[19] Preparation of Functional Ribosomal Complexes and Effect of Buffer Conditions on tRNA Positions Observed by Cryoelectron Microscopy

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Introduction

Electron microscopy and immunoelectron microscopy have played an important role in establishing the gross shape of ribosomes and in localizing of proteins within the ribosome during the 1970s and 1980s.[1-3] Due to the development of three-dimensional cryoelectron microscopy (3-D cryo-EM) of single particles[4-6] the ribosome is now seen with much higher quality: reconstructions of the highly asymmetric particles reached a resolution of 20–25 Å.[7,8] In the light of this new resolution it was possible to study functional complexes, i.e., ribosomes carrying tRNAs,[9,10] and elongation factors such as EF-Tu[11] and EF-G.[12] A further improvement in resolution was achieved by using programmed ribosomes with high occupancy at the P site (80%) with fMet-tRNA: for the first time, a large portion of the tRNA was directly visible, thus allowing an unambiguous localization and orientation of the tRNA within the intersubunit space. A large part of

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this improvement in resolution has been attributed to the high degree of conformational stability of the ribosome–tRNA complex.[13]

This article covers the isolation of the ribosomes and the preparation of functional complexes followed by a short overview of the possibilities for analyzing ribosomal complexes. Further, we summarize and discuss the results of recent cryoelectron microscopy studies that reflect the effect of buffer conditions. Although previous studies have established that the ribosome has three tRNA binding sites, 3-D cryo-EM has surprisingly revealed five different tRNA positions on the ribosome, classified as A, P, P/E, E, and E2.[14–16] The occupancy of some of these positions strongly depends on the buffer conditions used and the charge state of the tRNA. We will show that in the presence of the polyamine buffer, mimicking the *in vivo* conditions, only occupancy of A, P, and E sites is observed in complexes of the initiating and elongating ribosomes.

Preparation of 70S Ribosomes

The procedure described here for the small-scale isolation of tightly coupled ribosomes yields highly active and intact ribosomes, an important prerequisite for the preparation of functional complexes. For large-scale isolation using zonal centrifugation see Bommer *et al.*[17] In addition we describe the isolation of ribosomal subunits that can be used to prepare reassociated ribosomes. Reassociated ribosomes show a more efficient tRNA binding, as compared to tightly coupled ribosomes, because saturation of tRNA binding is reached at molar ratios slightly above stoichiometric ones. This can be attributed to at least two factors, (1) a selective pressure for active particles in the reassociation step and (2) the loss of residual amounts of tRNAs (about 0.8 per 70S ribosomes)[18] and of mRNA fragments.

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Materials

To minimize damage by RNases, we use the strain CAN/20-E12 derived from *Escherichia coli* K12, which is deficient in RNases BN, II, D, I.[19]

Bacto-tryptone and yeast extract, DIFCO Laboratories (Detroit, MI); Alcoa A-305, Serva (Heidelberg, Germany); sucrose (ultrapure), GibcoBRL (UK); all other chemicals mentioned are from Merck (Darmstadt, Germany) and Roche Diagnostics (Mannheim, Germany).

Buffers

Ribosome buffer $H_{20}M_6N_{30}SH_4$: 20 m*M* HEPES–KOH, pH 7.5, at 4°, 6 m*M* magnesium acetate, 30 m*M* NH_4Cl, and 4 m*M* 2-mercaptoethanol. (Tightly coupled ribosomes withstand these conditions, whereas loosely couple ribosomes dissociate into subunits. Tightly coupled ribosomes are functionally competent in contrast to the loosely coupled 70S.)[20]

Dissociation buffer $H_{20}M_1N_{200}SH_4$: 20 m*M* HEPES–KOH, pH 7.5, at 4°, 1 m*M* magnesium acetate 200 m*M* NH_4Cl, and 4 m*M* 2-mercaptoethanol (lowering Mg^{2+} and raising monovalent ions cause dissociation of ribosomes).

