Catalytic Activation of Transfer Ribonucleic Acid by a Mammalian Protein†

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ABSTRACT: A tRNA activator has been isolated from mammalian organs which increases the capability of tRNA to accept certain amino acids through the action of mammalian aminoacyl-tRNA synthetases. This activity may be separated from the aminoacyl-tRNA synthetases for isoleucine, leucine, lysine, serine, and methionine by fractionation of liver or pancreas cytosol with ammonium sulfate or by chromatography over Sephadex G-200. The tRNA activating material is nondialyzable and is destroyed by trypsin or short heating. It acts catalytically. A molecular weight of approximately 45 000 was obtained by chromatography of tRNA activator on a calibrated Sephadex G-150 column. Activator increases acceptance of yeast tRNA for the amino acids isoleucine, leucine, lysine, serine, and methionine. It shows higher activity on liver $tRNA^{Met}_f$, $tRNA^{Met}_m$, and $tRNA^{Lys}$ than on unfractionated liver tRNA. Removal of protein from mammalian tRNA by extra phenol extractions, chromatography, or proteinase treatment increases its response to activator.

Makman and Cantoni (1966) reported the presence of a substance in a yeast extract which increased the rate of formation of Ser-tRNA from (*Escherichia coli*) $tRNA^{Ser}$ with purified yeast serine-tRNA synthetase. Enhanced activity was not demonstrable, however, when (yeast) $tRNA^{Ser}$ served as substrate. Cantoni later tested inorganic pyrophosphatase and found that it did increase Ser-$tRNA^{Ser}$ formation by yeast enzyme similar to that obtained with "enhancing factor" (cited by Jacobson, 1971). Pearlman and Bloch (1967) observed that crude preparations of tyrosine-tRNA synthetase from calf liver catalyzed the formation of Tyr-tRNA from yeast tRNA, whereas purified fractions of the enzyme were inactive. They were able to separate a "factor" from the crude enzyme which apparently modified yeast tRNA and restored its acceptor activity. The liver factor exerted no enhancing effect when liver tRNA served as substrate. Neither of these "factors" was characterized except in a preliminary manner.

In the present work we report on a protein from mammalian organs with similar properties to the "factors" mentioned above. It activates yeast tRNA in a catalytic manner to improve its acceptor activities for the amino acids isoleucine (Ile), leucine (Leu), lysine (Lys), serine (Ser), and methionine (Met). It activates chromatographed liver tRNA for Met and Lys to a greater extent than unfractionated liver tRNA. This tRNA activator can be separated from the high molecular weight aminoacyl-tRNA synthetases of a liver cytosol extract by chromatography over Sephadex G-200, or by fractionation with ammonium sulfate. Some of the properties of this tRNA activation factor are described.

Materials and Methods

Materials. ^{3}H-Labeled amino acids were purchased from New England Nuclear Inc. Specific activities were: L-methionine 190 mCi/mmol; L-lysine, 2.2 Ci/mmol; L-leucine, 5 Ci/mmol; L-isoleucine, 65 Ci/mol; L-serine, 17 Ci/mmol; L-valine, 16 Ci/mmol; and L-phenylalanine, 12 Ci/mmol. The latter six were diluted with the corresponding ^{1}H-labeled amino acid to furnish approximately the same yield of [^{3}H]aminoacyl tRNA as with [^{3}H]Met-tRNA in the standard assay. ATP, DTT,[1] $PhCH_2SO_2F$, lysozyme, and bovine serum albumin were obtained from Sigma. Ultrapure ammonium sulfate was purchased from Schwarz/Mann. Yeast-tRNA was from Calbiochem. Trypsin and soybean trypsin inhibitor were obtained from Worthington Biochemical Corp. Proteinase K was purchased from E. Merck. Sephadex G-200, G-150 superfine, and DEAE-Sephadex were obtained from Pharmacia Inc. and Bio-Gel HTP and BD-cellulose from Bio-Rad. All other chemicals were reagent grade. Deionized water was routinely used. tRNA was isolated from dog or rabbit liver by a modification of the procedure of Rogg et al. (1969). The chromatographic step over DEAE-cellulose was omitted. The preparation was next chromatographed over BD-cellulose according to Petrissant et al. (1970). Three samples were obtained by this procedure: (1) a rapidly eluted fraction which is enriched in $tRNA^{Met}_f$; (2) an intermediate fraction which is enriched in $tRNA^{Lys}$; (3) a broad slow-running fraction which contains $tRNA^{Met}_m$ and tRNAs for many other amino acids. The $tRNA^{Met}_f$ sample was further purified by chromatography over DEAE-Sephadex (Petrissant et al., 1970).

Assays. (a) Aminoacyl-tRNA Synthetase. The incubation mixture (0.1 ml) in a plastic tube (8 mm, i.d., × 75 mm)[2] contained 5 mM Tris, pH 7.05 at 37 °C, 100 mM KCl, 10 mM Mg^{2+}, 5 mM dithiothreitol, 5 mM ATP, 0.1 mM yeast or liver tRNA, approximately 2.6 μM (0.05 μCi) ^{3}H-labeled L-amino acid, and enzyme (1–10 μl). Solutions were incubated for 15 min at 37 °C and then quickly chilled, and 75 μl of the solution was transferred to a Whatman 3 MM paper circle, 23-mm diameter. This was immediately plunged into ice-cold 5% Cl_3CCOOH. After 15 min, the pad was washed thoroughly with cold 5% Cl_3CCOOH, followed by ethanol–ether (1:1). The pad was immersed in cold ether for 15 min, then dried, and placed in a vial, and 10 ml of a scintillation cocktail was added, and the ^{3}H was counted in a Nuclear Chicago liquid scintillation spectrometer. The amount of product formed under

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[1] Abbreviations used are: Cl_3CCOOH, trichloroacetic acid; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; BD-cellulose, benzoylated DEAE-cellulose; DTT, dithiothreitol.

[2] We thank Dr. W. E. Stirewalt for suggesting the use of plastic tubes.

