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EVIDENCE THAT LYSYL- AND/OR ARGINYL-tRNA SYNTHETASES FROM RAT LIVER CONTAIN CARBOHYDRATE

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Summary

Lysyl- and arginyl-tRNA synthetases have been found to exist in multiple molecular weight forms in rat liver. The small molecular weight forms of lysyl- and arginyl-tRNA synthetases copurify throughout a five step chromatographic procedure resulting in a purification of 370- and 140-fold, respectively. The enzymes appear to be homogeneous on Sephadex G-200 and elute at an apparent molecular weight of 240,000. Gas chromatography reveals that the synthetases contain nearly 14% carbohydrate by weight. The carbohydrates present are: mannose, fucose, glucose, galactose, N-acetylglucosamine and N-acetylgalactosamine. This is the first report that aminoacyl-tRNA synthetases may exist as glycoproteins.

In the early 1970's, Bandyopadhyay and Deutscher (1) found all 18 aminoacyl-tRNA synthetases from rat liver exist in a large molecular weight complex. Since this initial observation other investigators working with mammalian cells have found that the aminoacyl-tRNA synthetases can be isolated in multiple forms with respect to their molecular weight (2-9) or association with other cellular constituents (10-12). In general it can be stated that the synthetases occur in three forms: associated with ribosomes and microsomes, in high molecular weight enzyme complexes (15-25S), and as single soluble enzymes (4-9S).

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Our laboratory has found that lysyl- and arginyl-tRNA synthetases exist in multiple molecular weight forms in rat liver[1]. Application of a crude extract to Sepharose 6B results in two peaks of equal activity. The large molecular weight form elutes at a size of 1.5×10^6 daltons while the smaller forms of arginyl- and lysyl-tRNA synthetase elute at 120,000 and 150,000 daltons (for arginyl and lysyl-tRNA synthetases, respectively). In this communication we report the purification of the small molecular weight forms of lysyl- and arginyl-tRNA synthetases and present evidence that either one or both of the enzymes may exist as glycoproteins.

Experimental Procedures

Materials. Long-Evans female rats (125-150g) were purchased from Charles River Laboratories, Inc. Radioactive amino acids were obtained from Schwarz/Mann. Fluorescamine (Fluram) was purchased from Roche Diagnostics and Nonidet P-40 from Particle Data Labs. Chromatographic materials were purchased from the following sources: hydroxylapatite (Bio-Gel HTP) and phosphocellulose, Cellex-P (hydrogen form) from Bio-Rad, DEAE-cellulose (DE-52) from Whatman and ω-aminohexane agarose from Sigma Chemical Co. Blue Sepharose was made according to the procedure of Ryan and Vestlig (13) using Blue Dextran and CNBr-activated Sepharose 4B obtained from Sigma Chemical Co. All other materials were purchased from Bio-Rad, Fisher, or Sigma Chemical Co.

Protein Determination. Protein content of the solutions during the purification procedure was determined by the method of Lowry et al. (14). Determination of the protein profile for Sephadex G-200 gel filtration was done by the fluorescamine protein assay (15).

Aminoacyl-tRNA Synthetase Assay. The enzyme assay used was the aminoacylation assay of Bandyopadhyay and Deutscher (1). The assay volume was 100 µl and contained 25 µmoles of Tris-acetate (pH 7.2), 0.5 µmoles of $MgCl_2$, 0.5 µmoles of ATP, 10 µg of bovine serum albumin, 200-250 µg of beef liver tRNA, 0.2 µmoles of disodium EDTA and 0.025 µmoles of amino acid (8-12 cpm/ pmole). The reaction was allowed to proceed for 5 minutes at 37°C and was stopped by the addition of 2.5 ml of ice cold 10% trichloroacetic acid (TCA) containing 20 mM sodium pyrophosphate. After 10 minutes on ice the TCA precipitable counts were collected on Whatman 934AH glass fiber discs and washed six times with 2 ml of 2.5% TCA containing 20 mM sodium pyrophosphate. The filters were oven dried, cooled, and then counted in a toluene based scintillation fluid using a Beckman LS-3133P Scintillation Counter.

