRS complex were also observed in previous experiments (*5*), in which an antibody against L5 was used to separate the MetRS complex fraction from rat liver cytosol (see Introduction for more details).

Table II(1) summarizes typical results of purification of the MetRS complex by Method I. The purification procedures described above resulted in approximately 1,500-fold enrichment of MetRS-activity with a recovery of about

TABLE I. **Effects of octylglucoside concentration and subsequent treatment on MetRS relative specific activity and MetRS activity released from the macromolecular ARS complex.** Concentrated Fraction I (1 ml, 1.8 mg protein) was treated with 1 or 2% octylglucoside in 0.2 M KCl. After standing at 0°C for 10 min, the mixture was treated with Bio-Beads SM-2 as described in "MATERIALS AND METHODS" or dialyzed for 2 h against Medium A at 4°C. The mixture (0.9 ml) was subjected to Sephadex G-200 column chromatography (1.5 × 40 cm) and the material was eluted with 2 mM DTT containing Medium A. Fractions of 2 ml were collected. The A_{280} of each fraction was measured. Samples of treated Fraction I (1 μl) and of each fraction (5 μl) on Sephadex G-200 chromatography were assayed for MetRS activity. The relative specific activity of MetRS is expressed as a percentage of the specific activity of the original concentrated Fraction I. The sum of the MetRS activities of the fractions located between the markers catalase (M_r 250,000) and lactate dehydrogenase (M_r 140,000) on Sephadex G-200 column chromatography is expressed as a percentage of the MetRS activity of Fraction I.

Octylglucoside (%)	Subsequent treatment	Relative specific activity	MetRS activity released
1	Bio-Beads	100	22
2	Bio-Beads	4	11
1	Dialysis, 2 h	60	11
2	Dialysis, 2 h	20	19

TABLE II. **Purification of the MetRS complex fraction from the macromolecular ARS complex in rat liver cytosol by Method I using octylglucoside (1) or by Method II using ω-aminooctyl agarose chromatography (2).**

(1) Method I

Exp. No.	Specific activity (pmol/mg/10 min) RT54	Specific activity (pmol/mg/10 min) RT57	Total activity (nmol/min) RT54
Cytosol	3.6×10^2 (×1)§	2.7×10^2 (×1)§	114 (100)
Fraction I	1.4×10^4 (×33)	4.3×10^3 (×16)	77 (67)
Sephacryl S-300	5.2×10^4 (×140)	3.5×10^4 (×128)	19 (16)
tRNA-Sepharose	6.0×10^5 (×1,686)	3.5×10^5 (×1,300) 11.1×10^5* (×4,140)*	15 (13)
Superdex™200	3.7×10^5* (×1,039)*	4.1×10^5* (×1,500)*	3.7 (3)

(2) Method II

Exp. No.	Specific activity (pmol/mg/10 min) RT56	Specific activity (pmol/mg/10 min) RT53	Total activity (nmol/min) RT56	Total activity (nmol/min) RT53
Cytosol	5.5×10^2 (×1)§	4.6×10^2 (×1)§	177 (100)	111 (100)
Fraction I	6.1×10^3 (×11)	2.3×10^3 (×5)	120 (67)	62 (56)
ω-Aminooctyl agarose		6.9×10^3 (×15)		
Gigapite	2.8×10^4 (×50)	2.1×10^4 (×45)	19 (11)	42 (4)
Superdex™200	1.9×10^5 (×350)	1.5×10^5 (×330) 5.1×10^5* (×1,140)*	1.1 (0.1)	2.7 (2.4)

*Specific activity calculated from specific activities of individual fractions on tRNA-Sepharose chromatography or Superdex™200 chromatography. §Purification factor.

(3) Restoration of MetRS activity by serum albumin

Fraction	Specific activity (pmol/mg/10 min) Albumin (−)	Specific activity (pmol/mg/10 min) Albumin (+)
Superdex™200	6.2×10^5 (×921)§	2.4×10^6 (×3,700)§
	1.1×10^6 (×1,700)	1.8×10^6 (×2,600)
tRNA-Sepharose	1.3×10^5 (×200)	6.5×10^5 (×970)
	1.4×10^5 (×102)	4.9×10^5 (×354)

The sample was diluted (tenfold) in 2% albumin-50% glycerol, 2.5 mM DTT (final concentration). §Purification factor.

13% at the tRNA-Sepharose chromatography step and about 3% at the final step. The specific activity did not increase on the final Superdex™200 chromatography owing to the instability of the MetRS complex fraction. It must be added that the specific activities calculated from the activities of the individual fractions freshly obtained on tRNA-Sepharose or Superdex™200 chromatography were usually higher than that of the fraction collected after 2 to 3 h. As shown in the experiment RT57 in the table, the specific activity calculated from individual fractions freshly obtained on tRNA-Sepharose chromatography was about 3 times higher than that of the fraction collected for further purification. Thus, owing to the instability of the complex, the figures in Table I may represent minimal purification values with the exception of those for cytosol and Fraction I. Furthermore, it was found that dilution of tRNA-Sepharose or Superdex™200 fraction in 2% serum albumin-50% glycerol-2.5 mM DTT (final concentration) increased the MetRS activity of these fractions, probably by restoring the conformation of a MetRS inactive form (Table II(3)). These findings are discussed later. It must be added that the yield of MetRS complex was rather low (about 3% at the final step in both methods). Since MetRS is exclusively present in macromolecular ARS complex, the low yield may reflect low efficiency of release of MetRS complex from the macromolecular ARS complex by octylglucoside in Method I and hydrophobic interaction chromatography in Method II, and instability of the complex, as shown by aggregation, dissociation and degradation of MetRS complex on Superdex™200 chromatography [Figs. 1(3) and 2(3)].

