protein synthesis, which is linear with time for at least 60 sec. The assay is performed in two steps. In the first step AcPhe-tRNA is bound to ribosomes at 6 mM Mg^{2+}, taking into account that tRNA binding requires higher Mg^{2+} concentrations than optimal poly(Phe) synthesis. The two incubations at 6 mM Mg^{2+} during the first step improve AcPhe-tRNA binding for unknown reasons. For poly(Phe) synthesis the Mg^{2+} concentration is decreased to 3 mM, which is required for an optimal rate of Phe incorporation under the applied conditions. It is important to use HEPES buffer instead of Tris, since HEPES has its maximal buffer capacity at pH 7.55 (Tris at pH 8.3) and hence shows fewer changes during temperature shifts. For example, a 10 mM HEPES (Tris) solution adjusted to pH 7.5 at room temperature (21°) measures at 37° 7.3 (Tris, 7.1) and at 0° 7.7 (Tris, 8.1). In this system the accuracy is impaired at a pH above 7.8 and below 7.3.

[45] Parameters for the Preparation of *Escherichia coli* Ribosomes and Ribosomal Subunits Active in tRNA Binding

By HANS-JÖRG RHEINBERGER, UTE GEIGENMÜLLER, MARKUS WEDDE, and KNUD H. NIERHAUS

Highly active ribosomes are a prerequisite in order to characterize quantitatively the interactions between transfer RNA, messenger RNA, and ribosomal particles. Preparation procedures as well as the binding capacities of ribosomal particles for all three kinds of tRNA involved in the elongation cycle—peptidyl-tRNA, aminoacyl-tRNA, and deacylated tRNA—have remained a matter of controversy until recently.[1-5] Much of the controversy is not only due to the fact that there are considerable differences in the ribosomal saturation levels for tRNA binding, but that, in addition, a correct interpretation of the results relies on an unequivocal site determination test. Further difficulties in evaluating ribosomal activity are based on the fact that: (1) There may be functional heterogeneity

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[1] H. J. Rheinberger, H. Sternbach, and K. H. Nierhaus, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 5310 (1981).

[2] M. Schmitt, A. Möller, D. Riesner, and H. G. Gassen, *Eur. J. Biochem.* **119**, 61 (1981).

[3] R. A. Grajevskaja, Y. V. Ivanov, and E. M. Saminsky, *Eur. J. Biochem.* **128**, 47 (1982).

[4] S. V. Kirillov and Y. P. Semenkov, *FEBS Lett.* **148**, 235 (1982).

[5] R. Lill, J. M. Robertson, and W. Wintermeyer, *Biochemistry* **23**, 6710 (1984).

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within a ribosomal population carrying a tRNA in a distinct site. Such heterogeneity would not be reflected in the mere binding numbers. (2) tRNA-binding capacity and overall elongation activity need not necessarily coincide.

In this chapter, first we report in detail a preparation procedure for tightly coupled 70S ribosomes of *Escherichia coli*, as well as for 30S and 50S subunits derived from tight couples. The preparation procedure has been developed by modifying a protocol of Hapke and Noll[6] and it reproducibly yields a high level of active ribosomes. Second, an *in vitro* system is described which allows the site-specific ribosomal tRNA-binding activity to be tested.

Preparation of Ribosomes

Materials

In order to minimize damage by RNases, strain D10 derived from *E. coli* K12 is used, which is deficient in RNase I.[7]

Bacto-Tryptone and yeast extract: Oxoid (Hamburg, FRG)
Alcoa A-305: Serva (Heidelberg, FRG)
Sucrose (ultrapure): BRL (Cambridge, UK)
All other chemicals mentioned are from Merck (Darmstadt, FRG) and Boehringer (Mannheim, FRG)

Buffers

$T_{10}M_6N_{30}SH_4$: 10 m*M* Tris-HCl, pH 7.5 (4°), 6 m*M* $MgCl_2$, 30 m*M* NH_4Cl, and 4 m*M* 2-mercaptoethanol
$T_{10}M_{10}N_{30}SH_4$: same as $T_{10}M_6N_{30}SH_4$ except that the Mg^{2+} concentration is 10 m*M*
$T_{10}M_3N_{100}SH_4$: 10 m*M* Tris-HCl, pH 7.5 (4°), 3 m*M* $MgCl_2$, 100 m*M* NH_4Cl, and 4 m*M* 2-mercaptoethanol
$T_{10}M_{10}N_{100}SH_4$: same as $T_{10}M_3N_{100}SH_4$ except that the Mg^{2+} concentration is 10 m*M*

