A FACTOR IN THE SALT WASH OF RETICULOCYTE RIBOSOMES THAT CAN INTERACT WITH ACETYLPHENYLALANYL-tRNA

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A factor removed from reticulocyte ribosomes by 0.5 M KCl solution can bind acetylphe-tRNA or phe-tRNA either independently to itself or to washed ribosomes. This binding is not dependent upon poly U or GTP. Acetylphe-tRNA bound by this mechanism appears not to be available directly for the reactions of peptide extension catalyzed by TI, TII, or the ribosomal peptidyl-transferase. The acetylphe-tRNA binding factor is coincident with phenylalanine activating enzyme activity over several purification steps. Prior incubation of the salt wash fraction with phenylalanine and ATP reduced the level of acetylphe-tRNA or phe-tRNA that can be bound to the salt wash fraction during a subsequent incubation. It is suggested that phe-tRNA synthetase may be involved in these binding phenomena.

Acetylphe-tRNA may be used as a functional analogue of formylmet-tRNA to study some aspects of peptide initiation in systems derived from bacteria Haenni and Chapeville (1) demonstrated poly U directed incorporation of acetylphenylalanine into the aminoterminal position of polyphenylalanine. Lucas-Lenard and Lipmann (2) observed that with initiation factors, f_1 and f_2, and GTP, acetylphe-tRNA can be bound into the donor ribosomal site at relatively low concentrations of Mg^{++}.

Initiation factors with activities for acetylphe-tRNA comparable to f_1 and f_2 originally were not detected in systems derived from mammals. Mosteller, Culp and Hardesty (3) did not detect incorporation of acetylphenylalanine into the aminoterminal position of phenylalanine peptides formed at low Mg^{++} concentrations on reticulocyte ribosomes. Reboud (4) observed binding of acetylphe-tRNA to reticulocyte ribosomes but concluded that it did not function as it did in systems derived from E. coli. Siler and

Moldave (5) found that at relatively high concentrations of Mg^{++} acetylphe-tRNA may be nonenzymatically bound first into the acceptor (aminoacyl-tRNA) ribosomal site then moved into the donor site by the action of TII with GTP.

Recently, several reports have indicated that an initiation factor from reticulocytes is active with acetylphe-tRNA. Prichard, Gilbert, Shafritz and Anderson (6) reported that a factor, designated M_1, binds acetylphe-tRNA to washed reticulocyte ribosomes, and that E. coli f_2 can partially replace M_1 in the reticulocyte poly U system. An earlier report (7) indicated that acetylphe-tRNA with M_1 and M_2 lowered the Mg^{++} optimum for the synthesis of polyphenylalanine.

Materials and Methods

The preparation of rabbit reticulocytes, regular ribosomes, ribosomes for binding or polymerization assays isolated from reticulocytes incubated with NaF then washed with deoxycholate, rabbit liver tRNA, and aminoacyl-tRNA were as described previously (8). Phe-tRNA and met-tRNA were acetylated with acetic anhydride by the method of Haenni and Chapeville (1). The salt wash fraction was prepared by a modified form of the procedure described by Miller and Schweet (9) as described in the legend to Table 1.

Results

As shown in Table 1, acetylphe-tRNA may be retained on nitrocellulose filters in the presence of the salt wash fraction removed from reticulocyte ribosomes with 0.5 M KCl. The addition of ribosomes, poly U or GTP in any combination does not appreciably affect the total amount of acetylphe-tRNA that may be bound. The amount of acetylphe-tRNA retained by the filters is not reduced appreciably by incubation at 0°. The specificity for the type of aminoacyl-tRNA that may be bound is indicated by the data presented in Table 2. Of the types tested, acetylphe-tRNA is bound most efficiently. Binding of acetylphe-tRNA has been consistently higher than binding of phe-tRNA in a number of experiments involving several preparations of tRNA.