Contamination of the Superdex™200 fraction by other ARSs presented in the macromolecular ARS complex and stimulated by 5SRNP (*3, 5*) was investigated. As shown in Table III(1), ArgRS, LysRS, and IleRS were found to be present in the Superdex™200 fraction, although their activities were significantly lower than that of MetRS owing to the use of tRNA-Sepharose chromatography based on partially purified yeast $tRNA^{met}$.

It is important to know whether octylglucoside releases other ARS complexes from the macromolecular complex or not. To examine this possibility, a large-scale experiment was done with Method I except that tRNA-Sepharose using unfractionated rabbit liver tRNA was employed. The activities of eight individual ARSs (except for GluRS) contained in the macromolecular ARS complex were measured in the fractions distributed in the region corresponding to catalase (M_r 250,000) on Superdex™200 chromatography. The results are shown in Table IV. Considering that the molecular weights of individual ARSs are distributed from 150K (GluRS, ProRS) to 57K (AspRS), they may be present as the complex form in this region. Although the extent of purification is variable among individual ARSs, Method I may be useful for the purification of individual ARS complexes from the macromolecular ARS complex. It must be mentioned that for this purpose, the use of an affinity column containing tRNA specific for individual amino acids would be necessary. The silver staining pattern on SDS-PAGE of a tRNA-Sepharose fraction showed nine bands corresponding to nine ARSs (lane 2 in Fig. 3B).

Purification of the MetRS Complex Fraction by ω-Aminooctyl Agarose Chromatography (Method II)—Partial dissociation of the macromolecular ARS complex by hydrophobic interaction chromatography has been reported (*7, 16, 17*). Therefore, we used ω-aminooctyl agarose chromatography to dissociate the MetRS complex from Fraction I. Figure 2(1) shows the elution pattern on ω-aminooctyl agarose chromatography. Peaks containing both the main protein components and high MetRS activity were immediately eluted with 0.3 M KCl, followed by a broad region with high levels of MetRS specific activity that was thought to contain the MetRS complex dissociated from Fraction I. Materials in this region were pooled and subjected to hydroxyapatite column chromatography using Gigapite. As shown in Fig. 2(2), after elution of the peak containing both the main protein components and MetRS activity, there is a region showing high levels of MetRS specific activity. The material in this region was immediately subjected to Superdex™200 column chromatography. As shown in Fig. 2(3), there is a fraction with definite MetRS activity in the region corresponding to the molecu-

TABLE III. **Contamination of Superdex™200 fractions purified by Method I or II by ARSs contained in the macromolecular ARS complex.** (1) 4.7 ng of protein (RT54) or (2) 3.3 ng of protein (RT49) of the Superdex™200 fraction was incubated with [^{14}C]amino acid under the conditions described in "MATERIALS AND METHODS."

Exp. No.	Specific activity (pmol/mg/10 min)			
	[^{14}C]Met	[^{14}C]Arg	[^{14}C]Lys	[^{14}C]Ile
(1) RT54 (Method I)	3.4×10^{5} (100)	8.7×10^{4} (25)	6.8×10^{4} (20)	1.5×10^{4} (8)
(2) RT49 (Method II)	1.6×10^{5} (100)	2.7×10^{4} (17)	5×10^{3} (4)	3×10^{3} (2)

TABLE IV. **Activity of eight kinds of ARS in the 250K (catalase) region on Superdex™200 chromatography.** Rat liver cytosol (33 ml) was subjected to Sephacryl S-300 column chromatography (5.0×90 cm). Fraction I was collected, concentrated on Diaflow ultafiltration membrane to 24 ml, and treated with 1% octylglucoside. After Bio-beads treatment, the material was applied to a Sephacryl S-300 column (2.6×90 cm). The fractions containing high MetRS activity and distributed in the region corresponding to a molecular weight from 140,000 (lactate dehydrogenase) to 250,000 (catalase) were collected and applied to a tRNA-Sepharose column (2.6×40 cm) in which non-fractionated tRNA was used. The column was eluted with 0.5 M NaCl and fractions eluted with lower concentrations than 0.4 M were collected and applied to a Superdex™200 column (2.6×40 cm). The peak fraction with high specific activity of MetRS which was present in the molecular weight region of 250,000 (catalase) was collected. The activity of eight kinds of ARS was measured after dilution (fivefold) in 0.01% bovine serum albumin-50% glycerol-2.5 mM DTT.

	Specific activity (pmol/mg/min)							
	MetRS (108K)	LysRS (76K)	ArgRS (73K)	AspRS (57K)	LeuRS (120K)	IleRS (130K)	GluRS (150K)	ProRS (150K)
Cytosol	4.6×10^{2}	3.4×10^{2}	2.1×10^{2}	1.5×10^{2}	5.6×10^{2}	2.0×10^{2}	2.7×10	2.2×10^{2}
Superdex™200 250K Fr.	3.7×10^{5} (×800)	1.4×10^{5} (×410)	2.9×10^{5} (×1,300)	4.5×10^{4} (×300)	1.0×10^{5} (×180)	3.9×10^{4} (×200)	3.4×10^{4} (×1,250)	7.7×10^{4} (×350)

lar weight of the MetRS-5SRNP-tRNA^{met} complex (M_r 200,000), suggesting the presence of the MetRS complex in this fraction. As in the case of Method I, aggregated, dissociated and degraded materials showing MetRS activity can also be observed.