Growth of Bacteria

Cells are grown in a 100-liter fermenter inoculated with 2.5 liters of an overnight culture of *E. coli* D10. The growth medium contains 1 kg Bacto-

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[6] B. Hapke and H. Noll, *J. Mol. Biol.* **105,** 97 (1976).
[7] R. F. Gesteland, *J. Mol. Biol.* **16,** 67 (1966).

tryptone, 0.5 kg NaCl, 0.5 kg yeast extract, and 1 liter of a 20% (w/v) glucose solution per 100 liters. Fermentation is performed under continuous aeration at 37°. Cell growth is stopped at 0.5 A_{650} units/ml, making sure that mid-log phase had not been passed. The activity of ribosomes depends considerably on the time at which cells are harvested. Early mid-log phase ribosomes have proved to be optimal in tRNA binding and elongation. After centrifugation for 15 min at 20,000 rpm, wet cells (usually 100–150 g per 100 liters) are shock-frozen and stored at −80°.

Isolation of Tight Couples

Three hundred grams of frozen cells are thawed in 600 ml of $T_{10}M_6N_{30}SH_4$ buffer. After resuspending, cells are centrifuged for 10 min at 10,000 rpm in a Sorvall GSA rotor, mixed with Alcoa A-305 (twofold cell weight), and ground in a Retsch mill for 25 min. The cell paste is homogenized with $T_{10}M_6N_{30}SH_4$ buffer (100 ml per 100 g cells) for 15 min. The homogenized cell paste is subjected to two low-speed centrifugations (10 min at 10,000 rpm in a Sorvall GSA rotor to remove Alcoa and 45 min at 15,000 rpm in a Sorvall SS34 rotor to remove cell debris). The resulting supernatant is centrifuged for 18 hr at 20,000 rpm in a 45 Ti rotor. Ribosomal pellets are rinsed with $T_{10}M_6N_{30}SH_4$ buffer and then resuspended in the same buffer with continuous gentle stirring for about 2 hr. The resuspended crude ribosomes are clarified (5 min at 5,000 rpm) and their concentration is determined. Usually, the yield amounts to 30,000 A_{260} units per 100 g of cells (concentration range 400 to 700 A_{260} units/ml). The whole procedure is performed on ice and/or at 4°, and sterilized glassware and tubes are used.

If possible, crude ribosomes are immediately subjected to a zonal centrifugation in a gradient of 0 to 40% sucrose in $T_{10}M_6N_{30}SH_4$ buffer for 18 hr at 21,000 rpm (about 7000 A_{260} units per run, Fig. 1A). In the first zonal centrifugation, 70S tightly coupled monosomes are separated from 30S and 50S subunits and (usually small amounts of) polyribosomes. 70S peaks are pooled and ribosomes pelleted out of the sucrose solution in a 45 Ti rotor for 24 hr at 22,000 to 24,000 rpm. The use of higher centrifugation speeds is not recommended in order to avoid pressure-induced dissociation of the ribosomes. Ribosomal pellets are resuspended in $T_{10}M_6N_{30}SH_4$ (1 ml per tube) by gentle shaking for about 30 to 60 min, and then the resuspended particles are clarified by a low-speed centrifugation, and their concentration measured at A_{260}. Resuspended ribosomes are (again, if possible, without prior freezing and storing) subjected to a second zonal run under the conditions of the first run, except that the centrifugation speed is now 18,000 rpm (Fig. 1B). The 70S peak is separated from 50S

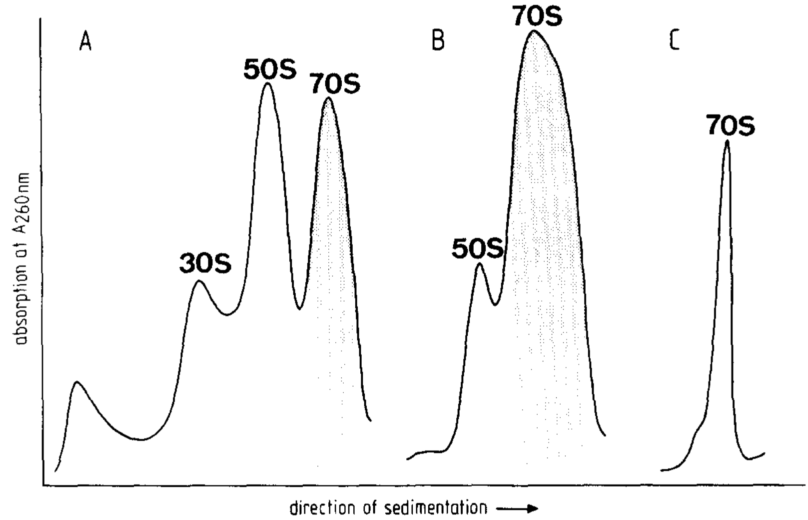

FIG. 1. Sucrose gradient profiles of tightly coupled ribosomes. A, First zonal run of crude tightly coupled 70S. The shaded area indicates the pooled 70S fractions subjected to a second zonal run (B). C, Control run of isolated tightly coupled 70S ribosomes in an SW 40 rotor.

