Plant Physiol. (1973) 52, 13–16

An Arginyl-Transfer Ribonucleic Acid Protein Transferase from Cereal Embryos[1]

Received for publication December 7, 1972

CHARLES O. MANAHAN AND ALVA A. APP
Boyce Thompson Institute, 1086 North Broadway, Yonkers, New York 10701

ABSTRACT

Embryos from rice (*Oryza sativa* L. var. Bluebonnet) and wheat (*Triticum aestivum* L.) contain an aminoacyl-tRNA protein transferase which transfers arginine from arginyl-tRNA to the N terminus of a protein acceptor. The activity was measured *in vitro* in a reaction mixture containing embryo supernatant fraction, buffer, sulfhydryl reagent, and arginyl-tRNA. It was not dependent on the usual cofactors for ribosomal protein synthesis, nor was it sensitive to cycloheximide or puromycin. However, the activity was inhibited by ribonuclease. The enzyme was purified 33-fold from a crude homogenate of rice embryos. An apparent endogenous substrate from rice embryos was prepared free of transferase activity; however, the transferase was not purified sufficiently to show absolute dependence on the presence of this endogenous substrate.

Aminoacyl-tRNA protein transferases catalyze the transfer of amino acids from aminoacyl-tRNA to the N-terminal ends of preformed protein acceptors (9, 14). They were first discovered by Kaji *et al.* in *E. coli* (5). They were subsequently found in rat liver (6), plasma cell tumors (18), sheep thyroid glands (16), and rabbit liver (15). These enzymes can be divided into two classes: those which are found in bacteria and transfer leucine and phenylalanine (8), and those found in eucaryotes which transfer arginine (6, 13).

The bacterial enzyme requires that an NH_2-terminal arginine be present on the protein acceptor (9, 10), and further that the tRNA moiety be intact, since no transfer occurs from the acylated 3′-pentanucleotide fragment of *E. coli* $tRNA^{Phe}$. The exact anticodon triplet present on the tRNA molecule does not seem to be the basis for specificity in the reaction, since valyl-$tRNA^{Val}$ will not serve as a substrate, but phenylalanyl-$tRNA^{Val}$ will (10).

The enzyme isolated from rabbit liver has a requirement for aspartic or glutamic acid as the NH_2-terminal amino acid of the acceptor protein (14).

MATERIALS AND METHODS

Embryo Isolation. Isolation of excised embryos of wheat (*Triticum aestivum* L.) and rice (*Oryza sativa* L. var. Bluebonnet) was performed by a modification of the organic solvent method of Johnston and Stern (4).

Enzyme Purification. The embryos were homogenized in 10 mM tris acetate, pH 7.8 (8 ml/g embryos) with a mortar and pestle at 0 C. The homogenate was centrifuged for 10 min at 27,000*g* at 3 C, and the supernatant fraction was decanted and subsequently centrifuged for 2 hr at 40,000 rpm in a Spinco Ti50 rotor at 2 C. The supernatant fraction was removed and designated as crude supernatant fraction.

Crude supernatant fraction was brought to pH 5.7 (temperature approximately 0 C) by the addition of 0.05 N HCl. The preparation was then centrifuged immediately at 15,000*g* for 10 min at 2 C. The supernatant fraction was decanted, adjusted to pH 4.8 by the addition of 0.05 N HCl, and again centrifuged as above. The material, which precipitated at pH 4.8, was saved and dissolved in 10 mM tris acetate, pH 8.2.

The preparation was then brought to 30% saturation by the addition of solid ammonium sulfate at 0 C. The suspension was immediately centrifuged at 15,000*g*. The supernatant fraction was decanted, brought to 60% saturation with solid ammonium sulfate, and immediately centrifuged as before. The second supernatant fraction was discarded, and the precipitated material was dissolved in a minimal volume of 10 mM tris acetate, pH 8.2.

An equal volume of 8.0 M urea containing 0.4% Triton X-100 and 20 mM potassium phosphate, pH 8.0, was added to the enzyme solution which was then allowed to stand for 10 min at 0 C. Four milliliters of this preparation were chromatographed at 2 C on a Sephadex column (2.5 × 45 cm) packed with Sephadex G-200 in the manner described by Sachs and Painter (12). The column was eluted with 4.0 M urea in 20.0 mM potassium phosphate, pH 8.0, buffer. The A_{254} was continuously monitored and 6.0-ml fractions were collected. The fractions were dialyzed separately overnight against 20 mM potassium phosphate, pH 8.0, at 0 C and then assayed.