Carbohydrate Analysis. The carbohydrate moiety associated with the purified synthetases was hydrolyzed with 0.6 N HCl at 100°C for 4 hours. The neutral sugars were separated from the protein and amino sugars by

1. R. H. Hilderman and T. C. Gilliam. Isolation of Multiple Forms of aminoacyl-tRNA Synthetases from Rat Liver. Submitted.

anion and cation exchange. The neutral sugars were then derivatized to their corresponding aldononitrile acetates by the method of Varma et al. (16). The derivatized sugars were analyzed on a 2% diethylene glycol adipate (DEGA) column in a Perkin-Elmer 3 Sigma gas chromatograph equipped with dual flame ionization detectors at a nitrogen flow rate of 24 ml/min. Amino sugars were hydrolyzed in 3 N HCl at 125°C and 15 psi for 45 min. They were then derivatized and analyzed according to Mawhinney and Feather.[2]

Results

A detailed account of the purification procedure will be published later. Table I gives a summary of the scheme which results in the purification of the small molecular weight forms of lysyl- and arginyl-

Table I

Purification of Lysyl- and Arginyl-tRNA Synthetases

Step		Total Units	Total Protein	Specific Activity	Yield	Purification
Crude Extract	Arg	1508 (604)	17066	0.088 (0.035)	100	
	Lys	1716 (628)		0.100 (0.037)	100	
ω-Amino Hexyl-Agarose	Arg	604 (604)	1972	0.31 (0.31)	40 (100)	3.5 (8.9)
	Lys	628 (628)		0.32 (0.32)	37 (100)	3.2 (8.6)
DEAE-Cellulose	Arg	360 (360)	779	0.46 (0.46)	24 (60)	5.2 (13.1)
	Lys	1083 (1083)		1.39 (1.39)	63 (172)	13.9 (37.6)
Hydroxyl-apatite	Arg	221 (221)	65.3	3.38 (3.38)	15 (37)	38.4 (96.6)
	Lys	389 (389)		5.96 (5.96)	23 (62)	59.6 (161)
Phospho-cellulose	Arg	127 (127)	40.3	3.15 (3.15)	8.4(21)	35.8 (90)
	Lys	367 (367)		9.10 (9.10)	21 (58)	91 (246)
Blue Sepharose	Arg	54 (54)	11.8	4.57 (4.57)	3.6(8.9)	52.3 (130.1)
	Lys	163 (163)		13.8 (13.8)	9.5(26)	137.5 (373)

A detailed account of the purification will be reported elsewhere. The fold purification and yield of the enzymes has been presented in two different ways. The numbers outside the parentheses were arrived at by calculating the yield and purification considering the total enzyme activity to be composed of both the large and small forms. However, we feel that the numbers in parentheses are a more accurate indication of the yield and purification. They have been calculated relative to the amount of activity that exists in the small molecular weight forms, which is the only activity we wish to purify. The ω-aminohexane agarose column has been shown to be an effective technique for separating the large molecular weight synthetases from the small molecular weight enzymes.[1] The small forms adhere to the colunn and the amount of activity recovered from the salt wash of the column is regarded 100% of the activity of the small molecular weight enzymes present in the crude extract.

2. T. P. Mawhinney and M. S. Feather. A Rapid and Quantitative Procedure for the Analysis of Amino Sugars. Submitted.

tRNA synthetases 370- and 140-fold pure, respectively. The final product is devoid of all other aminoacyl-tRNA synthetases.

An interesting aspect of the purification is the copurification of the small molecular weight enzymes. In addition to the procedures used in the purification, CM-agarose and preparative isoelectric focusing also failed to separate the activities. The association of lysyl- and arginyl-tRNA synthetases with each other has also been documented by other workers. In rabbit reticulocytes, Irvin and Hardesty (11) found these enzymes to be associated with ribosomes and with each other in a 14S enzyme complex. From rat liver, Goto and Schweiger (17) purified lysyl-tRNA synthetase 440-fold and found that arginyl-tRNA synthetase copurified during the procedure 260-fold. More recently, out of rat liver, Dang and Yang (9) were unable to separate the enzymes by affinity chromatography on a lysine-Sepharose column. Whether or not the two activities exist on the same or separate polypeptides cannot yet be stated. It must be noted, however, that one group (18) using rat liver has purified arginyl-tRNA synthetase 100-fold having only 3% lysyl-tRNA synthetase still present.

In order to determine purity and an apparent molecular weight, a sample of the purified synthetases was chromatographed on Sephadex G-200 as described in Figure 1. A single protein peak emerged from the column, and it coincides precisely with both enzyme activities. The specific activities of the enzymes were constant throughout the peak, with lysyl-tRNA synthetase varying from 13 to 16.1 units/mg and arginyl-tRNA synthetase from 4.8 to 5.5. units/mg, providing evidence for the homogeneity of both activities. The enzymes eluted from the column at an apparent molecular weight of about 240,000.