contamination and the pooled material pelleted as described. The pellets are resuspended in $T_{10}M_{10}N_{30}SH_4$, which is the storage buffer for tightly coupled ribosomes. The particles are clarified and their concentration adjusted to 500–700 A_{260} units/ml. Small aliquots of 200 μl each are shock-frozen in liquid nitrogen and stored at −80°.

Routinely, the quality of the preparation is checked by three tests. (1) An SW 40 run was performed (gradient of 10 to 30% sucrose in $T_{10}M_{10}N_{30}SH_4$, 18 hr at 18,000 rpm and 4°) in order to test the intactness of the tight couples. They contain barely detectable amounts of 30S and less than 5% 50S (Fig. 1C). (2) RNA gels were run in order to test the intactness of the ribosomal RNA. 16S and 23S RNA have to be free from breaks. (3) A poly(U)-dependent poly(Phe) synthesis assay was performed under standard conditions.[8]

There are some critical points which have to be carefully observed during preparation:

1. Cells should not be harvested as late as possible, in order to maximize the amount of ribosomes. Concerning the activity of ribosomes the optimal harvesting point is early mid-log phase.

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[8] K. H. Nierhaus, K. Bordasch, and H. E. Homann, *J. Mol. Biol.* **74**, 587 (1973).

2. Any RNase contamination has to be strictly avoided (e.g., it is not recommended to add DNase before or during the grinding procedure, because trace amounts of RNase can spoil the preparation).
3. All work should be done preferentially on ice, but by no means at temperatures higher than 4°.
4. Ribosomes should be passed through the two zonal cycles as fast as possible (optimally without freezing between the zonal runs).
5. Ribosomes must not be salt washed (e.g., with 500 m*M* or higher concentrations of NH_4Cl), since the salt washing procedure removes ribosomal proteins and generates functional heterogeneity.
6. $T_{10}M_6N_{30}SH_4$ buffer should be used during the 70S isolation procedure, since 30 m*M* NH_4Cl is the optimal monovalent ion concentration for both the stability of tightly coupled ribosomes and the association of the subunits in the presence of 6 to 10 m*M* Mg^{2+}.

Isolation of 30S and 50S Subunits

The most active ribosomal subunits are obtained from tight couple preparations. For subunit isolation, the pellets after the first zonal run containing the 70S peak material are resuspended in the dissociation buffer $T_{10}M_3N_{100}SH_4$ and subjected to a second zonal run under the same ionic conditions. The separated subunits are pelleted for 24 hr at 24,000 rpm in a 45 Ti rotor, resuspended in $T_{10}M_{10}N_{100}SH_4$, clarified, and stored in small portions at −80°. In order to get optimal activity, it is essential to avoid precipitation of subunits either with polyethylene glycol or with ethanol. The former spoils some ribosomal activities (e.g., EF-G-dependent GTPase activity), the latter removes some ribosomal proteins and alters the ribosomal conformation drastically.

Preparation and Purification of Ac[^{14}C]Phe-tRNA

Materials

$tRNA^{Phe}$ from *E. coli*: Boehringer (Mannheim, FRG), Cat. No. 109681, Phe acceptance 1150 to 1600 pmol/A_{260} unit
[^{14}C]Phe: Amersham (UK), Cat. No. CFB 70, specific activity >450 mCi/mmol (>16.6 GBq/mmol)
ATP: Boehringer (Mannheim, FRG), 0.2 *M*, dissolved in H_2O
S150: prepared and freed from endogenous RNA as described below
Benzoylated DEAE cellulose: Boehringer (Mannheim, FRG)

Buffer 1: 50 m*M* sodium acetate, pH 5.0, 10 m*M* $MgCl_2$, 500 m*M* NaCl
Buffer 2: 50 m*M* sodium acetate, pH 5.0, 10 m*M* $MgCl_2$, 800 m*M* NaCl
Buffer 3: 50 m*M* sodium acetate, pH 5.0, 10 m*M* $MgCl_2$, 2 *M* NaCl, 20% (v/v) ethanol