Enzyme Assay. The enzyme usually was assayed by incubation with ^{14}C-arginyl-tRNA, 3.0 mM dithiothreitol, 20 mM tris acetate, pH 8.1, and 0.2 to 1.0 mg of rice protein substrate in a volume of 0.5 ml at 30 C. The protein substrate was prepared by heating the crude rice supernatant fraction for 5 min on a boiling water bath and dialyzing at 2 C for 48 hr against four changes of 200 volumes of 20 mM potassium phosphate pH 7.8. The procedure for preparation of ^{14}C-arginyl-tRNA has been described (1).

The enzyme activity was also assayed where indicated by incubation with 40 mM tris acetate, pH 8.1, 3.0 mM dithiothreitol, 3.5 mM Mg acetate, 0.2 μc/ml of ^{14}C-amino acids, 0.1 mM GTP, 4.0 mM creatine phosphate, 0.030 mg/ml of creatine kinase, 1.0 mM ATP, and 24 mM KCl in a volume of 0.5 ml.

The amount of radioactivity insoluble in trichloroacetic acid was determined as previously described (1). All incorporation

[1] This research was supported in part by Grant GB 19914 from the National Science Foundation.

data reported are total counts corrected for background (42 cpm).

N-Terminal Analyses. A large amount of the ^{14}C-arginyl product was prepared using the ATP generating system reaction conditions given under "Enzyme Assay" above. The reaction mixture was dialyzed twice at 2 C against 200 volumes of 20 mM potassium phosphate buffer, pH 7.8, for 4 hr, once against the same buffer containing 1.0 mM ^{12}C-arginine, and finally twice against the buffer alone. The product was stored in liquid N_2 until needed. A portion of radioactive product was reacted with fluorodinitrobenzene as described by Block *et al.* (2), and the purified product of this reaction hydrolyzed in a sealed ampoule in 6.0 N HCl for 18 hr at 105 C. The resulting free amino acids and free dinitrophenylated amino acids were chromatographed on Whatman No. 1 paper in the following two solvent systems: acetone–water–urea, 60:40:0.5, and water saturated 1-butanol. Chromatography was carried out at 25 C.

Another portion of radioactive product was used for the cyanate amino terminal analysis. For this purpose the mixture was reacted with sodium cyanate and hydrolyzed as described by Stark and Smyth (17). The resulting hydrolysate was chromatographed on Whatman No. 1 paper with 70% aqueous acetone in addition to the solvents described above.

Chromatograms were dried, cut into 1.0-cm strips perpendicular to the direction of solvent flow, and counted in a liquid scintillation counter using 10 ml of scintillation fluid per strip. A toluene based scintillation fluid consisting of 3.0 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-2-(4-methyl-5-phenyloxazole)-benzene per liter of toluene was used.

Miscellaneous. Protein concentrations were determined by either the Biuret method (7) or the Lowry *et al.* method (11).

Ammonium sulfate precipitations were carried out at 0 C with solid ammonium sulfate using the nomograph of di Jeso (3).

RESULTS

^{14}C-Amino acid incorporation by rice supernatant fraction was not dependent on ribosomes. In addition, neither GTP nor a significant concentration of monovalent cation was required (Table I). However, incorporation was dependent on the presence of Mg^{2+} and ATP.

Since the results presented in Table I were obtained by employing a mixture of ^{14}C-amino acids, ^{12}C-amino acids were added singly to the reaction mixture to determine which ^{14}C-amino acids were responsible for the incorporation (Table II). Arginine was the only amino acid which significantly decreased

Table I. *^{14}C-Amino Acid Incorporation by Crude Supernatant Fraction from Rice*

The complete reaction mixture used was that given under "Materials and Methods;" 0.2 ml of crude supernatant fraction (15 mg/ml protein) and 3.0 A_{360} units of ribosomes were also present. Incubation time was 1 hr.

Experimental Conditions	Incorporation
	cpm
Complete reaction mixture	1602
− Ribosomes	1863
− GTP	1714
− Mg^{2+}	23
− ATP, cretaine phosphate and creatine kinase	32
− K^+	1577

Table II. *Isotopic Dilution of ^{14}C-Amino Acid Incorporation by Crude Supernatant Fraction from Rice*

Reaction mixture is that described under "Materials and Methods," except that 1.0 mM of the above listed amino acids was present as noted. The control value (100% incorporation) was 2572 cpm. All percentage values are ±4%.

^{12}C-Amino Acid Present	Incorporation
	%
None	100
Alanine	100
Arginine	39
Aspartic acid	100
Glutamic acid	100
Glycine	100
Histidine	95
Isoleucine	95
Leucine	93
Lysine	100
Phenylalanine	97
Proline	99
Serine	92
Threonine	100
Tyrosine	100
Valine	97

Table III. *^{14}C-Arginine Incorporation by Crude Supernatant Fraction from Rice and Employing Arginyl-tRNA as Substrate*

There were 3400 cpm of ^{14}C-arginyl-tRNA per tube. Ribonuclease concentration was 10.0 μg/ml. Incubation time was 1 hr.