TABLE 1

BINDING OF ACETYLPHE-tRNA ON NITROCELLULOSE FILTERS

Conditions	Acetylphe-tRNA bound, pmoles - ribosomes	+ ribosomes
Standard	13.7	15.0
+ poly U	11.0	12.2
+ GTP	13.2	14.8
+ poly U, + GTP	13.0	12.0
- salt wash	0.7	0.8
0°, 5 min	12.7	14.0

The standard reaction mixture contained in a total volume of 0.5 ml the following: 0.02 M Tris-HCl (pH 7.5); 5 mM $MgCl_2$; 0.5 mM dithiothreitol; 0.5 mg of salt wash protein and 50 pmoles of acetylphe-tRNA. Where indicated 0.5 mg of ribosomes, 100 µg of poly U and 40 µM GTP also were added to the reaction mixture. After 5 min of incubation at 37° the reaction mixtures were diluted with 5 ml of cold buffer containing the same salts as the reaction mixture then filtered through a 27 mm nitrocellulose filter (Millipore Corporation-type HA). The salt wash fraction was prepared by addition of 4.0 M KCl to a solution of regular ribosomes at a concentration of 20 mg/ml in 0.25 M sucrose, 0.1 mM EDTA (pH 7.5), and 1 mM dithiothreitol to give a final KCl concentration of 0.5 M. The solution was stirred at 0° for 15 min. Ribosomes were removed by centrifugation at 50,000 rpm for 2 hr (50 rotor, Beckman Instruments, Inc.). The salt wash fraction (supernatant) was used as obtained or dialyzed overnight against 50 mM KCl, 10 mM Tris-HCl (pH 7.5) and 1 mM dithiothreitol. The salt wash fraction was stored at -90° until it was used.

TABLE 2

AMINOACYL-tRNA SPECIFICITY FOR BINDING

Aminoacyl-tRNA	Aminoacyl-tRNA Binding pmoles
acetylphe-tRNA	12.4
phe-tRNA	7.5
acetylmet-tRNA	1.7
met-tRNA	0.3
his-tRNA	>0.2
leu-tRNA	>0.2
val-tRNA	>0.2
lys-tRNA	>0.2
ile-tRNA	>0.2

Binding was carried out in the standard reaction mixture and by the procedure described for Table 1 except that 50 pmoles of the indicated form of aminoacyl-tRNA was substituted for acetylphe-tRNA and the reaction mixtures were incubated at 0° for 5 min.

The data presented represent these results; however, some variability in absolute amounts and relative proportion of binding has been observed. Furthermore, other experiments indicate that binding of acetylphe-tRNA or phe-tRNA is inhibited by addition of additional uncharged tRNA to the binding reaction mixture. The relationship appears similar to that described by Yarus and Berg (10) for binding of ile-tRNA and uncharged $tRNA^{ile}$ to its synthetase. These considerations indicate that caution is warranted in interpreting the relative binding affinities of phe-tRNA and acetylphe-tRNA. The other forms of aminoacyl-tRNA tested are bound in relatively low amounts. Acetylmet-tRNA appears to be bound in relatively low but significant amounts. This binding of acetylmet-tRNA is considered with the data of Figure 1.

The salt wash fraction also can promote binding of acetylphe-tRNA or phe-tRNA to ribosomes as shown in Table 3. For these experiments, ace-

TABLE 3

REQUIREMENTS FOR BINDING ACETYLPHE-tRNA TO RIBOSOMES

Conditions	Aminoacyl-tRNA Bound pmoles
Standard	5.3
+ poly U	5.9
+ GTP	6.1
+ Poly U, + GTP	5.7
phe-tRNA*	3.4
- salt wash fraction	>0.2
- ribosomes	>0.2
0°, 5 min	5.0

* 50 pmoles of phe-tRNA substituted for acetylphe-tRNA

The indicated components were incubated at 37° for 5 min in the standard reaction mixture and at the concentrations described for Table 1. After incubation the ribosomes were pelleted by centrifugation (angle rotor, 50,000 rpm, 23°, 2 hr) through a discontinuous gradient of 8% to 28% glycerol solution containing salts at the concentrations of the reaction mixture. The pellets were suspended in 0.50 ml of 0.01 M KCl and counted by liquid scintillation.