Procedure

Fifty A_{260} units of $tRNA^{Phe}$, dissolved in 500 μl H_2O, are mixed with 150 μCi [^{14}C]Phe (= 300 nmol) in 3 ml H_2O containing 2% ethanol, 375 μl 1 *M* Tris-HCl (pH 7.5 at 4°), 750 μl 1 *M* KCl, 75 μl 1 *M* magnesium acetate, 120 μl 0.2 *M* ATP, and 30 μl 1.4 *M* 2-mercaptoethanol. The pH of the solution is adjusted at 7.5 using 1 *M* KOH, then H_2O and optimized amounts (usually about 1 ml) of S150 enzymes (in 10 m*M* Tris-HCl, pH 7.5 at 4°, 10 m*M* $MgCl_2$, 6 m*M* 2-mercaptoethanol) are pipetted into the reaction mixture to a final volume of 7.5 ml. After 15 min incubation at 37°, the mixture is cooled on ice and made acidic with 375 μl of 20% sodium acetate, pH 5.5. Then 7.5 ml of phenol (25% v/v H_2O) is added and after vigorous shaking (10 min) and phase separation (10 min, 7,000 rpm, HB4 Sorvall rotor), the upper water phase is withdrawn and kept at 0°. The phenol phase is washed with 7.5 ml 20% sodium acetate, pH 5.5, and 1 ml H_2O as described above. Both water phases are combined and tRNA is precipitated by the addition of 2 volumes ethanol. After 3 hr at −20°, tRNA is pelleted (15 min, 7,000 rpm), dried in a lyophilizer, and resolved in 1 ml H_2O.

Acetylation is essentially according to Haenni and Chapeville.[9] Two hundred and eighty microliters of 1 *M* sodium acetate, pH 5.5, is added, followed by four 28-μl aliquots of acetic acid anhydride every 15 min at 0°. Then the tRNA is precipitated again with 2 volumes ethanol and kept at −20° overnight. After centrifugation (15 min, 7,000 rpm) the pellet is washed once with ethanol, dried in a lyophilizer, and resuspended in 1 ml H_2O. Acetylation is 100% as judged by thin-layer chromatography. The charging degree is usually 70% (about 1100 pmol/A_{260} unit).

Ac[^{14}C]Phe-tRNA is freed from deacylated tRNA by BD-cellulose chromatography. A column (1 × 15 cm) of benzoylated DEAE-cellulose is equilibrated with buffer 1 (500 m*M* NaCl) until the absorbance of the eluate at 260 nm is below 0.03. Up to 100 A_{260} units of charged tRNA are applied, the column is washed with 50 ml of the same buffer, then with

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[9] A.-L. Haenni and F. Chapeville, *Biochim. Biophys, Acta* **114,** 135 (1966).

150 ml of buffer 2 (800 m*M* NaCl) in order to remove deacylated tRNA. The charged tRNA is eluted with buffer 3 (2 *M* NaCl, 20% v/v ethanol) and appears in 4–5 fractions (fraction size 50 drops; elution rate 30 ml/hr). AcPhe-tRNA is precipitated with 2 volumes ethanol (−20°, overnight), pelleted by low-speed centrifugation, washed twice with 70% ethanol and once with 100% ethanol, dried (1 min) in a lyophilizer, and resuspended in 1 ml H_2O. The concentration is adjusted to about 30 pmol/μl and the preparation stored in small portions at −80°. The preparations usually contain 1400 to 1500 pmol phenylalanine per A_{260} unit.

Preparation of tRNA-Free S150 Enzymes

Materials

DEAE-cellulose: DE-52, Whatman (Maidstone, Kent, UK)
$T_{10}M_{10}K_{150}$: 10 m*M* Tris-HCl, pH 7.5 (4°), 10 m*M* $MgCl_2$, 150 m*M* KCl
$T_{10}M_{10}K_{200}$: 10 m*M* Tris-HCl, pH 7.5 (4°), 10 m*M* $MgCl_2$, 200 m*M* KCl
$T_{10}M_{10}K_{500}$: 10 m*M* Tris-HCl, pH 7.5 (4°), 10 m*M* $MgCl_2$, 500 m*M* KCl
$T_{10}M_{10}SH_6$: 10 m*M* Tris-HCl, pH 7.5 (4°), 10 m*M* magnesium acetate, 6 m*M* 2-mercaptoethanol