Reassociation buffer $H_{20}M_{20}K_{30}SH_4$: 20 m*M* HEPES–KOH, pH 7.5, at 4°, 20 m*M* magnesium acetate 30 m*M* KCl, and 4 m*M* 2-mercaptoethanol.

Growth of Bacteria

1 liter of LB media [10 g Bacto-tryptone, 5 g yeast extract, 5 g NaCl, and 25 ml of a 20% (w/v) glucose solution per 1 liter] is inoculated with 10 ml of an overnight culture of *E. coli* CAN/20-E12. Fermentation is performed under continuous agitation at 37°. Cell growth is stopped at 0.5 A_{560} units per milliliter, ensuring that mid-log phase had not been passed. The activity of ribosomes depends considerably on the time at which the cells are harvested. Early mid-log phase ribosomes have proved to be optimal in tRNA binding and elongation. After centrifugation for 15 min at 10,000 rpm (about 30,000*g* at 4°) in a Sorvall GSA rotor, wet cells (usually 1–1.2 g per 1 liter) can be stored at −80°.

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Isolation of Crude 70S

The following procedure is performed on ice or at 4°, and sterilized glassware and tubes are used. The cells are resuspended in 30 ml of ribosome buffer $H_{20}M_6N_{30}SH_4$ and centrifuged for 15 min at 10,000 rpm (about 30,000*g*) in a Sorvall GSA rotor, mixed with Alcoa A-305 (twofold cell weight) and ground in a prechilled mortar and pestle for 2 min. The cell paste is resuspended in ribosome buffer $H_{20}M_6N_{30}SH_4$ (2 ml per 1g cells). The paste is subjected to two low-speed centrifugation steps (20 min at 12,000 rpm in a Sorvall SS34 rotor to remove the Alcoa A-305 followed by 60 min at 16,000 rpm in a Sorvall SS34 rotor to remove the cell debris; 15,000 and 30,000*g*, respectively). The resulting supernatant is centrifuged for 17 hr at 40,000 rpm (about 110,000*g*) in a Beckman 70.1 Ti rotor. The crude ribosomal pellet is rinsed with ribosome buffer $H_{20}M_6N_{30}SH_4$ buffer when tightly coupled 70S are to be isolated, or with dissociation buffer $H_{20}M_1N_{200}SH_4$ when ribosomal subunits are to be isolated. The pellet is then resuspended in the same buffer with continuous shaking for about 1 hr. The resuspended crude ribosomes are clarified (5 min at 10,000 rpm in a Eppendorf centrifuge 5415) and their concentration is determined. Usually, the yield is 300–400 A_{260} units per gram of cells and the concentration is between 500 and 1,000 A_{260} units per milliliter.

Isolation of Tight-Coupled 70S

The crude ribosomes in ribosome buffer $H_{20}M_6N_{30}SH_4$ are loaded on a sucrose gradient (10–30% sucrose; about 100 A_{260} units per bucket) in ribosome buffer $H_{20}M_6N_{30}SH_4$ and centrifuged for 16 hr at 19,000 rpm (about 48,000*g* at 4°) in a Beckman SW 28 rotor. After centrifugation the tightly coupled 70S ribosomes are separated from the subunits. The gradient is fractionated and the fractions of the 70S peak are pooled and pelleted (24 hr at 24,000 rpm, about 47,000*g*, in a 55.2 Ti rotor at 4°).

Ribosomal pellets are resuspended in ribosome buffer $H_{20}M_6N_{30}SH_4$ (100 μl per tube) by gently shaking for about 60 min at 4°. The resuspended particles are clarified by a low-speed centrifugation, and their concentrations are determined from the A_{260}.