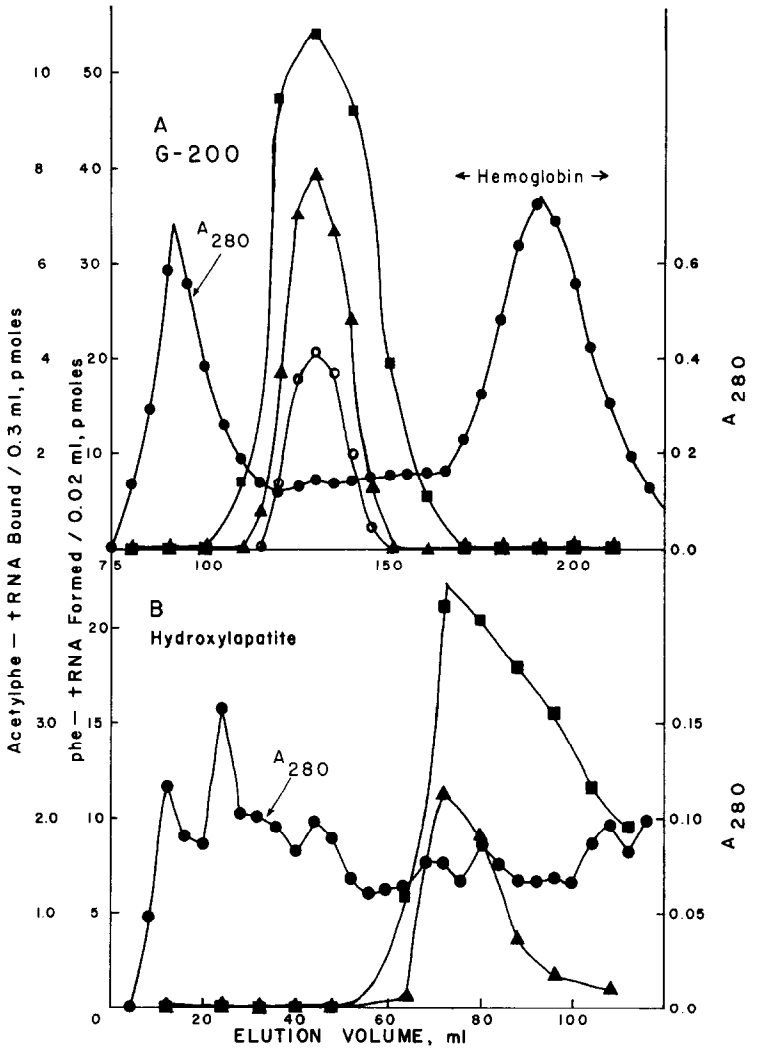

Figure 1. Chromatography of the Salt Wash

A. G-200 Sephadex. Salt wash fraction protein (2.0 ml containing 38 mg of protein/ml) was applied to a 2.5 x 50 cm G-200 Sephadex column and eluted with buffer containing 5 mM $MgCl_2$, 120 mM KCl, 0.01 M Tris-HCl (pH 7.5) and 1.0 mM β-mercaptoethanol. After chromatography the optical density at 280 mμ was determined for each 5.0 ml fraction collected. The fractions (0.3 ml from each) were assayed for binding of acetylphe-tRNA, phe-tRNA and acetylmet-tRNA by the nitrocellulose filter procedure described for Table 1 and for binding of phe-tRNA and acetylphe-tRNA to ribosomes by the procedure described for Table 3. Phe-tRNA synthetase activity in 0.020 ml of each fraction was determined by the procedure described previously (13).

B. Hydroxylapatite. The fractions between 115 ml and 145 ml from G-200 Sephadex were pooled and concentrated to 4 ml by ultrafiltration then layered on a 1.0 x 10 cm hydroxylapatite column (Clarkson Chemical Company) equilibrated with a solution containing 50 mM potassium phosphate, pH 7.5, and 10^{-3} M β-mercaptoethanol. The column was washed with 16 ml of the same buffer then eluted with a linear gradient from 0.05 M to 0.5 M potassium phosphate, pH 7.5, with 10^{-3} M β-mercaptoethanol. Aliquots of four ml fractions were assayed for binding, phe-tRNA synthetase and A_{280} as described above.

■ - Acetylphe-tRNA binding to nitrocellulose filters

▲ - phe-tRNA binding to nitrocellulose filters

○ - acetylphe-tRNA binding to ribosomes

tylphe-tRNA, salt wash fraction and ribosomes were incubated together, then the ribosomes with any bound acetylphe-tRNA were collected by centrifugation. Under these conditions binding of acetylphe-tRNA to the ribosomes is not promoted by poly U or GTP but is dependent upon the salt wash fraction. This binding occurs to a near maximum extent during incubation at 0°. In other extensive experiments not reported here, it has not been possible to form appreciable amounts of acetylphenylalanylpuromycin or hot TCA insoluble polyphenylalanine containing acetylphenylalanine from acetylphe-tRNA bound to the ribosomes with the salt wash fraction. In these experiments acetylphe-tRNA has been bound to ribosomes with salt wash fraction in the presence or absence of poly U and GTP then reincubated with all combinations of TI, TII, poly U, GTP and unlabeled phe-tRNA. Acetylphenylalanylpuromycin can be formed from acetylphe-tRNA bound nonenzymatically to ribosomes at relatively high concentrations of $MgCl_2$. Formation of acetylphenylalanylpuromycin from nonenzymatically bound acetylphe-tRNA is dependent on TII and GTP.