Further attempts to ascertain the purity of the enzymes by native gel electrophoresis 6% acrylamide showed no entry into the gels (data not shown). Kane et al. (19) were unable to get histidinyl-tRNA syn-

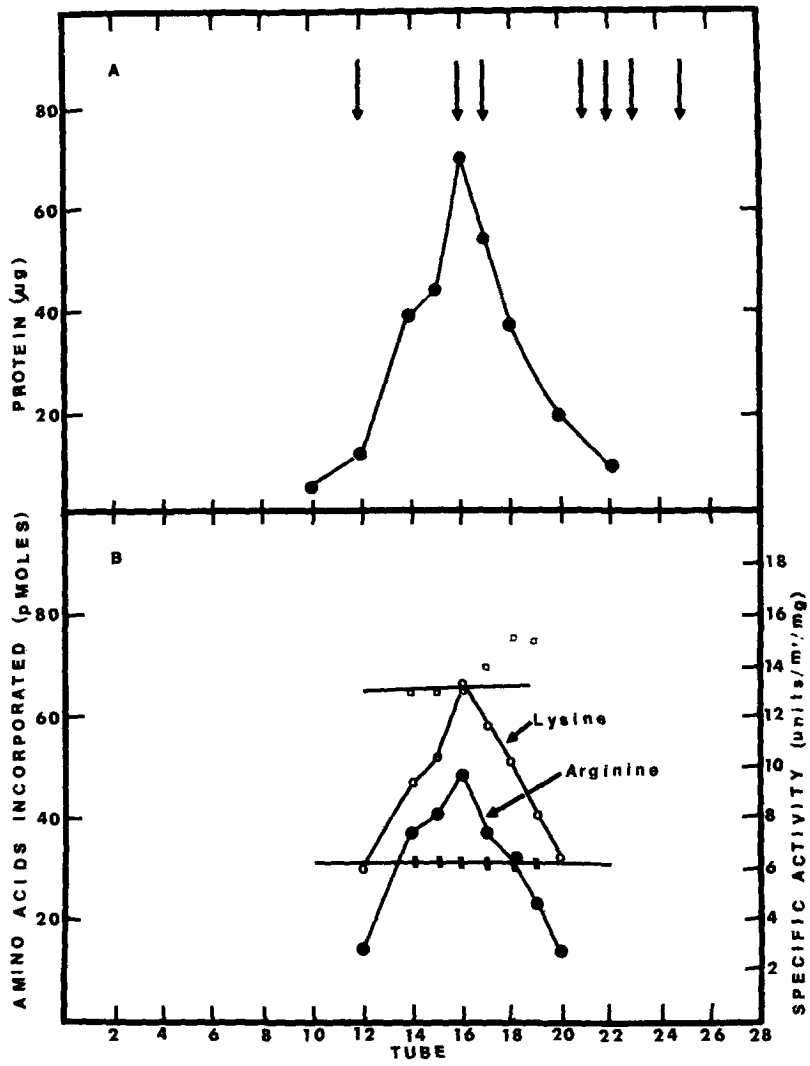

Figure 1. Gel Filtration of the Purified Enzymes on Sephadex G-200. A 0.75 ml aliquot of the concentrated enzyme solution was applied to a Sephadex G-200 column (0.9 x 54 cm) that had been equilibrated at a flow rate of 4 ml/hr with a buffer containing 20 mM potassium phosphate (pH 7.0), 10 mM β-mercaptoethanol, 8 mM $MgCl_2$, 10% glycerol (v/v) and 0.1% Nonidet P-40. A. The arrows indicate the elution volume of proteins used to standardize the column. From left to right the arrows represent thyroglobulin (669,000), catalase (240,000), aldolase (158,000), hemoglobin (64,000), ovalbumin (43,000), chymotrypsinogen A (25,000), and ribonuclease A (13,700). Fluorescamine protein assay of the fractions reveals that the purified synthetases elute from the Sephadex G-200 at an apparent molecular weight of 240,000. B. Aminoacyl-tRNA synthetase activity was determined as described in Experimental Procedures. Both enzyme activities peak at a molecular weight of 240,000 and the specific activities remain constant throughout the protein profile. ○-○-○ specific activity of lysyl-tRNA synthetase, ●-●-● specific activity of arginyl-tRNA synthetase.

thetase to enter native gels and attributed it to aggregation. However, our purified enzymes have entered 4.5% acrylamide gels, but only weakly stained, diffuse bands could be seen with either Coomassie Blue G-250 or R-250 protein stain.

This has also been the case in gel electrophoresis under denaturing conditions. Gel electrophoresis in the presence of sodium dodecyl sulfate reveals multiple bands which are also broad, weakly stained, and of varying intensities. This could be due to aggregation or perhaps microheterogeneity of carbohydrate moieties.

In addition to the anomalous electrophoretic data, a precipitate results when the purified synthetases, which have been extensively dialyzed against deionized distilled water, are assayed for protein content by the method of Lowry et al. (14). Since the alkaline copper solution of the Lowry is similar to Benedict's sugar test, the results are consistent with the fact that carbohydrate may be present. These results suggested to us that the synthetases may be glycoproteins. To further investigate the possibility that carbohydrate may be present, gas chromatography was used.