Isolation of 30S and 50S Subunits

The crude ribosomes are loaded on a sucrose gradient (10–30% sucrose; about 100 A_{260} units per bucket) in dissociation buffer $H_{20}M_1N_{200}SH_4$ and centrifuged for 17 hr at 19,000 rpm (about 48,000*g* at 4°) in a Beckman SW 28 rotor. The centrifugation separates the subunits from tRNA (4 to 5S) and mRNA fragments. The gradient is fractionated and the 50S and 30S

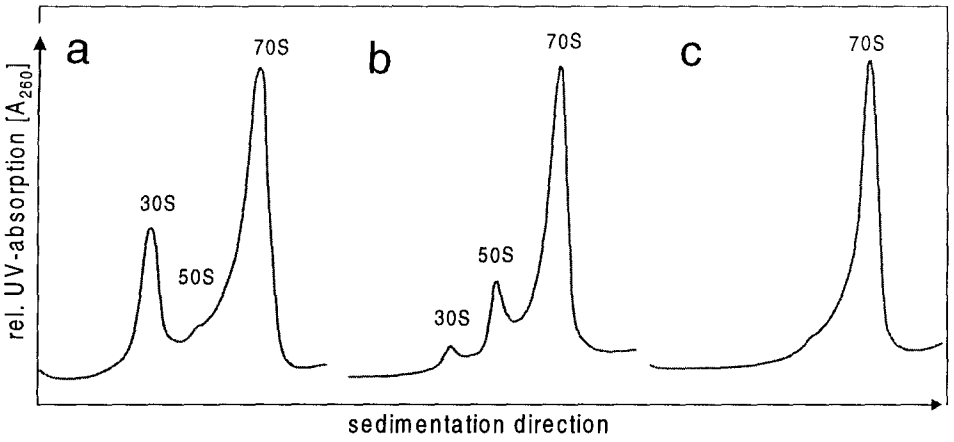

FIG. 1. Analytical sucrose gradient runs in reassociation buffer $H_{20}M_{20}K_{30}SH_4$. (a) A_{260} profile after incubating the 30S plus 50S mixture, (b) after isolating the 70S peak from the sucrose gradient, and (c) after a final incubation at 40° for 20 min.

peaks are individually pooled and pelleted (20 hr at 48,000 rpm, about 190,000*g*, in a 55.2 Ti rotor at 4°).

Ribosomal pellets are resuspended in reassociation buffer $H_{20}M_{20}K_{30}SH_4$ (100 μl per tube) by gently shaking for about 60 min at 4°. The resuspended particles are cleared by a low-speed centrifugation and their concentrations are determined from A_{260}.

Preparation of Reassociated 70S Ribosomes

The 50S and 30S subunits are mixed in a 1 : 1 ratio of A_{260} units, diluted to a final concentration of 40–140 A_{260}/ml in reassociation buffer $H_{20}M_{20}K_{30}SH_4$, and incubated for 40–60 min at 40° (Fig. 1a). By using an excess of 30S the amount of free 50S is minimized, thus improving the separation of the reassociated 70S ribosomes from the 50S subunits in the following gradient centrifugation. After incubation (10 min at 4°) the particles are subjected to a gradient centrifugation (10–30% sucrose; about 100 A_{260} units per bucket) in reassociation buffer $H_{20}M_{20}K_{30}SH_4$ and centrifuged for 17 hr at 18,000 rpm (about 35,000*g* at 4°) in a Beckman SW 28 rotor. The gradient is fractionated and the 70S peak is pooled and pelleted by centrifugation in a 50.2 Ti rotor for 27 hr at 21,000 rpm, about 40,000*g* at 4°. The use of higher centrifugation speeds is not recommended because it may lead to pressure-induced dissociation of the ribosomes.[21] The ribosomal

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pellets are resuspended in reassociation buffer $H_{20}M_{20}K_{30}SH_4$ (Fig. 1b) and incubated 20 min at 40° (Fig. 1c). After the solution has been cleared by low-speed centrifugation, the concentration is determined from the A_{260}. Small aliquots (50 μl) are shock frozen in liquid nitrogen and stored at −80°. A homogenous population of reassociated ribosomes is obtained with a yield of around 75% (100% is the input of 50S in A_{260} units multiplied with 1.5). When using reassociated ribosomes in the poly(U)-dependent poly(Phe) synthesis or in the preparation of functional complexes, the ionic conditions have to be adapted to the ribosome buffer $H_{20}M_6N_{30}SH_4$.