Procedure

Fifteen grams of DEAE-cellulose is dissolved in 300 ml $T_{10}M_{10}K_{500}$ and incubated for 30 min at 90°. After cooling down to 4° the matrix is washed 4 times with 300 ml $T_{10}M_{10}K_{150}$. After the last washing step, 150 ml of S150 enzymes[10] is added to the cellulose and thoroughly mixed. The mixture is added to centrifuge tubes, kept for 2 hr at 4° and then centrifuged for 30 min at 8000 rpm. Supernatant I is withdrawn and the procedure is repeated three times, adding 20 ml $T_{10}M_{10}K_{150}$ (supernatant II), $T_{10}M_{10}K_{200}$ (supernatant III), and $T_{10}M_{10}K_{500}$ (supernatant IV), respectively. The supernatants are dialyzed overnight against $T_{10}M_{10}SH_6$. The absorption is measured at 230, 260, and 280 nm and the supernatants are tested for their tRNA content in a ligase assay under the ionic conditions described for tRNA charging in the preceding section. Supernatants II and III are essentially free from tRNA and stored in small portions at −80°.

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[10] K. H. Nierhaus and F. Dohme, this series, Vol. 59, p. 443.

Testing the Binding Activity of the Ribosome Preparations

Materials

Mix 1: 230 m*M* Tris-HCl, pH 7.5 (4°), 30 m*M* magnesium acetate, 740 m*M* NH_4Cl, and 14 m*M* 2-mercaptoethanol

Mix 2: 62 m*M* Tris-HCl, pH 7.5 (4°), 25 m*M* magnesium acetate, 200 m*M* NH_4Cl, and 6 m*M* 2-mercaptoethanol

Mix 3: 210 m*M* Tris-HCl, pH 7.5 (4°), 53 m*M* magnesium acetate, 720 m*M* NH_4Cl, and 21 m*M* 2-mercaptoethanol

$T_{50}M_{15}N_{160}SH_4$ (binding buffer): 50 m*M* Tris-HCl, pH 7.5 (4°), 15 m*M* magnesium acetate, 160 m*M* NH_4Cl, 4 m*M* 2-mercaptoethanol

Poly(U): Boehringer (Mannheim, FRG); 3.75 mg/ml H_2O

Energy mix: GTP (Boehringer, Mannheim, FRG), 2 m*M*; phosphoenolpyruvate (Boehringer, Mannheim, FRG), 20 m*M*, solved in mix 3 and adjusted to pH 7.5 at 4° (1 volume), plus pyruvate kinase (Boehringer, Mannheim, FRG), 0.1 mg/ml H_2O (2 volumes)

Puromycin: Serva (Heidelberg, FRG); 10 m*M*, dissolved in $T_{50}M_{15}M_{160}SH_4$ (binding buffer), adjusted to pH 7.5 at 4° with unbuffered Tris

$tRNA^{Phe}$ from *E. coli*: Boehringer (Mannheim, FRG); Phe acceptance 1150 to 1600 pmol/A_{260} units, dissolved in H_2O to a suitable concentration (usually 75 pmol/μl)

Ac[^{14}C]Phe-tRNA: prepared as described above, charging degree 1400 to 1500 pmol [^{14}C]Phe/A_{260} unit, dissolved in H_2O to a concentration of 30 to 60 pmol/μl

Elongation factor G (EF-G): prepared according to Leberman *et al.*,[11] stored in a buffer containing 10 m*M* Tris-HCl pH 7.5 (4°), 10 m*M* magnesium acetate, 100 m*M* KCl, 10 m*M* 2-mercaptoethanol, and 20% v/v glycerol

Sodium acetate, 0.3 *M*, pH 5.5/$MgSO_4$ saturated

Ethyl acetate

Nitrocellulose filters: Sartorius No. 11 306 (Göttingen, FRG)

All buffers, poly(U), energy mix, $tRNA^{Phe}$, EF-G are stored at −20°, Ac[^{14}C]Phe-tRNA and ribosomes are stored at −80°. Puromycin was prepared immediately before use.

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[11] R. Leberman, B. Antonsson, R. Giovanelli, R. Guariguata, R. Schumann, and A. Wittinghofer, *Anal. Biochem.* **104,** 29 (1980).

Procedure

We use an *in vitro* system based on Watanabe[12] which allows—in the absence of any factors—the binding of peptidyl-tRNA specifically either to the P site or the A site of programmed ribosomes. The test is performed in four steps.