Table II(2) summarizes typical results of the MetRS complex purification by Method II. The purification procedures described above resulted in about 350-fold enrichment of MetRS. In the experiment RT53 in this table, the specific activity calculated from individual fractions freshly obtained on Superdex™200 chromatography was about 3.5 times higher than that of the collected fraction after 2 or 3 h. Therefore, the figure in the table is the minimal value

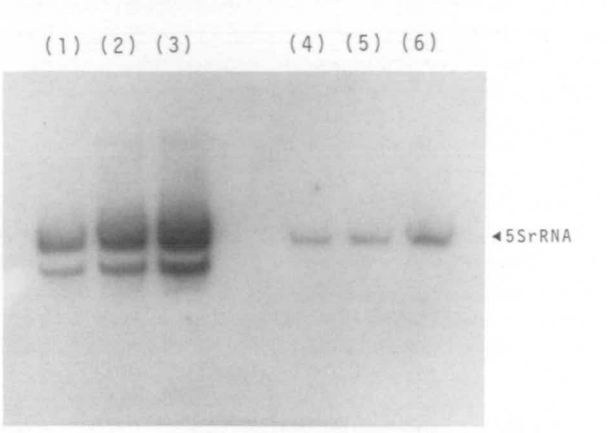

Fig. 4. **Northern blot analysis of the tRNA-Sepharose fraction.** Northern blot analysis was carried out as described previously (*2*), using a double-stranded 70 mer cDNA corresponding to the 5′-terminus of mammalian 5SrRNA. The specific activity of the probe DNA used for hybridization was $1 \times 10^9/\mu g$. RNA was subjected to PAGE under non-denaturing conditions and then transferred to a Zeta-probe blotting membrane. The pre-hybridization buffer was 5× SSC, 50 mM sodium phosphate (pH 6.5), 10×Denhardt's solution, 50% formamide, and 1 mg/ml of *E. coli* tRNA (*3*). Lanes 1, 2, and 3: 0.2 μg, 0.5 μg, and 1 μg of rat liver 5SrRNA. Lanes 4, 5, and 6: RNA prepared by SDS-phenol from 35 μg, 70 μg, and 140 μg of protein of the tRNA-Sepharose fraction. (Since the tRNA-Sepharose chromatography fraction was used, it was impossible to determine the amount of RNA of the sample.)

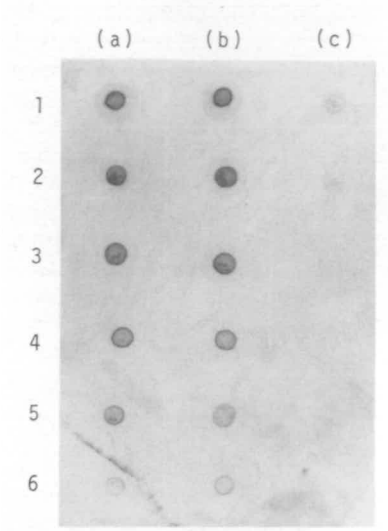

Fig. 5. **Immune dot blot analysis of protein in the Superdex™-200 fractions prepared by Method I or II.** Immune dot blot analysis was carried out using an ECL Western blotting system as described previously (*2, 3*). An IgG fraction of rabbit antiserum against L5 was used as the primary antibody. (a) 1-6: 7.5, 3.4, 1.7, 0.8, 0.4, and 0.2 μg of protein of the Superdex™200 fractions prepared by Method I. (b) 1-6: about 15, 7.5, 3.4, 1.7, 0.8, and 0.4 μg of protein of the Superdex™200 fractions prepared by Method II. (c) 1-5: about 25, 12.5, 6.2, 3.1, and 1.6 ng of protein of 5SRNP.