TABLE I: Separation of tRNA Activator from Aminoacyl-tRNA Synthetases with Ammonium Sulfate and Sephadex G-150.

Amino Acid	Ammonium Sulfate Fraction[a] (%)	Aminoacyl-tRNA Synthetase Act. (pmol/tube)	Activator Response Ratio[b]		Aminoacyl-tRNA Synthetase Act.[c] (pmol/tube)	Activator Response Ratio[b]
Met	0–5	20	2	Seph. eluate	22	3
	0–45 + 45–65[d]	66		Seph. eluate + 45–65[d]	86	
Leu	0–45	2	8	Seph. eluate	9	9
	0–45 + 45–65[d]	18		Seph. eluate + 45–65[d]	94	
Lys	0–45	10	4	Seph. eluate	89	2
	0–45 + 45–65[d]	46		Seph. eluate + 45–65[d]	269	
Ile	0–45	8	3	Seph. eluate	26	3
	0–45 + 45–65[d]	35		Seph. eluate + 45–65[d]	105	
Ser	0–45	2	5	Seph. eluate	5	5
	0–45 + 45–65[d]	11		Seph. eluate + 45–65[d]	33	
Val	0–45	8	1	Seph. eluate	27	1
	0–45 + 45–65[d]	19		Seph. eluate + 45–65[d]	57	
Phe				Seph. eluate	19	0
				Seph. eluate + 45–65[d]	20	

[a] The 0–45% and 45–65% $(NH_4)_2SO_4$ fractions of a dog pancreas cytosol were tested for aminoacyl-tRNA synthetase activity, separately and as a mixture, with yeast tRNA and the ^3H-labeled amino acids listed. [b] tRNA activator response ratio = [pmol of product (synthetase + activator) − pmol of product (synthetase alone)]/pmol of product (synthetase alone). [c] A dog liver cytosol was passed through a column of Sephadex G-150 superfine (0.9 × 52 cm) which had been equilibrated with buffer (0.01 M P_i, pH 7.5, 2 mM mercaptoethanol, 20% glycerol). The A_{280} excluded peak tube was tested. [d] This fraction had been treated with 5% Cl_3CCOOH and exhibited no significant aminoacyl synthetase activity toward the listed amino acids.

these conditions is proportional to time and to the amount of enzyme added. A synthetase enzyme unit is defined as that amount which forms 1 μmol of [^{3}H]aminoacyl-tRNA min^{-1} (mg protein)$^{-1}$.

(b) tRNA Activator. The assay components and conditions are the same as above except that 1–10 μl of solution which may contain activator was also included and the incubation time was 30 min. tRNA activation is calculated as picomoles of [^{3}H]aminoacyl-tRNA formed by the mixture of synthetase and activator minus the sum of [^{3}H]aminoacyl-tRNA formed by these two proteins in separate incubations. In many experiments an activator was used which had been previously warmed at 50 °C for 10 min or treated with 5% Cl_3CCOOH. Both of these treatments inactivated aminoacyl-tRNA synthetases.

Analytical Determinations. Protein concentration in solutions was estimated either by A_{280}:A_{260} measurements (Layne, 1957) or by a modification of the Lowry procedure (Campbell and Sargent, 1967) using beef serum albumin as standard.

Preparative Procedures. Rabbit Liver Cytosol. Fresh rabbit liver was chilled in ice, trimmed, cut into small pieces, and homogenized in two 30-s treatments at medium speed in a Waring Blendor with 3 volumes of ice-cold buffer (0.25 M sucrose–50 mM P_i, pH 8.0). All subsequent procedures were carried out at 0–4 °C unless otherwise specified. The suspension was centrifuged for 45 min at 95 000*g*. The supernatant solution below the fatty layer was removed with a syringe and centrifuged at 95 000*g* for 120 min. The clear, red supernatant solution (cytosol) was also removed with a syringe and adjusted to pH 7.0. It is stable for approximately 3 months at −20 °C. The same procedure was used to prepare cytosol from dog pancreas, dog liver, or rat liver.

tRNA Activator. (a) Direct Precipitation with Ammonium Sulfate. One hundred milliliters of cytosol was rapidly adjusted to 45% saturation with saturated ammonium sulfate, pH 7.0 (Green and Hughes, 1955), allowed to stand 1 h at 3 °C, and centrifuged 20 min at 95 000*g* in a Beckman 35 rotor. The supernatant was then brought to 65% saturated ammonium sulfate and treated as above. The 45–65% precipitate was dissolved in 0.05 M P_i–10% glycerol buffer, pH 7.0, and adjusted to 5% Cl_3CCOOH. The turbid solution was centrifuged at 20 000*g* for 30 min, and the precipitate was homogenized in 0.25 M P_i (pH 6.5)–10% glycerol. The suspension was centrifuged as above and the extraction repeated. This solution was tested for tRNA activation activity and for aminoacyl-tRNA synthetase activity with the following ^{3}H-labeled L-amino acids: methionine, lysine, isoleucine, leucine, serine, valine, and phenylalanine. As shown in Table I, the 0–45% fraction demonstrated significant aminoacyl-tRNA synthetase activity for six amino acids. The Cl_3CCOOH-treated 45–65% fraction possessed no aminoacyl-tRNA synthetase activity itself but stimulated those of the 0–45% fraction from a minimum effect with valine to a maximum of eight times with leucine. In order to evaluate activation with a completely different source of enzymes, a dog liver cytosol was chromatographed on Sephadex G-150 superfine, and the excluded fraction was tested for aminoacyl-tRNA synthetase activity with seven amino acids. The results with tRNA activator were similar to those with the ammonium sulfate fraction in that valine showed the lowest stimulation and leucine the highest. It is noteworthy that phenylalanine-tRNA formation did not increase in this experiment.[3]

(b) Chromatographic Separation from Aminoacyl-tRNA Synthetases on Sephadex G-200. Forty milliliters of rabbit liver cytosol which had been adjusted to 20% glycerol was added to a 2.5 × 90 cm column of Sephadex G-200 prewashed with buffer T (10 mM, Tris, pH 7.4, 20 mM KCl, 10 mM Mg^{2+}) adjusted to 20% glycerol. The material was washed through the column at 10–20 ml/h.