Step 1. In a normal assay comprising nine aliquots, 45 μl mix 1, 45 μl poly(U), 45 μl H_2O, and 90 μl of $T_{10}M_{10}N_{30}SH_4$ buffer containing 90 to 450 pmol 70S ribosomes are preincubated for 10 min at 37°. The final ion concentrations at this step are 50 m*M* Tris-HCl, 10 m*M* Mg^{2+}, 160 m*M* NH_4^+, and 4 m*M* 2-mercaptoethanol. If subsequent A-site binding of peptidyl-tRNA is to be achieved, deacylated $tRNA^{Phe}$ dissolved in H_2O is added in addition in a molar ratio of 1.5 to 2.0 per 70S.

Step 2. In the second step, 180 μl mix 2 is added. In order to test the binding capacity of the 70S particles, 45 μl H_2O containing N-acetylated Phe-tRNA in increasing amounts is added either under P-site or A-site binding conditions. The final ion concentrations are the same as in step 1, except that Mg^{2+} is raised to 15 m*M*. Samples are incubated for 30 min at 37°. After incubation, two 50-μl aliquots of the nine-aliquot sample are removed, diluted with 2 ml of ice-cold binding buffer ($T_{50}M_{15}N_{160}SH_4$), filtered through nitrocellulose filters, rinsed two times with 2 ml of the same buffer, dried under an infrared lamp, and counted.

Step 3. In order to test the site location of the Ac[^{14}C]Phe-tRNA bound in the second step, the remaining 350 μl is supplied with 70 μl of an energy regeneration mix containing GTP, phosphoenol pyruvate, and pyruvate kinase (final concentrations 0.1 m*M*, 1 m*M*, and 10 μg/ml, respectively) and then divided into six 60-μl aliquots. Four aliquots receive 5 μl EF-G storage buffer, the remaining two substoichiometric amounts of EF-G (ratio of EF-G to 70S = 0.2) in EF-G storage buffer. Samples are incubated for 10 min at 37°. P-site bound Ac[^{14}C]Phe-tRNA is expected not to be affected by the addition of EF-G, whereas A-site bound Ac[^{14}C]Phe-tRNA undergoes translocation.

Step 4. In the last incubation step, two aliquots without and two aliquots with EF-G receive 5 μl of a puromycin solution (final concentration 0.7 m*M*), and two aliquots receive 5 μl $T_{50}M_{15}N_{160}SH_4$ buffer without puromycin serving as a background control for the subsequent extraction procedure. Puromycin exclusively reacts with P-site bound Ac[^{14}C]Phe-tRNA and forms Ac[^{14}C]Phe-puromycin. After 30 to 90 min incubation at 0°, 65 μl of 0.3 *M* sodium acetate $MgSO_4$ saturated is added to the samples, and the product is separated from the unreacted material by extrac-

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[12] S. Watanabe, *J. Mol. Biol.* **67**, 443 (1972).

tion with 1 ml of ethyl acetate. Samples are vigorously shaken for 1 min. Phase separation is allowed to take place for 15 min at 0°, and then the upper 0.7 ml of the organic phase is withdrawn, mixed with scintillation cocktail, and counted. Usually, incubation at 0° for 30 min yields no quantitative puromycin reaction. The site location of the Ac[^{14}C]Phe-tRNA bound to the ribosome is estimated in the following way: the values of the puromycin reaction in the absence of EF-G (originally P-site bound material) are divided by the values of the puromycin reaction in the presence of EF-G (originally P-site bound material plus translocated material). A ratio of 1 indicates 100% P-site location, whereas a ratio of 0 indicates 0% P-site (= 100% A-site) location in the binding reaction (second step).

Cautions Concerning the Puromycin Reaction

The puromycin reaction only gives a reliable picture of the site location of bound peptidyl-tRNA, if certain restrictions are observed.[13]

1. It is not recommended to perform the puromycin reaction at room temperature or even at 37°, if a significant pool of free peptidyl-tRNA is present in solution, as is usually the case under saturation conditions. Under such conditions, even in the absence of EF-G, the puromycin reaction will become repetitive due to spontaneous translocation, the extent being dependent on the incubation time. The repetitive reaction is even more pronounced if EF-G is present. Therefore, the ratio of the puromycin values in the absence and presence of EF-G no longer indicates the site location of the originally bound peptidyl-tRNA.
2. Under saturation conditions, the puromycin reaction will become repetitive even at 0° in the presence of stoichiometric excess of EF-G at longer incubation times. Therefore the translocation reaction should be performed with *catalytic* amounts of EF-G. Quantitative translocation can be achieved at a ratio of EF-G to ribosomes not larger than 0.2 : 1.
3. For the puromycin incubation, the pH should be adjusted to between 7.5 and 8.0. At pH values lower than 7.0 the puromycin reaction becomes very slow at 0°.