Activity for acetylphe-tRNA or phe-tRNA binding to nitrocellulose filters or ribosomes appears to be coincident with phe-tRNA synthetase activity in the eluant of the salt wash fraction chromatographed on G-200 Sephadex and then hydroxylapatite. Typical elution profiles are shown in Figure 1. In all fractionation procedures employed, activities for binding either acetylphe-tRNA or phe-tRNA to nitrocellulose filters in the presence or absence of ribosomes, or binding to ribosomes as measured by centrifugation, and phe-tRNA synthetase activity have been coincident, as indicated, and appear to follow approximately proportional changes in specific activities. A relatively low level of nitrocellulose filter binding activity for acetylmet-tRNA is found in the void volume from G-200, in the eluant between 85 ml to about 105 ml. This activity for acetylmet-tRNA could not be detected in the eluant from hydroxylapatite. Components required for acetylmet-tRNA binding appear to have been excluded from the fractions that

TABLE 4

THE EFFECT OF PHENYLALANINE AND ATP ON ACETYLPHE-tRNA BINDING

Conditions	Nitrocellulose Filter Binding, pmoles: Phenylalanine Bound	Acetylphe-tRNA Bound
None	-	19.1
ATP	-	14.8
Phenylalanine	0.2	12.9
Phenylalanine + ATP	14.0	9.5
Methionine	-	19.3
Leucine	-	18.7

Binding of phenylalanine or acetylphe-tRNA was measured as described for Table 1. Where indicated the reaction mixtures were modified to contain 2 mM ATP and 20 mM phenylalanine, methionine, or leucine. The reaction mixtures were incubated at 37° for 5 minutes, 50 pmoles of acetylphe-tRNA was added and then the incubation was continued for an additional 5 min at 37°. Phenylalanine binding was measured before the addition of acetylphe-tRNA.

contain activity for binding and synthesis of phe-tRNA.

A further indication that phe-tRNA synthetase may be involved in binding of acetylphe-tRNA or phe-tRNA to nitrocellulose filters or ribosomes is presented in Table 4. For these experiments the salt wash fraction was incubated with phenylalanine and ATP as indicated before addition of phe-tRNA or acetylphe-tRNA to the reaction mixture. Incubation with phenylalanine and ATP at either 0° or 37° results in the retention of phenylalanine by the nitrocellulose filters, apparently as phe-tRNA synthetase·AMP·phenylalanine complex. Formation of the complex appears to reduce the amount of phe-tRNA or acetylphe-tRNA that may be bound to the salt wash fraction during a subsequent incubation. Methionine or leucine cannot substitute for phenylalanine for inhibition of acetylphe-tRNA binding to the salt wash fraction. We have observed repeatedly a somewhat variable effect of phenylalanine without added ATP on binding of acetylphe-tRNA. This may reflect a direct effect of phenylalanine on the tRNA binding site similar to that described by Yarus and Berg (14) for the ile-tRNA synthetase system.

Discussion

The formation of stable complexes between tRNA and its cognate aminoacyl-tRNA synthetase have been demonstrated in a number of laboratories (15). Yarus and Berg (10, 14, 16) have studied in detail the dynamic interaction of tRNA with ile-tRNA synthetase and retention of several aminoacyl-tRNA synthetase complexes on nitrocellulose filters. The results and interpretation outlined here appear to be consistent with these studies. It is not surprising that binding of acetylphe-tRNA or phe-tRNA to phe-tRNA synthetase might occur with approximately the same efficiency or that formation of phenylalanine·AMP·synthetase complex would reduce acetylphe-tRNA in the nitrocellulose filter binding assay. That a component of the salt wash fraction, presumably phe-tRNA synthetase, can bind these tRNA's to nitrocellulose filter or to ribosomes without codon direction appears to be a novel observation.

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