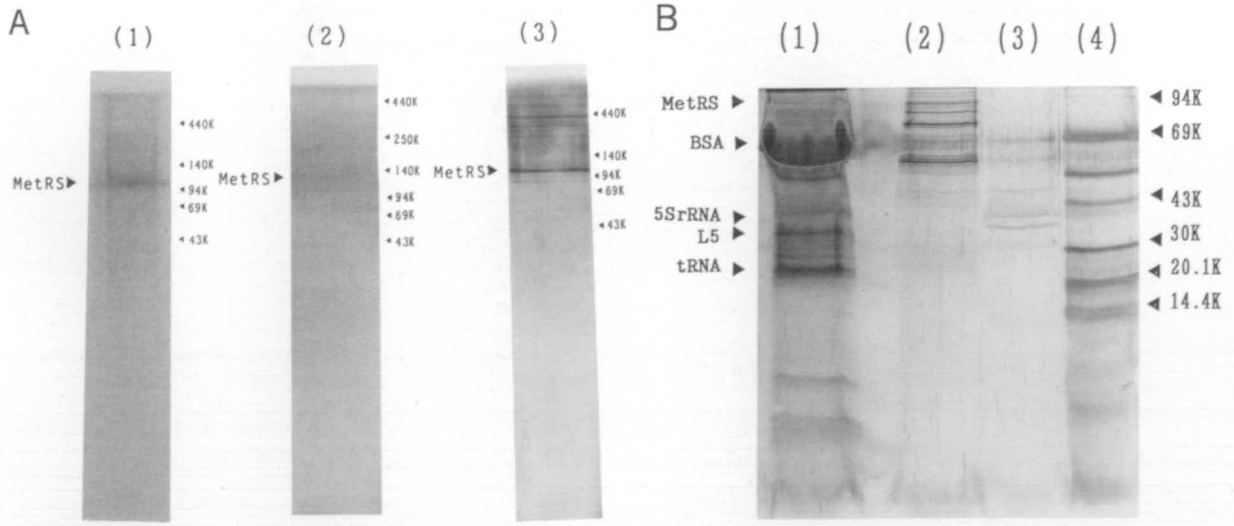

Fig. 3. **A: Protein silver staining patterns on SDS-PAGE of the MetRS complex fraction.** MetRS complex fractions were concentrated by lyophilization and subjected to SDS-PAGE, and silver staining of the protein on the gel was carried out. Lane(1), Superdex™-200 chromatography fraction prepared by Method I; 0.7 μg of protein was used. Lane (2), Superdex™200 chromatography fraction prepared by Method II; 0.8 μg of protein was used. Lane (3), tRNA-Sepharose chromatography fraction prepared by Method I; 4.5 μg of protein was used. **B: Silver staining pattern on SDS-PAGE of MetRS complex fraction.** MetRS complex fractions were concentrated in microconcentrators. Lane (1), Superdex™200 chromatography fraction prepared by Method I concentrated after addition of serum albumin. 3.1 μg of protein was used. Lane (2), tRNA-Sepharose chromatography fraction concentrated without addition of serum albumin. 0.34 μg of protein was used. Lane (3), 5SRNP (0.2 μg of protein). Lane (4), low weight markers (Pharmacia). 0.5 μg of each protein was used.

owing to instability of the MetRS complex, as in the case of Method I. These findings are discussed later. Contamination of the purified product by ArgRS, LysRS, and IleRS is shown in Table III(2), although the degree of contamination was rather low.

SDS-PAGE of Superdex™200 Chromatographic Fractions Prepared by Methods I and II—The silver staining patterns of protein on SDS-PAGE of fractions purified by Superdex™200 column chromatography in Methods I and II are shown in Fig. 3, A and B, respectively. In the case of Method I only one main protein band representing MetRS (M_r 108,000) (*13*) is observed, while in Method II two bands are seen, of which the slow-migrating one may represent MetRS; the fast-migrating band appears to represent LysRS (M_r 76,000) or ArgRS (M_r 74,000) (*13*), or both, since their activities are seen in this fraction. These results indicate that the Superdex™200 fractions contain pure MetRS, especially that prepared by Method I. It was expected that the L5 band would be present on the gel. However, only a weak band was observed in the region of L5, probably because of the notorious insolubility of L5.

The insolubility of L5 can cause many difficulties in interpretation of experimental results, as discussed later. In our experiments the MetRS fraction was extensively dialyzed against water and lyophilized. The lyophilized sample was dissolved in a small volume of water and subjected to SDS-PAGE. This method is efficient for concentration of the enzyme fraction. In Fig. 3B, 6 μg/4 ml of Superdex™200 fraction was concentrated to 110 μl with a protein yield of 40%. It was found, however, that 5SRNP-(L5) adhered to the dialysis membrane during dialysis and to the glass vessel during lyophilization. For example, 5SRNP solution (0.8 μg/100 μl) was dialyzed overnight in dialysis tubing, and the dialyzed solution and SDS-extract (0.2% SDS at 90°C, 2 min) of the tube membrane was subjected to SDS-PAGE. The silver staining patterns showed that the L5 band was detected only in the extract (data not shown). Therefore, it is reasonable that the L5 band is hardly detectable on the gel in the case of the lyophilized sample.

Since it was necessary to see the L5 band, the following experiments were carried out. To prevent the dissociation of 5SRNP from MetRS complex fraction, bovine serum albumin (about 10-fold excess) was added to the fraction on Sephacryl S-300 chromatography of octylglucoside-treated Fraction I, and the tRNA-Sepharose chromatographic fraction. To minimize the fraction volume, the tRNA-Sepharose column was eluted with 0.5 M NaCl and the fraction eluted up to 0.4 M NaCl was collected and applied to a small Superdex™200 column (1.5×29 cm). For concentration of the final sample obtained on Superdex™200 chromatography, serum albumin was added to the sample to give a concentration of 10 μg/0.1 ml. Then concentration of the sample was carried out with a microconcentrator (Centricon™) treated with 1% serum albumin. As shown in Fig. 3B, the silver staining pattern after SDS-PAGE shows a distinct L5 band, the intensity of which is comparable to that of the MetRS band. 5SRNA is also detectable as a yellow band. A broad and thick band of serum albumin (added to the sample) was also observed.