Routinely, the quality of the preparation is checked using three assays: (1) A SW 40 run is performed (gradient of 10–30% sucrose in reassociation buffer $H_{20}M_{20}K_{30}SH_4$, 16 hr at 22,000 rpm, about 34,000*g*, and 4°) in order to test the homogeneity of the reassociated 70S (see Fig. 1c). (2) RNA gels are run in order to test the intactness of the ribosomal RNA. 16S and 23S RNA have to be essentially free from breaks. (1) and (2) establish the structural integrity of the particle. (3) The activity in the poly(U)-dependent poly(Phe) synthesis system is an important criterion for estimating the activity of the reassociated 70S preparation. Bommer *et al.*[17] describe this assay system in detail: it uses the postribosomal supernatant (S-100) as the source of enzymes, i.e., tRNA synthetases, the elongation factors EF-Tu, EF-G, and EF-Ts, etc. The ionic conditions are identical to those of the binding buffer $H_{20}M_6N_{150}SH_4Sp_{0,05}Spd_2$ that are also used for the preparation of functional complexes.

Preparation of Defined Functional Complexes

The two main states of the ribosome in the ribosomal elongation cycle, namely, the state before translocation or PRE state and the state after translocation or POST state,[22] are prepared and assayed in an experimental approach based on a procedure described by Watanabe in 1972.[23] The ability to produce homogeneous PRE and POST states is due to the high energy barrier between the two states. During elongation EF-Tu and EF-G catalyze the transition from POST to PRE and from PRE to POST, respectively.[22] Some antibiotics can fix the elongation factors on the ribosome. In particular, kirromycin stalls EF-Tu on the ribosome[11] and fusidic acid prevents dissociation of EF-G after translocation.[12]

The reaction scheme in Fig. 2 allows a controlled stepwise execution of one complete elongation cycle, thus providing a tool for the precise determination of the occupation of the three tRNA binding sites on the

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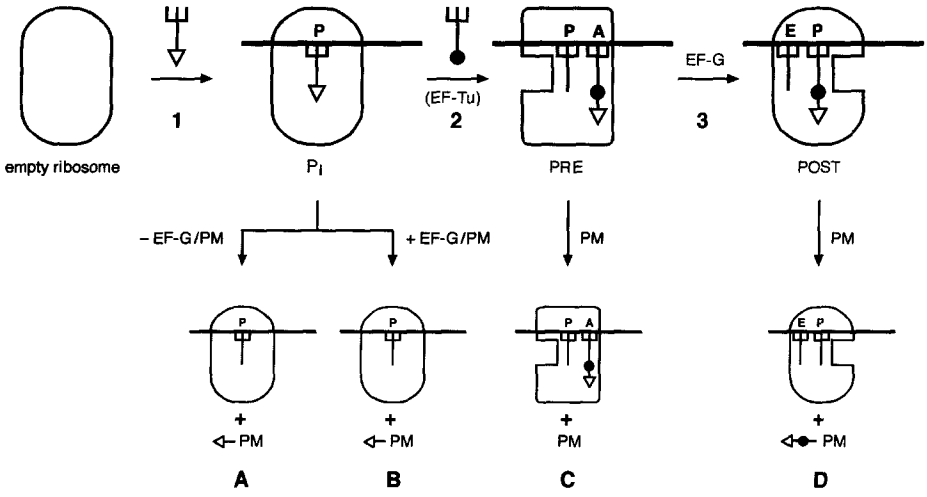