The purified synthetases were lyophilized and analyzed for carbohydrate content as described in Experimental Procedures. Table II shows the results from two separate enzyme preparations. The carbohydrates found with the synthetases are mannose, fucose, glucose, galactose, N-acetylglucosamine, and N-acetylgalactosamine. Table II also shows that the carbohydrates comprise nearly 14% of the enzymes by weight.

In support of a glycoprotein nature for the enzymes is the fact that they adsorb to Concanavalin A-Sepharose (data not shown), suggesting that the synthetases are associated with the carbohydrate. Although Concanavalin A-Sepharose has the capacity to bind proteins by hydrophobic interactions, it is unlikely that this is the case here, since both the buffer used to equilibrate the column and the sample applied

Table II

Carbohydrate Composition of Purified Synthetases.
Analysis of Two Different Enzyme Preparations.

Carbohydrate	µg CHO/mg Protein	
	Preparation 1	Preparation 2
Mannose	70.5	69.3
Fucose	29.4	29.7
Glucose	18.8	18.4
Galactose	0.97	0.96
N-Acetylglucosamine	11.1	12.4
N-Acetylgalactosamine	5.7	5.1
Carbohydrate Percentage	13.7	13.6

Lysyl- and Arginyl-tRNA synthetases from two different preparations analyzed for carbohydrate content as described in Experimental Procedures.

contained 0.1% Nonidet P-40. The periodic acid-Schiff's base glycoprotein stain has been applied to samples electrophoresed in 4.5% acrylamide native gels, and a weakly staining band coinciding with the Coomassie Blue band was observed. The reason that protein and glycoprotein stains are taken up only slightly is not known.

Amino acid composition (data not shown) gave a minimum molecular weight of 22,400. The best fit of this to the Sephadex G-200 data, taking into account that almost 14% of the synthetases are carbohydrates, yields a molecular weight of 233,000 for the glycoprotein. From Table II the number of carbohydrate residues per 233,000 entity were calculated and found to be as follows: mannose, 78.2; fucose, 36.2; glucose, 20.8; galactose, 1.09; N-acetylglucosamine, 10.7; and N-acetylgalactosamine, 4.9. Since the number of carbohydrate molecules are near integral, we feel this helps substantiate the 233,000 molecular weight.

Discussion

The small molecular weight forms of lysyl- and arginyl-tRNA synthetases from rat liver have been purified 370- and 140-fold, respectively. The synthetases exhibit an apparent molecular weight of 233,000. Gas chromatographic analysis reveals that the purified synthetases contain nearly 14% carbohydrate by weight. The carbohydrates present are mannose, fucose, glucose, galactose, N-acetylglucosamine, and N-acetylgalactosamine. Although the data suggests that the synthetases are glycoproteins, the possibility that these enzymes are associated with a glycoprotein has not been ruled out. However, we feel that both the high degree of reproducibility and the larger amount of carbohydrate argue against a contaminating glycoprotein.

In addition to their role in protein biosynthesis, the aminoacyl-tRNA synthetases have been postulated to interact with the amino acid transport system in mammalian cells (20,21). These workers, along with those who find the synthetases with microsomes (10,22), have raised the possibility that the enzymes may be membrane-associated. Since glycoproteins are known to be components of membranes, a glycoprotein nature for the synthetases enhances the arguement that these enzymes are membrane associated. Membrane associated synthetases could also facilitate protein biosynthesis *in vivo*. If the synthetases are bound in close proximity to membrane-bound polysomes, this could minimize the diffusion necessary for the aminoacylated tRNA to reach the ribosomes. Also the association of synthetases with elongation factors (23) and tRNA processing enzymes (24,25) suggests a high degree of supramolecular structure.

Other characteristics of lysyl- and arginyl-tRNA synthetases also suggest that the enzymes may be membrane bound. Calculations from the amino acid composition according to the method of Capaldi and Vanderkooi (26) show the enzymes to possess a polarity of 51.7%, which agrees with known extrinsic membrane proteins. Also, the purification of the enzymes

is greatly enhanced by the addition of a non-ionic detergent, Nonidet P-40. Pan et al. (27) used 0.1% Brij-58 in their purification of cysteinyl-tRNA synthetase.

The possibility that lysyl- and/or arginyl-tRNA synthetases may exist as membrane-associated glycoproteins could explain why these enzymes have been found in multiple molecular weight forms and in association with other cellular constituents. Lysyl- and arginyl-tRNA synthetases have been found to be associated with ribosomes (6,11,28), microsomes (10,22,29), and in high molecular weight complexes (1,3,4,6-9). The aggregation seen during electrophoresis by us and others, as well as the tendency for these enzymes to aggregate with themselves, other synthetases, and still other cellular components may simply be due to the physical properties of the enzymes.

Work is now underway in our laboratory to determine the subunit structure of the synthetases (if any exists), the function of the carbohydrate moiety, and the possibility that the enzymes are membrane associated.

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