Presence of 5SrRNA in the tRNA-Sepharose Fraction

TABLE V. **Changes in the relative specific activity of MetRS incubated with and without the addition of tRNA during purification.**

(1) Method I

Exp. No. / tRNA	Relative specific activity			
	RT54		RT47	
	(+)	(−)	(+)	(−)
Cytosol	1	1	1	1
Fraction I	18	9	12	29
Sephacryl S-300	145	86	168	2,666
tRNA-Sepharose	1,686	3,857	1,130	6,200

(2) Method II

Exp. No. / tRNA	Relative specific activity	
	RT49	
	(+)	(−)
Cytosol	1	1
Fraction I	11	11
Aminooctyl agarose	16	100
Gigapite	373	928
Superdex™200	353	8,571

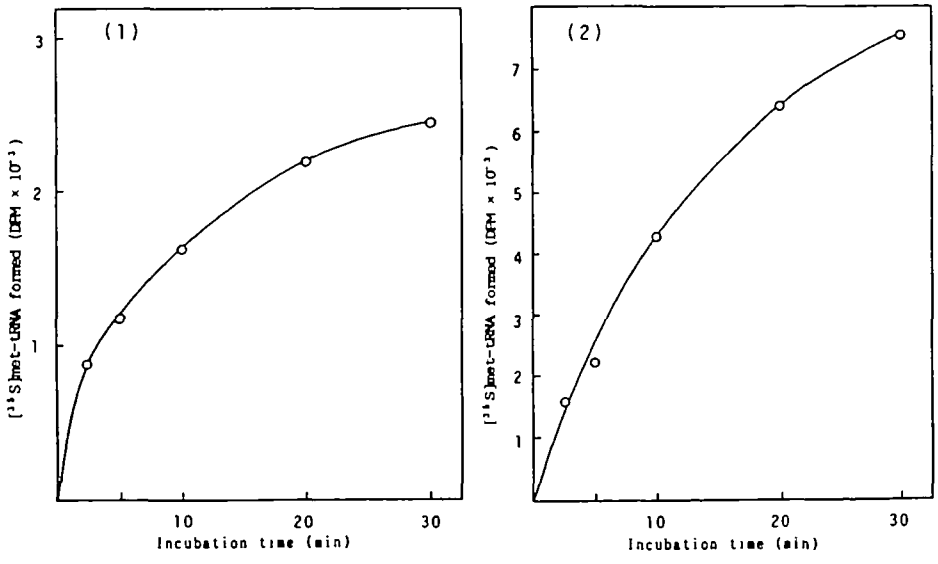

Fig. 6. **Time courses of the incorporation of [^{35}S]methionine into endogenous tRNA by the MetRS complex fraction on Superdex™200 chromatography.** (1) 7 ng of the MetRS complex fraction on Superdex™200 chromatography of the material prepared by Method II was incubated at 37°C in the reaction mixture without the addition of both tRNA and carrier methionine, as described in "MATERIALS AND METHODS." (2) Time course of the complete reaction mixture (+tRNA) containing the same enzyme fraction (7 ng of protein) incubated as described above.

and Protein L5 in the Superdex™200 Fractions—Since the amount of Superdex™200 fraction obtained in this experiment was too small for the preparation of RNA, we used the combined tRNA-Sepharose fractions which were prepared by Method I, and pooled in liquid nitrogen. The specific activity of this fraction is about 1,500-fold higher than that of the liver cytosol [Table II(1)] and the SDS-PAGE pattern shows one discrete band in the region corresponding to the molecular weight of MetRS, although several faint bands can be observed, especially in the higher-molecular regions. Some of them may be bands of ARSs contained in the macromolecular ARS complex [Fig. 3A lane (3) and Fig. 3B lane (2)].

The results of Northern blot analysis of the RNA in this fraction are shown in Fig. 4, in which ^{32}P-labeled 70-mer DNA corresponding to the 5′ terminus of 5SrRNA was used (*3*). The RNA gives one radioactive band at the same position as the slow-moving band of the marker 5SrRNA. The intensity of the band increases, depending on the concentration of the RNA. These results confirm the presence of 5SrRNA in our MetRS complex fraction.

It is of importance that this pattern for 5SrRNA differs from that of a Northern blot of 5SrRNA from ribosomal 60S subunits or from the macromolecular ARS complex, which gives two bands (*3*). Since two bands of 5SrRNA were reported to correspond to different conformations of 5SrRNA (*18, 19*), it appears that the conformational changes of 5SrRNA occurred during the preparation of the MetRS complex fraction.

Immune dot blot analyses of the Superdex™200 fractions prepared by Methods I and II were performed using an ECL Western blotting system (Amersham) as described previously (*3, 4*). As the primary antibody, we used an IgG fraction of rabbit polyclonal antiserum against L5 protein of *Artemia salina*, which has been shown to cross-react with rat L5 (*20*). 5SRNP was used as a reference. As shown in