Experimental Condition	Incorporation
	cpm
Complete reaction mixture	986
− Ribosomes	998
− Mg^{2+}	934
− ATP and ATP generating system	957
+ Ribonuclease	3

the incorporation obtained with the crude supernatant fraction. It appears therefore that the soluble system is specific for arginine.

As expected, employing ^{14}C-arginine as the sole labeled substrate gave good rates of incorporation, and this incorporation could be substantially reduced (3182 cpm to 25 cpm) by the addition of ^{12}C-arginine. Leucine and serine somewhat depressed this ^{14}C-arginine incorporation. This was probably due to trace amounts of ^{12}C-arginine in these amino acids, since the use of either ^{14}C-leucine or ^{14}C-serine alone did not result in significant incorporation by the rice supernatant fraction.

If ^{14}C-arginyl-tRNA was employed as the substrate in the assay, Mg^{2+}, ATP, and the ATP generating system were not necessary (Table III). These components apparently were needed to synthesize arginyl-tRNA *in vitro*. As expected, the reaction was abolished by ribonuclease.

The time course of the arginine incorporation was examined with the ^{14}C-arginyl-tRNA as substrate. The data in Figure 1 indicate a linear response for the first 10 to 15 min, and then a gradual decline in the rate of incorporation over the next 45 to 50 min.

The ^{14}C-arginine incorporation by the rice or wheat supernatant fraction was not sensitive to either puromycin or cycloheximide (Table IV).

It may be possible to separate the endogenous acceptor substrate from transfer activity by heating the crude supernatant fraction as described in Methods. While all enzymatic activity was lost after this treatment, the heat-treated preparation could still stimulate the incorporation by the partially purified enzyme. Unfortunately, we were not able to separate completely enzymatic activity from endogenous substrate. The partially purified enzyme preparation which had the highest specific activity (G-200 column fraction) showed good incorporation in the absence of added substrate. However, its activity was doubled in the presence of heat treated supernatant fraction (500 to 1200 cpm). Since it has been reported that heated rabbit liver supernatant can serve as a substrate for the liver enzyme, we suggest that the heated rice preparation may serve in a similar manner here (13).

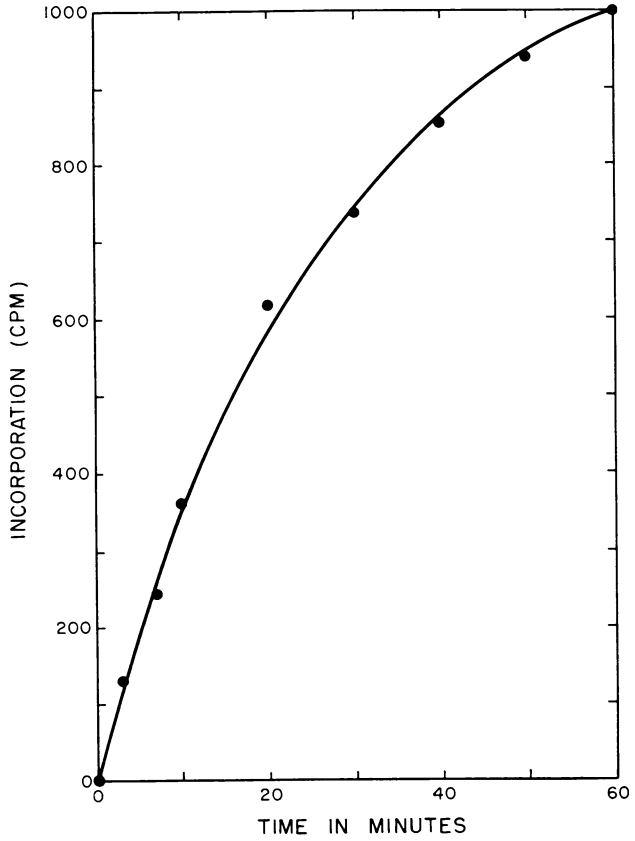

FIG. 1. Time course of ^{14}C-arginine incorporation by crude supernatant fraction from rice. Crude supernatant fraction contained 5.0 mg/ml protein. Each tube contained 3700 cpm of ^{14}C-arginyl-tRNA (2.4 μg).