The elution patterns of total protein (A_{280}), methionine-tRNA synthetase activity, and tRNA activator are included

[3] Preliminary results with tryptophan and arginine suggest little or no activator effect with these tRNAs. Other amino acids are being tested.

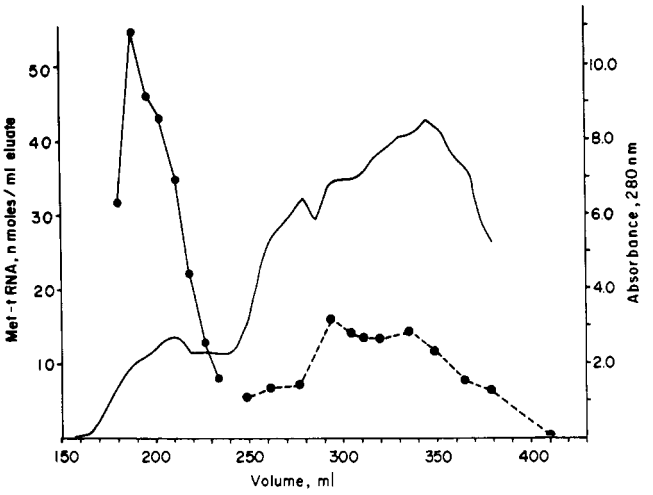

FIGURE 1: Separation of aminoacyl-tRNA synthetase activity from tRNA activator by Sephadex G-200. (—) A_{280}; (● — ●) activated Met-tRNA synthetase activity; (● - - - ●) tRNA activator.

in Figure 1. The peak of synthetase activity is eluted at void volume, whereas most proteins and tRNA activator are eluted in a broad, diffuse band in the region 250–400 ml. Similar results were obtained if the columns were equilibrated and the proteins eluted with 0.05 M P_i–20% glycerol. These data suggest that the synthetase is either a very large protein or occurs as part of a complex (Vennegoor and Bloemendal, 1972; Som and Hardesty, 1975). The eluates collected between 290 and 340 ml were combined and brought to 65% saturation with $(NH_4)_2SO_4$. The precipitate was centrifuged and redissolved in 2.5 ml of buffer T. This solution was frozen and thawed repeatedly with no loss in tRNA activation activity over a 2-year period.

Purification of Aminoacyl-tRNA Synthetases on Hydroxylapatite. Bio-Gel HTP was suspended in 0.05 M potassium phosphate, pH 7.0, which contained 20% glycerol (buffer P). A sample of rabbit liver cytosol (106 ml) which contained 20% glycerol was chromatographed over a 7.5 cm diameter 5 cm height column of HTP at a rate of 200 ml/h and eluted in a stepwise fashion with potassium phosphate buffers, pH 7.0, containing 20% glycerol as follows: wash 1, 0.2 M; wash 2, 0.25 M; wash 3, 0.3 M. The washes were pumped through the column at a rate of 400 ml/h. Because of the large amounts of protein, the eluates were monitored semiquantitatively at A_{260}, separate fractions were pooled, and the A_{280} was determined on the pooled fractions. The tRNA synthetase activities for Met, Lys, Leu, and Ile were eluted with 0.25 M P_i. This procedure resulted in approximately 20-fold purification for Met and Lys-tRNA synthetases in 65–70% yield. This will be referred to as the HA enzyme and was used for most of the experiments reported.

Results

Methionine-tRNA Synthetase and tRNA Activation Factor Concentration Curves. The synthetase activity was determined on a HA eluate by itself and in the presence of a constant ratio of tRNA activator. Figure 2 demonstrates approximately a tenfold increase in Met-tRNA formation in the presence of activation factor. The data also show that tRNA activation can be assayed quantitatively under these conditions (Figure 2B). The relatively low plateau of the tRNA activation factor concentration curve in this experiment is due to the amount of synthetase becoming limiting. The plateau demonstrates,

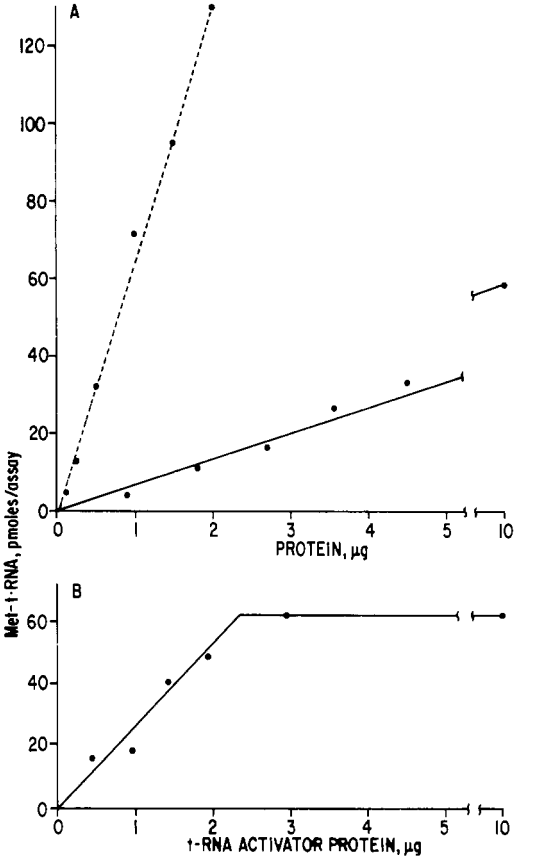

FIGURE 2: Methionine-tRNA synthetase and tRNA activator concentration curves. Assays were run under standard conditions. (a) Methionine-tRNA synthetase (—); methionine-tRNA synthetase + tRNA activator in constant ratio of 1:10, 0–2 µg of synthetase, 0–20 µg of tRNA activator (···); (b) tRNA activator, 0–10 µg, with 1 µg of synthetase (—).