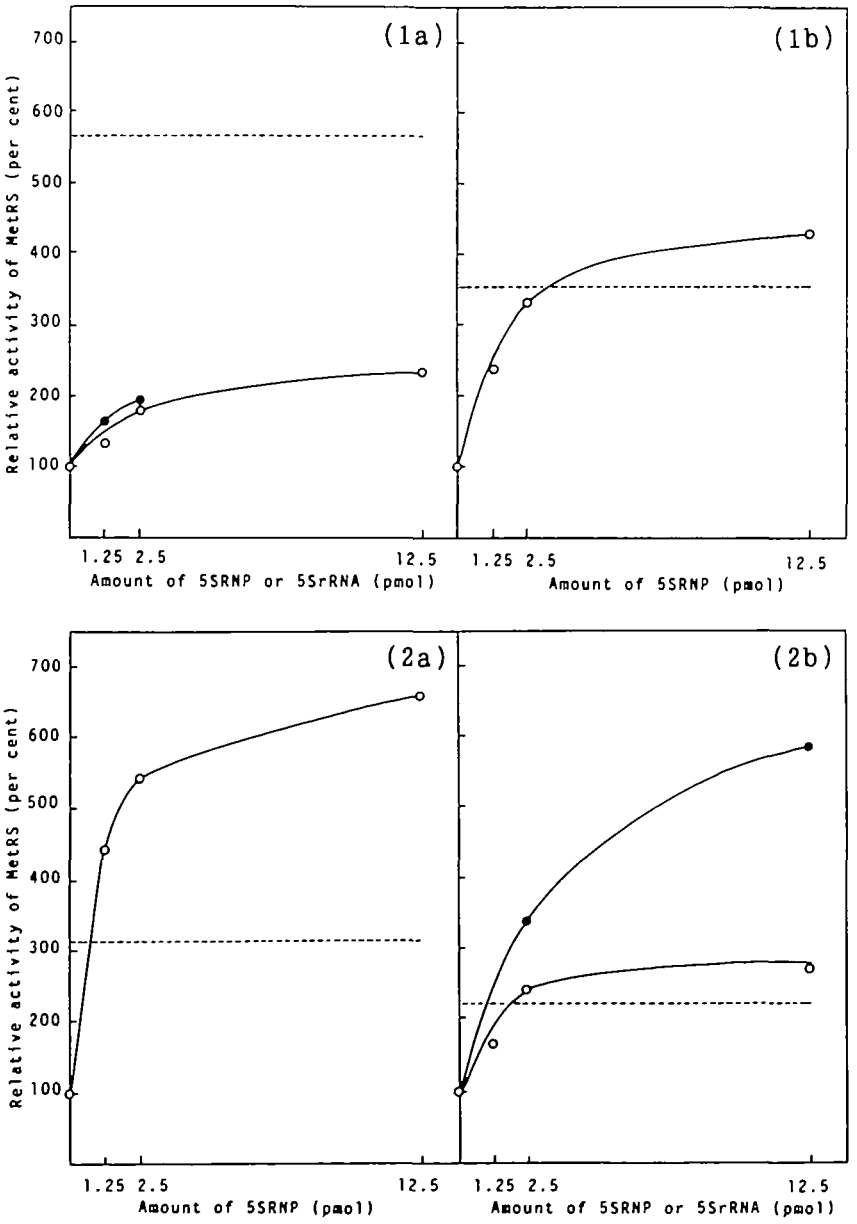

Fig. 7. **Effects of 5SRNP on MetRS activity of the Superdex™200 fraction prepared by Method I or Method II.** The reaction mixture (total volume, 50 μl) was as follows: 0.01 mM [^{35}S]methionine (0.75 μCi), 3 mM ATP, 35 μg of deacylated yeast tRNA freed from 5SrRNA (*4*), 80 mM KCl, 7 mM $MgCl_2$, 0.2 M sucrose, 3 mM DTT, and 40 mM Tris-HCl (pH 7.6), and 18 ng of protein of the ternary complex fraction on Superdex™200 chromatography prepared by Method I or 12 ng of protein of the same fraction prepared by Method II. The reaction mixture was incubated at 37°C for 10 min with various amounts of 5SRNP or 5SrRNA prepared as described previously (*3*). The values are the means for 3 or 4 experiments. The samples in (1a) and (1b) were prepared by Method I and stored in liquid nitrogen. The samples in (2a) and (2b) were prepared by Method II, and used after standing for several hours at 0°C after fractionation. ○, 5SRNP; ●, 5SrRNA; ----, activity of freshly prepared original fraction.

Fig. 5, positive signals were observed for the series of dots of the Superdex™200 fraction prepared by Methods I and II. This antiserum did not react to IgG from normal rabbit serum or to 5SrRNA [see Fig. 4(2) in Ref. *3*]. The result indicates the presence of an immunologically identical protein to L5 in our MetRS complex fraction.

Taking all the above results together, we conclude that 5SRNP is present in the MetRS complex fraction.

Presence of tRNA^{met} in the MetRS Complex Fraction—To provide evidence for the presence of tRNA^{met} in the MetRS complex fraction, the complex fraction at each step of the purification was incubated with [^{35}S]methionine in the reaction mixture without the addition of both tRNA and carrier methionine, and the specific activity of MetRS was measured. Figure 6 shows the time course of the reaction. Incorporation of [^{35}S]methionine into endogenous tRNA^{met} by the fraction obtained by Superdex™200 chromatography proceeds almost linearly for at least 30 min at 37°C, as in the case of incubation with added tRNA. It is of interest that the specific activity of the MetRS complex fraction incubated without the addition of tRNA increases during the purification procedures in a similar manner to that of the same fraction with the addition of tRNA. Furthermore, the extent of the specific activity increase without tRNA is markedly higher than that with tRNA (Table V). The higher degree of specific activity without tRNA may be partially ascribed to the decrease in the amount of free methionine in the fractions occurring during the purification procedures.