Table IV. *^{14}C-Arginine Incorporation by Wheat and Rice Supernatant Fractions in the Presence of Cycloheximide or Puromycin*

Cycloheximide concentration was 1.0 mM; puromycin, 1.0 mM; rice supernatnt fraction, 12 mg protein/ml; and wheat supernatant fraction, 15 mg protein/ml. Each tube contained 3500 cpm of ^{14}C-arginyl-tRNA.

Experimental Conditions	Wheat Supernatant Fraction	Rice Supernatant Fraction
	cpm	
Complete reaction mixture	377	353
As above + puromycin	391	441
As above + cycloheximide	388	342

Table V. *N-Terminal Analysis of ^{14}C-Arginyl-acceptor from Rice*

Chromatograms usually contained between 350 and 450 cpm.

Solvent System	N-Terminal Assay	Cpm as Arginine	Cpm as N-Terminal Arginine
		%	
1-Butanol, water saturated	FDNB	51	43
Acetone, H_2O, urea	FDNB	33	51
Acetone, H_2O, urea	Cyanate	0	84
Aqueous acetone (70%)	Cyanate	3	79

Table VI. *Purification of Arginyl-tRNA Protein Transferase from Rice Embryos*

Each tube contained 2100 cpm of ^{14}C-arginyl-tRNA. Purification is given in multiples of the crude supernatant fraction. All actual cpm observed were between 500 and 1400.

Fraction	Specific activity	Purification	Recovery
	cpm/mg		%
Crude supernatant fraction	198	1.0	100
pH precipitate	1188	6.0	48
$(NH_4)_3SO_4$ precipitate	2433	12.3	37
G-200 column fraction	6573	33.2	21

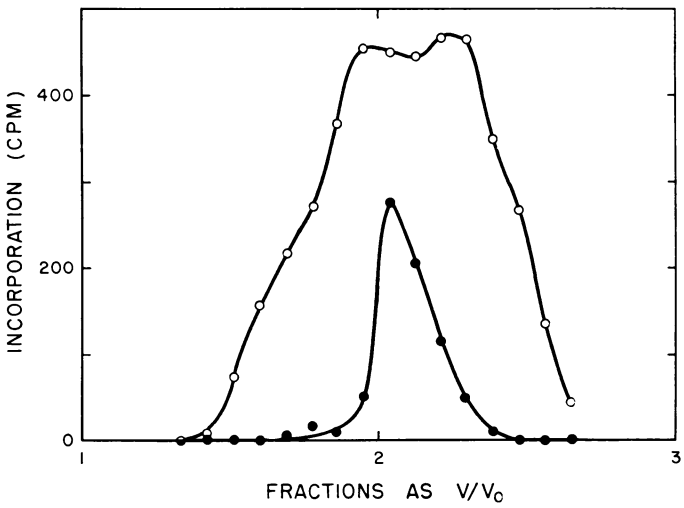

FIG. 2. Arginyl-tRNA protein transferase activity distribution with agarose gel filtration. Column dimensions were 45 cm × 2.5 cm diameter. Buffer used was 20 mM potassium phosphate, pH 7.8. Fraction volume given as ratio of the elution volume (including the void volume) to the void volume (69 ml). Activity distribution in 20 mM phosphate (○); activity distribution in buffer containing 4.0 M urea (●).

The results of the N terminal analyses indicated that most of the incorporated ^{14}C-arginine was in the N-terminal position (Table V). The FDNB[2] data revealed that only half of the incorporated ^{14}C-arginine formed the dinitrophenylated derivative. This suggested that perhaps the enzyme was adding more

[2] Abbreviation: FDNB; fluorodinitrobenzene.

than one arginyl residue per polypeptide chain, or that there was an incomplete reaction with FDNB. Since the cyanate method showed approximately 80% of the ^{14}C-arginine to be N-terminal, it seems more likely that the reaction with FDNB was not complete. Further, since virtually none of the ^{14}C-arginine liberated by the cyanate procedure was recovered as free arginine, the 20% not accounted for as the hydantoin derivative may have been lost through some side reaction such as photodegradation.

An attempt was made to purify the transferase from rice embryos. The maximum observed specific activity was obtained by using the procedure described under "Materials and Methods" (Table VI). Attempts to purify the enzyme further were unsuccessful. The broad range of molecular size, as measured by the agarose gel column chromatography, was decreased considerably in 4.0 M urea (Fig. 2). This decrease in size in the presence of urea could be due to inactivation of certain forms of the enzyme or to disruption of large aggregates.