FIG. 2. Reaction scheme for the preparation of ribosomal functional complexes carrying tRNAs in defined positions. The P_i complex carries only one tRNA, namely, a peptidyl-tRNA in the P site, the PRE complex has a deacyl-tRNA in the P site and a peptidyl-tRNA in the A site, which move to the E and P sites on translocation (3), respectively. (A–D) Products of the puromycin reaction.

various complexes. In the first step, a 70S · mRNA · tRNA complex is formed, in which the tRNA is located in the ribosomal P site (note that the first tRNA to be bound to ribosomes usually occupies the P site). Either AcPhe-$tRNA^{Phe}$ or fMet-$tRNA^{fmet}$ are used, the α-amino group of which are blocked by an acetyl and formyl group, respectively, and therefore behave like a peptidyl-tRNA. A programmed ribosome that carries only one tRNA occupying the P site mimics an initiation complex and is called P_i complex (**i** for **i**nitiation). The P_i complex is assayed with and without EF-G in the puromycin reaction. No difference in reactivity with puromycin (Fig. 2, products A and B) indicates 100% P-site occupation. A similar highly defined P_i complex carrying an fMet-$tRNA^{fMet}$ led to a cryoelectron microscopy reconstruction of the ribosome with the highest resolution obtained so far with a functional complex (better than 15 Å).[13]

In the second step the A site is filled with the corresponding cognate aminoacyl-tRNA. Peptidyl transfer is a fast step in the elongation cycle and occurs immediately. The PRE complex is translocated in the third step by the addition of EF-G. In the POST complex the tRNAs are now located in the P and E sites, respectively. The efficiency of the translocation reaction and the binding state of the tRNAs can be determined by means of the puromycin reaction: the P site bound peptidyl-tRNA will react (Fig. 2,

product D), while the peptidyl-tRNA in the A site will not (Fig. 2, product C). PRE and POST states show typically about 80% homogeneity.[24] Binding values (tRNAs per ribosome) for each complex are assayed via nitrocellulose filter binding.

Single Reactions of the Elongation Cycle Produce Defined Functional States

We use a fully homologous system with respect to the source of ribosomes, tRNAs, factors, and enzymes in order to avoid ambiguities concerning tRNA binding features. The use of heteropolymeric mRNAs containing unique codons is to ensure unequivocal assignments of the bound tRNA to the various ribosomal sites,[25] although homopolymeric mRNAs, e.g., poly(U), in general yield higher binding values for tRNAs. Design and synthesis of heteropolymeric mRNAs are described by Bommer *et al.*[17] Near-physiologic conditions (polyamine buffer) with respect to the concentrations of the important ions are essential (see later discussion). The different mixes and buffers listed next are used to maintain the conditions of the binding buffer $H_{20}M_6N_{150}SH_4Sp_{0,05}Spd_2$ during the assay.

Buffers

Mix I: $H_{60}M_{18}N_{690}SH_{12}Sp_{0.25}$ Spd_{10}: 60 m*M* HEPES–KOH (pH 7.5 at 0°), 18 m*M* $MgCl_2$, 690 m*M* NH_4Cl, 12 m*M* 2-mercaptoethanol, 0.25 m*M* spermine, 10 m*M* spermidine

Mix II: $H_{100}M_{30}N_{750}SH_{20}Sp_{0.25}Spd_{10}$: 100 m*M* HEPES–KOH (pH 7.5 at 0°), 30 m*M* $MgCl_2$, 750 m*M* NH_4Cl, 20 m*M* 2-mercaptoethanol, 0.25 m*M* spermine, 10 m*M* spermidine

Mix IIE (**E** for **e**nzymatic A-site occupation): $H_{40}M_{12}N_{300}SH_8$ $Sp_{0.125}Spd_5$: 40 m*M* HEPES–KOH (pH 7.5 at 0°), 12 m*M* $MgCl_2$ 300 m*M* NH_4Cl, 8 m*M* 2-mercaptoethanol, 0.125 m*M* spermine, 5 m*M* spermidine