From these results, it is reasonable to consider that endogenous tRNA^{met} exists stably in the MetRS complex and is not released during purification. Previously, we found by UV-cross-linking of the MetRS complex fraction prepared with an antibody against L5 that 5SRNP was associated with methionyl-tRNA^{met} in the MetRS complex (*6*).

Effects of 5SRNP(A) on MetRS Activity—Previously, we reported that 5SRNP and 5SrRNA enhance the activity of the macromolecular ARS complex for the attachment of [^{35}S]methionine to tRNA and for methionine-dependent ATP-PP$_i$ exchange (*5*). Therefore, we examined the effect of 5SRNP or 5SrRNA on the attachment of [^{35}S]methionine to yeast tRNA freed from 5SrRNA (*5*) by the Superdex™200 chromatography fraction of the material prepared by Methods I and II. We determined the attachment of [^{35}S]methionine to tRNA as trichloroacetic acid-insoluble radioactivity, because it gave a similar value to that of the RNA fraction purified by the SDS-phenol method in the case of the macromolecular ARS complex, as reported previously (*5*).

As shown in Fig. 7, the MetRS activity was significantly increased by 5SRNP and 5SrRNA, depending on their concentration. The degree of stimulation was found to be higher than that observed in the purified macromolecular ARS complex (*5*). It should be noted that the specific activities of MetRS of the pooled fractions used in these experiments were lower when compared with those of original freshly prepared fractions, probably owing to conformational changes induced by standing at 0°C for several hours or storage in liquid nitrogen. 5SRNP or 5SrRNA appears to increase the MetRS activity by restoring the inactive conformation of MetRS generated during preparation, storage at 0°C or in liquid nitrogen to an active one, as discussed below. The reasons why 5SrRNA stimulated the activity more markedly than 5SRNP in the case of Fig. 7(2b) are unknown. In the case of the macromolecular ARS complex (Fraction B), the degree of stimulation of MetRS activity by 5SrRNA was similar to that by 5SRNP (*5*).

DISCUSSION

Previously, we incubated rat liver cytosol with a trace amount of ^{32}P-labeled 5SrRNA and [^{35}S]methionine, and investigated the components containing ^{32}P-labeled 5SRNP and [^{35}S]methionyl-tRNA by using an antibody against ribosomal protein L5. It was shown that 5SRNP and tRNA associated with MetRS on Sephadex G-150 chromatography. We assumed that MetRS complex containing the two components was present in the macromolecular ARS complex.

To obtain more direct evidence for this assumption, we isolated the MetRS complex from the macromolecular ARS complex fraction (Fraction I) of rat liver cytosol (*3*). In Method I, incubation of Fraction I with 1% octylglucoside in 0.2 M KCl followed by complete removal of the detergent with Bio-Beads SM-2 dissociated the MetRS complex with the maintenance of MetRS activity. Size fractionation on Sephacryl S-300 of the product, followed by tRNA-Sepharose chromatography in which partially purified tRNA^{met} was used, resulted in the 1,500-fold enrichment of MetRS activity.

It is of interest that octylglucoside released not only the MetRS complex but also other ARS complexes from the macromolecular ARS complex. By using tRNA-Sepharose in which unfractionated rabbit liver tRNA was used, eight ARSs were purified. Since the fraction was distributed in the region corresponding to M_r 250,000 (catalase), it was suggested that eight ARSs are present as the complex form, considering the molecular weight of individual ARS (from 150,000 to 57,000). Therefore, using tRNA-Sepharose with tRNA specific for individual amino acids, the ARSs contained in the macromolecular ARS complex can be obtained in the complex form. Furthermore, we could detect L5 band on SDS-PAGE of the tRNA-Sepharose fraction (data not shown). Therefore, it is possible that ARSs is stimulated by 5SRNP in macromolecular ARS and in cytosol contain 5SRNP. Further study, however, should be done to check the presence of 5SRNP in each individual ARS complex. Further, complexes containing two kinds of ARS or dimers of ARS may be present, as shown previously (*4*). In Method II, hydrophobic interaction chromatography using ω-aminooctyl agarose of Fraction I was used to dissociate the MetRS complex. The product was purified by hydroxyapatite chromatography. Partial dissociation of the macromolecular ARS complex by hydrophobic interaction chromatography has been reported (*7, 16, 17*). The analysis of the final products obtained by both methods by Superdex™200 chromatography suggests the presence of the MetRS-tRNA^{met}-5SRNP complex, although aggregation, dissociation and degradation of the complex were observed.

Most of the difficulties in the experiments arose from the fact that MetRS activity was unstable after dissociation from macromolecular ARS, as reported by other investigators (*7, 14*). Decrease in activity appeared to be marked

and very rapid in a dilute solution. The specific activity of MetRS calculated from the freshly prepared individual fractions on tRNA-Sepharose or Superdex™200 chromatography was usually markedly higher than that of the fraction collected after 2 to 3 h [Table II (RT57 and RT53)].

The addition of serum albumin to the pooled MetRS complex fraction restored MetRS activity. In connection with this, Jakubowski reported that dilution of the solution containing lupin seed ARSs resulted in complete loss of activity at 25°C. Addition of a high concentration of serum albumin partially prevented it (*22*). Therefore, the extent of purification of the MetRS complex of the final products may be higher than the increase of specific activity shown in Table II and is thought to be high enough for further analysis. The silver staining pattern on SDS-PAGE (Fig. 3) seems to confirm a high degree of purification.