To avoid the difficulties mentioned, the puromycin reaction should, as a rule, be carried out at 0°. For substoichiometric amounts of peptidyl-tRNA bound, 30 to 90 min of incubation will suffice to get a near quantitative reaction. Under saturation conditions, a quantitative reaction requires up to 50 hr of incubation. Nevertheless, a reliable picture of the site

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[13] U. Geigenmüller, Th. P. Hausner, and K. H. Nierhaus, *Eur. J. Biochem.* **161,** 715 (1986).

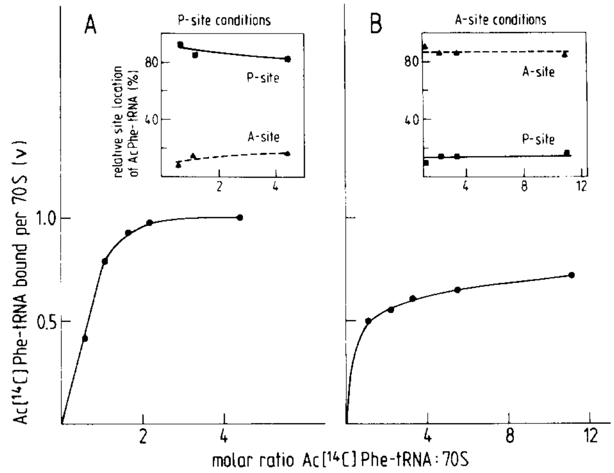

FIG. 2. Saturation of tight couples under P-site (A) and A-site conditions (B); (●) Binding curves. The inserts show the relative site location of AcPhe-tRNA as measured by the puromycin reaction (30 min at 0°). (■) P-site location; (▲) A-site location. Aliquots contained 13.8 pmol 70S.

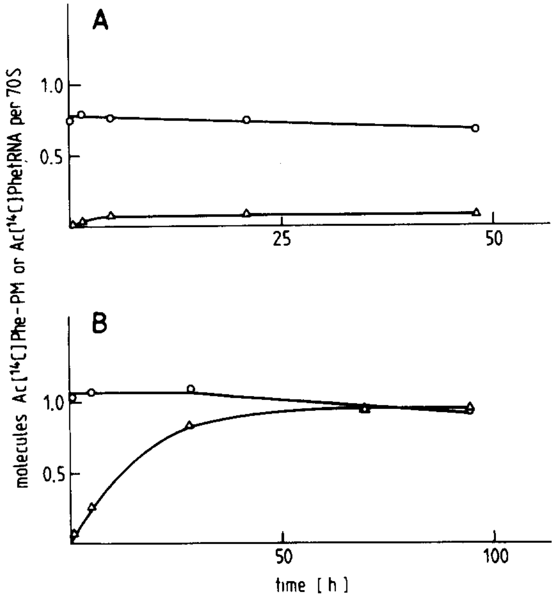

FIG. 3. Quantitative puromycin reaction of A-site bound (A) and P-site bound (B) AcPhe-tRNA (ratio of added AcPhe-tRNA to 70S = 8 and 3, respectively). (○) binding of AcPhe-tRNA; (△) puromycin reaction. After the binding reaction had been performed, one half of the sample was used for the puromycin reaction (incubation time at 0° as indicated), the other half was kept at 0° and the binding controlled at the indicated times. Aliquots contained 6.15 pmol 70S. From Geigenmüller *et al.*[13]