TABLE II: Effect of Trypsin on $tRNA^{Met}$ Activator Activity.[a]

Preincubation Components			
tRNA Activation Preparation (µl)	Trypsin (µg)	Trypsin Inhibitor (µg)	Met-tRNA Synthesis (pmol/tube)
			4.4
100			64
100	11		9.5
100	11	11	64

[a] One hundred microliters of 45–65% fraction tRNA activation preparation was preincubated for 60 min at 37 °C with 11 µl of trypsin (1 mg/ml). Eleven microliters of soybean trypsin inhibitor (1 mg/ml) was added as indicated. One microliter of each preincubation solution was added to the yeast Met-tRNA synthetase assay medium.

in addition, that excess activator does not inhibit the reaction.

Characteristics of the tRNA Activator. (1) Chemical Nature. As shown in Table II, when the activator was incubated with trypsin, it was inactivated. This inactivation was completely prevented by the inclusion of soybean trypsin inhibitor in the mixture. These data indicate that the effectiveness of the activator is dependent on peptide linkages. The macromolec-

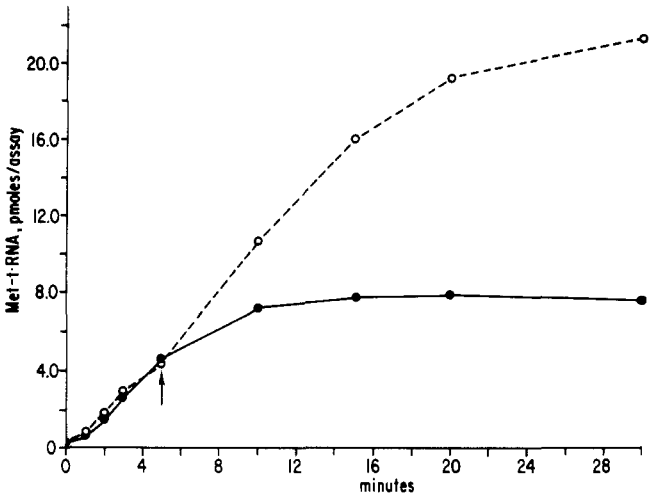

FIGURE 3: Catalytic effect of tRNA activator. Two assay mixtures, 1 ml each, were incubated at 37 °C. All components were present at their usual concentrations except that yeast tRNA was reduced to 7.1 μg and activation factor to 0.6 μg. After 5 min of incubation, 28 μg of yeast tRNA was added to one tube. Aliquots (75 μl) were removed from both tubes as indicated, processed by the regular procedure, and [^{3}H]Met-tRNA determined.

ular nature of the tRNA activator is shown by its nondialyzability through a hollow fiber 80 membrane. This suggests a minimum molecular weight of 30 000. It is inactivated after 2 min exposure to 93 °C and is partially inactivated by exposure to 6 M urea for 60 min at 2 °C.

(2) Component Affected. The two components in the aminoacyl-tRNA synthetase reaction most likely to be altered by the activator are the synthetase itself or the tRNA. Since enzymes in this group of mammalian aminoacyl-tRNA synthetases are very unstable, it might be postulated that the activator stabilized the enzyme(s) and thereby increased the rate of aminoacyl-tRNA formation. However, when the solution was heated at 50 °C for periods up to 10 min, the loss in aminoacyl-tRNA synthetase activity for Ile, Leu, Lys, and Met was as great in the presence of activator as it was in its absence.

The possibility that activation occurred merely as a result of general stabilization of Met-tRNA synthetase was also considered. The following proteins were added to the assay solution as activator substitutes: beef serum albumin, lysozyme, myokinase, soybean trypsin inhibitor, and 40S dog pancreas ribosomal subunits. The results were uniformly negative.

As mentioned previously, our enzyme preparation exhibited synthetase activity for at least four amino acids. The possibility that the activating material was acting as a subunit of an enzyme and thereby increased its activity was considered. When a mixture of the two proteins was sedimented through a sucrose gradient, no evidence of an association was observed.[4] Similarly, preincubation of the HA synthetase solution with the activating material did not increase aminoacyl-tRNA synthesis above that found when activator was added directly to the assay solution. To summarize, we have obtained no evidence that the "activator" is activating a synthetase directly.

On the other hand, preincubation of yeast tRNA with the activating protein and reisolation of the tRNA resulted in as much synthesis of Met-tRNA as when the activator was added directly to the assay (Table III). The activator–tRNA mixture retained activity even when it was heated to 93 °C for 2 min. These conditions had been established previously as sufficient

[4] S. R. Dickman and D. J. Boll, unpublished.

TABLE III: Substrate Effectiveness of Yeast tRNA Pretreated with Activator Protein.[a]

Preincubation Yeast tRNA with Activator Protein	Activator Protein Present in Assay	Met-tRNA Synthesis (pmol/tube)
−	−	4.8
−	+	34
+	−	36
+	+	38
+[b]	−	33

[a] Yeast tRNA (11.4 mg/0.5 ml) was preincubated with and without liver activator protein in 5 mM Tris, pH 7.0, at 37 °C, 10 mM Mg^{2+} buffer for 15 min. The solution was chilled and 2 volumes of cold 95% ethanol added. After 1 h at −20 °C, the solution was centrifuged 15 min at 6000*g*. The precipitate was dissolved in 2.0 ml of 0.2 M potassium acetate, pH 6, clarified by centrifugation, and reprecipitated with ethanol at −20 °C. The precipitates were washed twice with 95% ethanol and once with ether and dried in vacuo. The residues were redissolved in H_2O (22.7 mg/ml) and 10 μl of each was incubated in the regular Met-tRNA assay with HA enzyme. [b] The preincubated, isolated tRNA sample was placed at 93 °C for 2 min and then cooled before being added to the assay solution.