Increasing concentrations of sulfhydryl reagent (2-mercaptoethanol) irreversibly denatured the enzyme but did not change its distribution upon fractionation with ammonium sulfate. This is in marked contrast to the behavior of the rabbit liver enzyme, where 100 mM 2-mercaptoethanol apparently removed the enzyme from an enzyme-macromolecular complex (13). Incidentally, increasing the KCl concentrations up to 2.0 M did not change the distribution of the enzyme during agarose (Bio Rad A-5 M) gel filtration.

DISCUSSION

Both wheat and rice embryos contain an arginyl-tRNA protein transferase. The enzyme appears to transfer arginine from arginyl-tRNA to the N terminus of a protein acceptor. The *in vitro* activity of the enzyme requires a reaction mixture containing a sulfhydryl reagent and arginyl-tRNA. Unlike ribosomal incorporating systems, there is no requirement for monovalent or divalent cations, ATP, GTP, or ribosomes. However, the enzyme can function under the conditions usual for *in vitro* ribosomal incorporation. For this reason, caution should be exercised in interpreting results from experiments employing ^{14}C-arginine (or amino acid mixtures containing ^{14}C-arginine) to measure *in vitro* protein synthesis.

The enzyme from cereal embryos is similar to the one found in rabbit liver inasmuch as it appears to be bound to a complex cellular fraction. However, in the case of the cereal enzyme, the bonding does not appear to be eliminated with sulfhydryl reagents.

The occurrence of this class of enzymes in bacteria, mammals, and higher plants suggests that they may serve an important function in modifying pre-existing proteins.

LITERATURE CITED

1. App, A. A., M. G. Bulis, and W. J. McCarthy. 1971. Dissociation of ribosomes and seed germination. Plant Physiol. 47: 81–86.
2. Block, R. J., E. L. Durrum, and G. Zweig. 1958. A Manual of Paper Chromatography and Paper Electrophoresis, Ed. 2. Academic Press, New York.
3. di Jeso, F. 1968. Ammonium sulfate concentration conversion nomograph for 0°. J. Biol. Chem. 243: 2022–2023.
4. Johnson, F. B. and H. Stern. 1957. Mass isolation of viable wheat embryos. Nature 179: 160–161.
5. Kaji, A., H. Kaji, and G. D. Novelli. 1963. A soluble amino acid incorporating system. Biochem. Biophys. Res. Commun. 10: 406–409.
6. Kaji, H., G. D. Novelli, and A. Kaji. 1963. A soluble amino acid-incorporating system from rat liver. Biochim. Biophys. Acta 76: 474–477.
7. Layne, E. 1957. Spectrophotometric and turbidimetric methods for measuring proteins. Methods Enzymol. 3: 447–454.
8. Leibowitz, M. J. and R. L. Soffer. 1970. Enzymatic modification of proteins. III. Purification and properties of a leucyl, phenylalanyl transfer ribonucleic acid-protein transferase from *Escherichia coli*. J. Biol. Chem. 245: 2066–2073.
9. Leibowitz, M. J. and R. L. Soffer. 1971. Enzymatic modification of proteins. VI. Site of acylation of bovine serum albumin in the leucine, phenylalanine-transfer reaction. J. Biol. Chem. 246: 4431–4438.
10. Leibowitz, M. J. and R. L. Soffer. 1971. Enzymatic modification of proteins. VII. Substrate specificity of leucyl, phenylalanyl-transfer ribonucleic acid-protein transferase. J. Biol. Chem. 246: 5207–5212.
11. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275.
12. Sachs, D. H. and E. Painter. 1972. Improved flow rates with porous Sephadex gels. Science 175: 781–782.
13. Soffer, R. L. 1970. Enzymatic modification of proteins. II. Purification and properties of the arginyl transfer ribonucleic acid-protein transferase from rabbit liver cytoplasm. J. Biol. Chem. 245: 731–737.
14. Soffer, R. L. 1971. Enzymatic modification of proteins. V. Protein acceptor specificity in the arginine-transfer reaction. J. Biol. Chem. 246: 1602–1606.
15. Soffer, R. L. and Horinishi. 1969. Enzymic modification of proteins. I. General characteristics of the arginine-transfer reaction in rabbit liver cytoplasm. J. Mol. Biol. 43: 163–175.
16. Soffer, R. L. and N. Mendelsohn. 1966. Incorporation of arginine by a soluble system from sheep thyroid. Biochem. Biophys. Res. Commun. 23: 252–258.
17. Stark, G. R. and D. G. Smyth. 1963. The use of cyanate for the determination of NH_2-terminal residues in proteins. J. Biol. Chem. 238: 214–226.
18. Weinstein, I. B. and E. F. Osserman. 1964. Amino acid incorporation into protein by a cell-free system from plasma cell tumors. Acta Univ. Int. Contra Cancrum 20: 932–936.