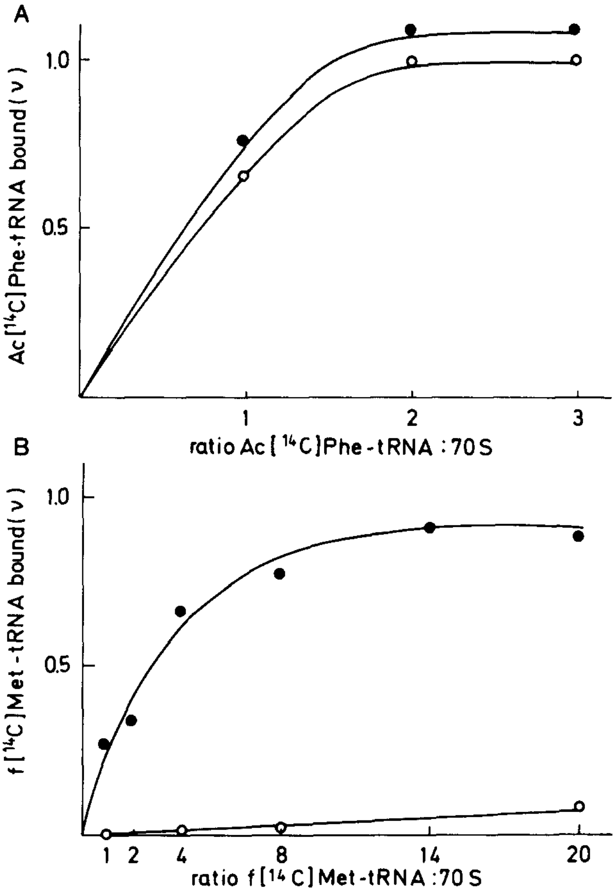

FIG. 4. (A) AcPhe-tRNA binding to poly(U)-programmed 70S ribosomes. Aliquots contained 8.9 pmol 70S ribosomes. (○) Ac[^{14}C]Phe-tRNA (1400 pmol per A_{260} unit) purified by BD-cellulose chromatography as described in this paper. (●) Ac[^{14}C]Phe-tRNA (1750 pmol per A_{260} unit) purified according to Odom *et al.*[14] on an HPLC column. (B) Initiation-factor dependent binding of f[^{14}C]Met-tRNA$_f^{Met}$ to 70S ribosomes in the presence of MS2 RNA. One aliquot (100 μl) contained 12 pmol 70S, 144 pmol MS2 RNA, 12 to 240 pmol f[^{14}C]Met-tRNA$_f^{Met}$ (150 cpm/pmol), and 5 μl of a crude initiation-factor preparation (142 A_{230}/ml) where indicated. The ionic conditions were 12 m*M* Mg^{2+}, 50 m*M* NH_4^+ and 30 m*M* K^+. The MS2-directed translation system has been described in Funatsu *et al.*[15] f[^{14}C]Met-tRNA$_f^{Met}$ has been prepared according to Kahn *et al.*[16] The preparation of initiation factors was according to Noll *et al.*,[17] except that the final precipitation step was omitted. Initiation factors were stored in a buffer containing 20 m*M* HEPES·KOH, pH 7.5, 1 m*M* EDTA, 0.5 m*M* DTE, 10 m*M* NH_4Cl and 10% glycerol. (○) binding in the absence of initiation factors, and (●) binding in the presence of initiation factors.

location (percentage of P-site and A-site bound material) equivalent to a quantitative reaction is obtained also with a short incubation time, if the precautions listed above are observed.

Results of the Activity Test

Site-Specific Saturation. Figure 2 shows site-specific saturation of 70S tight couples with Ac[^{14}C]Phe-tRNA. Under P-site conditions (Fig. 2A), exactly one Ac[^{14}C]Phe-tRNA molecule per 70S can be bound, whereas under A-site binding conditions (P site blocked with deacylated tRNA, Fig. 2B) the saturation level is somewhat lower (0.75 for the concentration range tested). The site specificity of the bound Ac[^{14}C]Phe-tRNA is better than 80% over the whole concentration range (see the puromycin reaction depicted in the inserts of Figs. 2A and B). Figure 3 demonstrates that all material bound under P-site conditions can react with puromycin, provided the incubation time is long enough (Fig. 3A), whereas A-site bound material remains essentially puromycin-insensitive during the same time of incubation at 0° (Fig. 3B).

For comparison, AcPhe-tRNA was purified on a HPLC column[14] (instead of by BD-column chromatography as described in this paper) yielding AcPhe-tRNA essentially free of contaminating deacylated tRNA (1750 pmol/A_{260} versus 1400 pmol/A_{260} of the BD-purified AcPhe-tRNA, see Fig. 4A). The same saturation level of about one AcPhe-tRNA per poly(U)-programmed 70S ribosome is obtained. This finding indicates that the content of contaminating deacylated tRNA (at least up to a contamination of 20%) does not affect the saturation level of AcPhe-tRNA in accordance with the exclusion principle for AcPhe-tRNA binding.[13]

The initiation-factor dependent binding of fMet-tRNA to 70S ribosomes in the presence of the natural mRNA MS2 also levels off at 0.9 fMet-tRNA per 70S ribosome (Fig. 4B).[15-17] The binding results clearly demonstrate that 90-100% of the ribosomes prepared observing the precautions described here participate in tRNA binding to the P site.

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