TABLE IV: Effect of Activation Protein on Aminoacylation of Liver and Yeast tRNAs.[a]

	AA-tRNA Formation			AA-tRNA Formation		
Amino Acid	Yeast tRNA (pmol)	Yeast tRNA + Activator (pmol)	Activator Response Ratio[b]	Liver tRNA (pmol)	Liver tRNA + Activator (pmol)	Activator Response Ratio[b]
Met	2.9	54	17	33	44	0.3
Lys	6.1	80	12	58	69	0.2
Leu	6.1	44	6	23	26	0.1
Ile	3.2	33	10	29	38	0.3

[a] Aminoacyl (AA)-tRNA synthetase activities from a hydroxylapatite column eluate were used to assay crude yeast or liver tRNA with four amino acids. tRNA activator, preheated at 50 °C for 10 min, was added as indicated. [b] Defined in Table I.

to inactivate the protein. This experiment established two points: (1) yeast tRNA exerts a strong adsorption toward liver activator; (2) the heat stability of activator protein is considerably increased when it is associated with tRNA compared with when it is not.

(3) Catalytic Action. To determine whether the activator protein was acting in a catalytic manner, the experiment described in Figure 3 was performed. Much smaller quantities of yeast tRNA and of activator than were usually present in the assay were added to each of two tubes. These quantities had been shown in a previous experiment to place both components at or close to limiting concentrations. After 5 min incubation, additional tRNA was added to one tube. The Met-tRNA synthesis rate immediately increased and continued at a linear rate for the next 15 min. These data demonstrate that the tRNA activation protein is able to increase the reaction rate

TABLE V: Effect of Deproteinization Procedures on Activator Response Ratio of Preincubated Yeast tRNA and Mammalian tRNA.[a]

	Protein Content (%)	Activator	Met-tRNA Formed (pmol/Assay)	Activator Response Ratio[b]
		Yeast tRNA		
Untreated	1.2	−	3	
		+	45	14
Preincubated with activator	2.7	−	25	
		+	30	0.2
Preincubated with activator, 2 × phenol extracted	1.0	−	14	
		+	41	2.0
Preincubated with activator, treated with proteinase K	3.1	−	9	
		+	33	2.7
		Mammalian tRNA		
Pancreas, 1 × phenol	4.6	−	33	
		+	44	0.3
Pancreas, 4 × phenol	1.6	−	19	
		+	48	1.5
Pancreas, 1 × phenol, then treated with proteinase K	3.7	−	3	
		+	18	5.0
$tRNA^{Met}_f$ (DEAE)	0.7	−	5	
		+	28	4.6

[a] Preincubation of yeast tRNA as described in Table III. Mammalian tRNA or preincubated yeast tRNA (20 mg) was treated with 0.5 mg of proteinase K for 60 min at 37 °C. It was precipitated at −20 °C with 2 volumes ethanol, centrifuged, redissolved, and reprecipitated with ethanol three additional times. The pellet was washed in ether and dried in vacuo. $PhCH_2SO_2F$ (8.5 μg) was added to the assay solutions of proteinase K treated tRNA. Phenol extractions were performed by shaking at 4 °C for 60 min each with phenol saturated with 0.2 M acetate, pH 6.0. After the final extraction, RNA was precipitated and dried with ethanol and ether as above. [b] Defined in Table I.

on the addition of more substrate; i.e., it is acting as a catalyst.

Activation of Yeast and Liver tRNA. The striking effects of the tRNA activator on methionine acceptance by yeast tRNA are shown in Table IV. Activation of yeast tRNA for Lys, Leu, and Ile is also demonstrated. In contrast, the activator response ratio for these four amino acids utilizing unfractionated liver tRNA was in the range 10–20%. This tRNA contained approximately 5% protein.

Chromatography of liver tRNA over BD-cellulose (Petrissant et al., 1970) gave three fractions: $tRNA^{Met}_f$, $tRNA^{Met}_m$, and $tRNA^{Lys}$. When these were tested for amino acid acceptance, the activator response ratio had increased to 1.4, 1.0, and 0.7, respectively. Analysis of these tRNAs showed that protein content had been decreased to about 0.6 by this procedure. It thus appears that activator protein may be associated with unfractionated liver tRNA as isolated in the laboratory by procedures which employ one phenol extraction.

This hypothesis was supported by the experiment described in Table V. Yeast tRNA, preincubated with activator, then precipitated with ethanol, washed with ether, and dried, responded to additional activator in the assay by only 20%. The large increase in Met-tRNA formed by the preincubated tRNA is apparently due to activator having been precipitated by ethanol and isolated in active form in association with the tRNA. The protein content of the preincubated tRNA increased to 2.7% by the treatment. The analysis shows that most of the protein in the activator preparation was precipitated with the tRNA. The protein content would have been 3.7% if all the protein added to the tRNA had remained in the sample. When this preincubated tRNA was extracted two times with phenol, the protein content fell to 1.0%, and the Met-tRNA formed dropped to 14 pmol unless additional activator was included. Correspondingly, the activator response ratio increased to 2.0.

Similarly, when preincubated tRNA was treated with proteinase K, only 9 pmol of Met-tRNA was formed in the assay unless additional activator was present. Under these conditions, the activator response ratio was 2.7. This sample, however, contained 3.1% protein. Much of this was demonstrated to be active proteinase K because the synthetase was completely inactivated in the assay unless the proteinase inhibitor $PhCH_2SO_2F$ was included. The total protein content of the activator–proteinase K–tRNA complex would have been 7.7% if no protein had been hydrolyzed.

Dog pancreas tRNA was similar to liver tRNA in that it contained considerable protein, 4.6%, and showed a low activator response ratio of 0.3. Three additional phenol extractions decreased protein content to 1.6% and concurrently Met-tRNA synthesis fell to 19 pmol unless activator was included in the assay. The activator response ratio with this tRNA rose to 1.5. Treatment of pancreas tRNA with proteinase K decreased protein to 3.7% and increased activator response ratio to 5.0. This ratio might even have been larger except for some inhibition of aminoacyl-tRNA synthetase by $PhCH_2SO_2F$.