It is expected that the L5 band is present on the gel. However, the band representing L5 was very faint (Fig. 3). We consider that this may be due to the notorious insolubility of L5 protein. Previously, using 5SrRNA immobilized Sepharose 4B column, Ulbrich and Wool failed to detect binding of L5 to 5SrRNA (*23*). The same result was obtained by nitrocellulose membrane assay (*24*). Later, the reason for the failure to detect the binding of L5 to 5SrRNA was concluded to be the poor solubility of L5 (*25*). For example, L5 remained insoluble at the top of the Sepharose column (*25*). Furthermore, 5SRNP is exchangeable with 5SrRNA in solution (*25, 26*). Since L5 is insoluble, it pulls the equilibrium towards the dissociation of 5SRNP. Therefore, 5SRNP itself is unstable. Previously, we reported that GTPase activity of 5SRNP prepared from 60S subunits by EDTA treatment was unstable (*27*).

In the experiment shown in Fig. 3 (*1*), we lyophilized the sample after extensive dialysis against water. This method is efficient to concentrate MetRS protein, but most of L5 was lost during dialysis and lyophilization owing to adhesion to the dialysis membrane and lyophilizing vessel. To detect L5 on the gel, the loss of 5SRNP was prevented by the specific procedures described in "RESULTS," using serum albumin-treated microconcentrators. Then, a distinct L5 band was detected, the intensity of which was comparable to that of the MetRS band (Fig. 3B), in addition to the 5SrRNA and $tRNA^{met}$ bands.

The presence of 5SrRNA in the MetRS complex was shown by Northern blot analysis of RNA prepared from the tRNA-Sepharose fraction. Only one band was observed against two bands of 5SrRNA from ribosomal subunit or from the macromolecular ARS complex (Fig. 5 in Ref. *3*). Since it has been reported that the two bands of 5SrRNA on PAGE may correspond to different conformations of 5SrRNA (*18, 19*), our results suggest that a conformational change of 5SrRNA itself occurs in the MetRS complex during the purification procedures.

The presence of L5 was also shown by immune dot blot analysis of the Superdex™200 fractions prepared by Methods I and II, using an ECL Western blotting system as described previously (*3, 4*). It should be noted that rabbit polyclonal antibody did not react to IgG from normal rabbit serum or to 5SrRNA [see Fig. 4(2) in Ref. *3*]. Furthermore, since it is very difficult for L5 to react to the antibody after denaturation with SDS, we could not identify L5 on SDS-PAGE by means of Western blot analysis (*3*).

Based on these findings, it is reasonable to consider that 5SRNP is present in the MetRS complex, although further study should be done to obtain quantitative data.

We found that the specific activity of MetRS complex fraction incubated without the addition of tRNA increased during the purification procedures, in parallel with that in the case of the addition of tRNA. Therefore, $tRNA^{met}$ exists stably in the MetRS complex. The association of 5SRNP with $tRNA^{met}$ in the MetRS complex was suggested by our previous UV-crosslinking experiment (*6*).

Conformational changes in the MetRS complex during the purification procedures, especially in dilute solution, are thought to be the main factor responsible for the MetRS inactivation described above. The following findings support the occurrence of conformational changes in the MetRS complex in a dilute solution. (1) A high concentration of DTT (10 mM) partially prevented rapid inactivation of MetRS. (2) Such inactivation was often observed after storage of the final products obtained on Superdex™200 chromatography in liquid nitrogen (Fig. 7). (3) Aggregation of MetRS was observed in the MetRS activity pattern of the eluate on Superdex™200 chromatography [Fig. 1(3), Fig. 2(3)]. (4) The addition of serum albumin restored the MetRS activity of the MetRS fraction described above.

In this respect it is important that 5SRNP(A) stimulated the MetRS activity of the MetRS complex fraction obtained on Superdex™200 chromatography (Fig. 7), and the degree of stimulation of MetRS activity by 5SRNP was markedly higher than that observed in the macromolecular ARS complex (*5*). Since the specific activity of the MetRS complex fraction used in this assay (without 5SRNP) was lower than that of the freshly prepared original fraction (Fig. 7), it appeared that added 5SRNP(A) enters the macromolecular ARS complex and restores the conformational change of MetRS in it, which occurs during preparation, storage at 0°C or in liquid nitrogen. Incorporation of ^{32}P-labeled 5SrRNA into the macromolecular ARS complex was demonstrated in our previous report (*6*). Thus, it is plausible that 5SRNP plays the role of a positive allosteric effector of MetRS in the MetRS complex, as suggested previously (*5*).

The results of the present experiments indicate that one of the functions of the macromolecular ARS complex is to stabilize the MetRS complex, as suggested by Jakubowski (*22*). Maintenance of 5SRNP in the complex may be important for the activity of MetRS.

We wish to thank to Dr. K. Kubota, Dobashi Kyoritsu Hospital, for his interest in this study and Dr. K. Iwasaki for suggesting the use of octylglucoside to dissociate macromolecular ARS. Thanks are also due to Dr. T. Ueda for providing benzoylated DEAE cellulose, and to Dr. Y. Endo for a critical reading of the manuscript.

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