Chromatography of the $tRNA^{Met}_f$ fraction from the BD-cellulose column over DEAE-Sephadex did not decrease protein content further but resulted in an activator enzyme response ratio of 4.6. It would appear that protein contamination of tRNA samples significantly affects their response to activator, but total protein content is not the sole determinant of this response.

Since a number of purine and pyrimidine bases are known to contribute to A_{660} in the Lowry protein determination (Layne, 1957), we assume that 0.6% calculated protein may be due to an artifact and this value may actually indicate a protein-free tRNA.

Effect of tRNA Activation on Kinetics of Met-tRNA Synthetase. When Met-tRNA synthetase (HA enzyme) was incubated with yeast tRNA, a constant rate of reaction was measured (Figure 4) of approximately 0.6 pmol of Met-tRNA/min. When tRNA activator was included, the rate of reaction was similar at first, then increased rapidly. After 10 min of incubation, the formation of Met-tRNA was about five times faster with activator than without.

Physical and Chemical Characteristics of tRNA Activation Protein. (a) Stability at 50 °C. A solution of 45–65% fraction activation protein in 0.05 M potassium phosphate–20% glycerol, pH 7.0, was placed in a 50 °C bath. Aliquots were removed at intervals and tested for aminoacyl-tRNA synthesis with the amino acids: [^{3}H]Ile, [^{3}H]Leu, [^{3}H]Lys, and [^{3}H]-Met. Activator activity remained almost constant for 10 min with all four tRNAs. In another experiment with methionine, the activation factor remained constant for 30 min but had decreased to 75% of the original after 60 min at 50 °C. The stability of activation factor at 50 °C in distinction to that of the aminoacyl-tRNA synthetase has been useful in obtaining

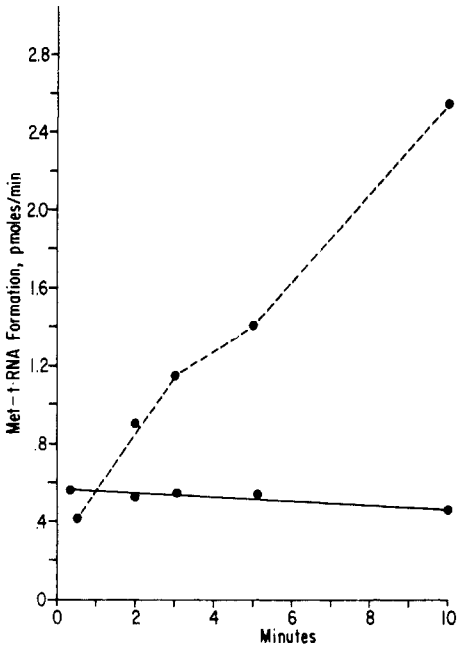

FIGURE 4: Effect of tRNA activation factor on kinetics of Met-tRNA synthetase. All components were premixed at ten times the usual amounts, and the reaction was started by the addition of Met-tRNA synthetase (hydroxylapatite eluate) (20 μl) or a mixture of Met-tRNA synthetase and tRNA activator, 4 μl plus 0.5 μl, respectively. The incubation was at 30 °C. Samples were removed at the indicated times, processed, and counted by the regular procedure. Met-tRNA synthetase (● — ●); Met-tRNA synthetase plus tRNA activation factor (● - - - ●).

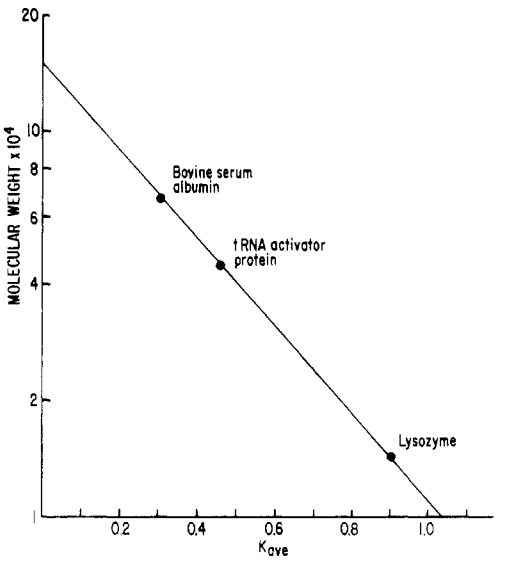

FIGURE 5: Estimate of molecular weight of tRNA activator protein. A Sephadex G-150 superfine column (0.9 × 43 cm) equilibrated with 0.25 M P_i (pH 6.0)–10% glycerol was calibrated with Dextran 2000, bovine serum albumin, and lysozyme. Two samples of (45–65%) tRNA activator in buffer were chromatographed; one had been treated with 5% Cl_3CCOOH. Aliquots (2 μl) were assayed for tRNA activator.

preparations that demonstrate considerable tRNA activation factor activity with no synthetase activity.

(b) Solubility as a Function of pH. Ten milliliters of 45–65% fraction activation factor in 0.05 M P_i–20% glycerol, pH 7.0, was adjusted to 5% Cl_3CCOOH, allowed to stand 15 min at 2 °C, and then centrifuged. The precipitate was suspended in 10 ml of cold 0.25 M P_i (pH 7.0)–20% glycerol. The suspension was at pH 6.3. HCl (10 N) was added dropwise to pH 3.1 and then alkali was slowly added and aliquots were removed at intervals. The suspensions were centrifuged at 2 °C and the supernatants tested for factor activity. The data obtained show that activator protein was insoluble at pH 3.1 but gradually increased in solubility to a maximum at pH 6.2. An overall purification of approximately threefold was obtained by this procedure with a small loss in total activator factor. The stability of activator protein to 5% Cl_3CCOOH is noteworthy.

(c) Dialysis. Activator protein (45–65%) was dialyzed against 0.25 M P_i (pH 7.0)–20% glycerol, for 4 h at 3 °C in a hollow fiber 80 device. No appreciable activity was lost. This result indicates that the minimum molecular weight of the activation protein is at least 30 000.

(d) Molecular Weight of tRNA Activator Protein. Two differently treated samples of tRNA activator protein were chromatographed on a calibrated Sephadex G-150 column. As shown in Figure 5, both the 45–65% ammonium sulfate fraction and the Cl_3CCOOH-treated sample exhibited a K_{av} which corresponded to a calculated molecular weight of 45 000.

(e) Isoelectric Point of tRNA Activator. Isoelectric focusing of activator protein in ampholyte buffers in the range pH 3–11 produced multiple bands. Factor activity was found in the region pH 5.8–6.0.

Possible Mechanisms of Activation. Effect of Deacylation of tRNA on Its Response to Activation Factor. Both yeast tRNA and liver tRNA were stripped of amino acids as described by Samuel et al. (1973). Aminoacylation was quite low with yeast tRNA in the absence of activation factor. Aminoacylation increased many fold in the presence of activator with both samples. With mammalian tRNA aminoacylation was greater with the stripped sample both with and without activation factor. With either sample, stripping had very little effect on its response to activation factor.

Effect of Denaturation of tRNA on Its Response to Activator. Conformational change is another possible modification of tRNA which may occur in conjunction with aminoacylation. Using the experiments of Lindahl et al. (1966) as a model, we heated yeast tRNA at 60 and at 93 °C in the presence and absence of 10 mM Mg^{2+}, and chilled each solution slowly or rapidly.[5] We also heated tRNA at pH 4.3 under the conditions reported by Bina-Stein and Crothers (1975). None of these treatments affected the activator response ratio of the treated tRNA for aminoacylation by methionine.

tRNA Nucleotidyl Transferase Activity. The differential effect of tRNA activator on yeast tRNA vs. unfractionated liver tRNA might be attributed to tRNA nucleotidyl transferase activity. Commercial yeast tRNA could be lacking terminal adenosine while the liver sample was not. This possibility was tested directly (Table VI). tRNA nucleotidyl transferase was assayed under two sets of conditions: (1) as described by Deutscher (1974), (2) utilizing our assay for tRNA activator except that [^{3}H]ATP was substituted for [^{3}H]methionine. Activity was found in liver cytosol and the 45–65% ammonium sulfate fraction. No significant tRNA nucleotidyl transferase activity was found, however, in the 45–65% ammonium sulfate fraction which had been heated

[5] Karpel et al. (1975) (*Fed. Proc., Fed. Am. Soc. Exp. Biol. 34*, 708) described RNA unwinding proteins, one of which can convert inactive, denatured $tRNA^{Leu}_3$ to an active form. Although the tRNA samples we have been using have not been knowingly denatured, the similarity in effects between unwinding proteins and tRNA activator protein is noteworthy and is being investigated. tRNA activator is different from the DNA relaxing enzyme described by Keller (1975), however, in that the latter is inactivated by heating to 50 °C for 5 min.

TABLE VI: Distinction between tRNA Activation Factor and tRNA Nucleotidyl Transferase.

Solution	tRNA Nucleotidyl Transferase[d]		
	Deutscher Assay Conditions[a]	AA-tRNA Activator Assay Conditions[b]	AA-tRNA Activator Assay[e]
Cytosol, dog pancreas	7.2	6.6	3.7
Cytosol, 50 °C, 10 min	2.0	2.1	3.2
45–65% $(NH_4)_2SO_4$	17		32.1
Cytosol, 50 °C, 10 min	0		33.5
Cytosol, 5% Cl_3CCOOH[c]	0	0	74

[a] pH 9.4; [^{3}H]ATP, 32 μg/100 μl; tRNA, 62.5 μg/100 μl; 10 min, 37 °C. [b] pH 7.0; [^{3}H]ATP, 320 μg/100 μl; tRNA, 250 μg/100 μl; 15 min 37 °C. [c] As described in Table I. [d] In nanomoles of product per milligram of protein. [e] In nanomoles of [^{3}H]Met-tRNA per milligram of protein.

at 50 °C for 10 min or had been exposed to 5% Cl_3CCOOH and redissolved. The data demonstrate that tRNA activator was stable to these latter two treatments.

Arginyl-tRNA Transferase Activity (Goz and Voytek, 1972). Although the specificity of the arginyl-tRNA transferase system would seem to rule it out as a general type of tRNA activator, dialyzed casein was added as a possible acceptor protein for Met. No increase in Met-tRNA synthetase activity was found.

In this experiment we also determined whether the product of the reaction was a nucleic acid or a protein by placing the [^{3}H]Met product in boiling 5% Cl_3CCOOH for 15 min before washing in the usual way. ^{3}H-Labeled material which remained on the filter pad was no higher than the control. This result excludes a ^{3}H-labeled protein as product.

Inhibition of Product Degradation. The quantity of aminoacyl-tRNA present at any one time is the resultant of the amount formed minus that which has been degraded. Bonnet and Ebel (1972) have observed considerable decreases in Val-tRNA in the presence of yeast purified Val-tRNA synthetase, AMP, and PP. We are investigating whether the protein described in this work may be acting as an inhibitor of a deacylation reaction in this mammalian aminoacyl-tRNA synthetase system.

Discussion

Of the previous groups who have utilized yeast tRNA as substrate with mammalian aminoacyl-tRNA synthetases, Pearlman and Bloch (1967) and Davies and Marshall (1975) have reported a "factor" or "activator" effect, the former with tyrosine, the latter with asparagine. Allende and Allende (1964), on the other hand, purified arginyl-tRNA synthetase 280 times from rat liver using yeast tRNA as substrate without mentioning such an effect. Similarly, Penneys and Muench (1974) purified human placental tryptophanyl-tRNA synthetase to homogeneity using commercial brewers yeast tRNA as substrate, and Nielsen and Haschemeyer (1976) have recently reported an approximately 800× purification of rat liver phenylalanine-tRNA synthetase in which they employed a pretreated yeast tRNA. These reports imply that tRNA activator does not increase amino acid acceptance of all yeast tRNAs. This conclusion is directly supported by our finding of no activation of $tRNA^{Phe}$ and quite low activation of a number of other yeast tRNAs (Table I). In our attempts to purify Met-tRNA synthetase with yeast tRNA substrate, however, the very low recovery of synthetase activity after a variety of first steps of purification suggested either that this enzyme was composed of extraordinarily labile protein(s) or that some essential component was being separated from the enzyme fraction.

As first demonstrated by Pearlman and Bloch with Tyr-tRNA synthetase and extended in this work for other aminoacyl-tRNA synthetases, the activation phenomenon exerts a small enough effect on unfractionated liver tRNA to have been easily missed. It appears that many mammalian tRNA preparations, like ours, contain more than trace amounts of protein. Our results suggest that one of these may be activating protein. This hypothesis would explain the greater difference in response to activator of yeast vs. mammalian tRNA. Even when the protein content of liver tRNA is reduced to very low levels by chromatography, however, its response to activation factor is not as great as that of untreated yeast tRNA. It is noteworthy, however, that yeast tRNA, preincubated with liver activator, then deproteinized with phenol or by treatment with proteinase, not only did not attain its original activator response ratio but now was in the same range as similarly treated liver tRNA. This comparison suggests that very small amounts of activator are shielded by tRNA against the attacking agents. Thus, there may be little difference between yeast and liver tRNA with regard to activator effectiveness.

Apparently tRNA remains activated only in the presence of the activator. If the latter is removed by phenol, by chromatography, or by proteinase action, the tRNA responds again to the addition of activator in the assay. These results suggest that permanent modification of the tRNA has not occurred. Thus enzymes which affect tRNA structure, such as nucleases, phosphatases, or nucleoside modifiers, can be eliminated as possible activators. Direct tests of some other enzymes have been mentioned previously.

Enzymes which remove product might enhance aminoacyl-tRNA synthetase activity. Inorganic pyrophosphatase, AMP deaminase, phosphatase, or 5′-nucleotidase fall in this category. These enzymes are considered unlikely candidates as activator for the following reasons: (1) Removal of product should be more effective at high synthetase activities than at low. tRNA factor activity often is larger with a low original synthetase activity rather than high. (2) The stability and solubility characteristics of the tRNA activator do not correspond with the known enzymes mentioned above. (3) Deproteinized liver tRNA does not respond to activator to as large an extent as yeast tRNA, even though the original synthetase activity may have been higher with liver tRNA. (4) The activator response ratio of yeast tRNA for four amino acids is not correlated with the amount of aminoacyl-tRNA formed.

Renaud et al. (1974) postulate two populations of yeast $tRNA^{Phe}$ based on kinetics of aminoacylation by yeast aminoacyl-tRNA synthetase. One type of tRNA was rapidly aminoacylated while the other was much slower. Preincubation of the $tRNA^{Phe}$ mixture with its corresponding aminoacyl synthetase transformed the slow into the rapidly reacting form. This is very similar to the results reported by us except that in the mammalian system a separate protein without aminoacyl-tRNA synthetase properties changes the less active to the more active form.

In conclusion, it should be recognized that the term "tRNA activator" summarizes a hypothesis. The data obtained to date support this idea more satisfactorily than they do other hy-

potheses, but no direct demonstration of conformational changes in responsive tRNAs has yet been secured.

Acknowledgments

We thank Sylvia Bjorge Berman for excellent assistance, David Peabody for isolation of mammalian tRNA, and Dr. Curtis Atkin for aid in trace-metal analysis.

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Formation of Anhydrosugars in the Chemical Depolymerization of Heparin†

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ABSTRACT: In the reactions used to break heparin down to mono- and oligosaccharides, anhydrosugars are formed at two stages. The first of these is the well-known cleavage of heparin with nitrous acid to convert the N-sulfated D-glucosamines to anhydro-D-mannose residues; this reaction has been studied in detail. It is demonstrated here that only low pH (<2.5) reaction conditions favor the deamination of N-sulfated D-glucosamine residues; the reaction proceeds very slowly at pH 3.5 or above. On the other hand, N-unsubstituted amino sugars are deaminated at a maximum rate at pH 4 with markedly reduced rates at pH 2 or pH 6. At room temperature solutions of nitrous acid lose one-fourth to one-third of their capacity to deaminate amino sugars in 1 h at all pHs. A low pH nitrous acid reagent which will convert heparin quantitatively to its deamination products in 10 min at room temperature is described, and a comparison of the effectiveness of this reagent with other commonly used nitrous acid reagents is presented. It is also shown that conditions used for acid hydrolysis of heparin convert approximately one-fourth of the L-iduronosyluronic acid 2-sulfate residues to a 2,5-anhydrouronic acid. This product is an artifact of the reaction conditions, and its formation represents one of several pathways followed in the acid-catalyzed cleavage of the glycosidic bond of the sulfated L-idosyluronic acid residues.

Because of the marked resistance of heparin and heparan sulfate to acid hydrolysis, structural studies on these polymers have relied heavily on cleavage of the polymers with nitrous acid to obtain oligosaccharides for further analysis. Nitrous acid cleaves 2-amino-2-deoxy-D-glucosidic bonds via a reaction sequence initiated by nitrosation of the amino group of the sugar followed by loss of N_2 with a ring contraction of the D-glucosamine residue to 2,5-anhydro-D-mannose coupled to elimination of the aglycone (for a review, see Horton and Philips, 1973). A consideration of data in the literature suggests that the yield of 2,5-anhydro-D-mannose may vary depending upon the 2-amino-2-deoxy-D-glucoside treated in the reaction (Shively and Conrad, 1970; Erbing et al., 1973) and the conditions under which the reaction is run (Lagunoff and Warren, 1962). Nitrous acid is usually generated in situ by addition of an inorganic nitrite salt to a solution of acid. A number of different formulations have been used to generate

† From the Department of Biochemistry, University of Illinois, Urbana, Illinois 61801. *Received March 29, 1976.* This research was supported by United States Public Health Service Grant AI 09551 from the National Institute of Allergy and Infectious Diseases. During the tenure of this work, J.E.S. was supported by a National Institutes of Health predoctoral traineeship on United States Public Health Service Training Grant GM 321.

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