Mix III: $H_{66.7}M_{20}N_{500}SH_{13.3}Sp_{0.25}Spd_{10}$: 66.7 m*M* HEPES–KOH (pH 7.5 at 0°), 20 m*M* $MgCl_2$, 500 m*M* NH_4Cl, 13.3 m*M* 2-mercaptoethanol, 0.25 m*M* spermine, 10 m*M* spermidine

HMK buffer $H_{20}M_6K_{150}SH_4$: 20 m*M* HEPES–KOH (pH 7.5 at 0°), 6 m*M* $MgCl_2$, 150 m*M* KCl, 4 m*M* 2-mercaptoethanol

Ribosome buffer $H_{20}M_6N_{30}SH_4$: 20 m*M* HEPES–KOH (pH 7.5 at 0°), 6 m*M* $MgCl_2$, 30 m*M* NH_4Cl, 4 m*M* 2-mercaptoethanol

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Binding buffer $H_{20}M_6N_{150}SH_4Sp_{0,05}Spd_2$: 20 m*M* HEPES–KOH (pH 7.5 at 0°), 6 m*M* $MgCl_2$, 150 m*M* NH_4Cl, 4 m*M* 2-mercaptoethanol, 0.05 m*M* spermine, 2 m*M* spermidine

P Site Binding: Construction of the Pi Complex

For construction of a P_i complex, incubate 110 pmol of 70S ribosomes (10 pmol per single determination) in 275 μl of binding buffer containing a 5- to 10-fold molar excess of heteropolymeric mRNA over ribosomes and fMet-tRNA in a 1.2- to 1.5-fold molar ratio to ribosomes for 30 min at 37°. The optimal amount of heteropolymeric mRNA and tRNA are determined according to their ability to saturate specific tRNA binding in filter binding assays. MF-mRNA is used as a heteropolymeric mRNA in many cases. This mRNA is 46 nucleotides long (a sequence of this length is covered by the ribosome) and contains in the middle the codons AUG (coding for Met)–UUC (Phe). We describe the preparation of 11 aliquots although only 10 will be used. The extra aliquot allows for minor pipetting imprecisions.

The 275 μl is pipetted in the following sequence:

27.5 μl of mix I $H_{60}M_{18}N_{690}SH_{12}Sp_{0.25}Spd_{10}$

27.5 μl of mix II $H_{100}M_{30}N_{750}SH_{20}Sp_{0.25}$ Spd_{10} (the separation into two mixes I and II is required for A-site binding as described in the next section; if only P-site binding is planned, these two mixes can be easily combined)

27.5 μl H_2O containing MF-mRNA

137.5 μl H_2O containing f[^{3}H]Met-tRNA^{fMet}

55 μl of ribosomes in ribosome buffer $H_{20}M_6N_{30}SH_4$

Incubate 30 min at 37° and then add 55 μl of a mix composed of mix III, H_2O, and a guanosine triphosphate (GTP) solution (2.5 m*M*, pH 6 with KOH) in a volume ratio of 3:4:3. Divide into 10 aliquots, 30 μl each. Continue as described in the Translocation section.

A-Site Binding: Construction of PRE Complex

When constructing a PRE complex the first site to be occupied with a tRNA is the P site. After this has been accomplished an acyl-tRNA is added in a 0.8- to 2-fold molar ratio to ribosomes in case of nonenzymatic A-site binding. For enzymatic A-site binding a ternary complex (aminoacyl-tRNA · EF-Tu · GTP) is formed immediately before its addition to the binding assay: aminoacyl-tRNA (1–2 pmol per picomole of 70S ribosomes), 0.5 m*M* GTP, and EF-Tu (1.2 pmol per picomole of aminoacyl-tRNA) is preincubated for 2 min at 37° under the ionic conditions of the binding buffer and is added to the reaction mixture.

First the P site is occupied by mixing

27.5 μl of mix I $H_{60}M_{18}N_{690}SH_{12}Sp_{0.25}Spd_{10}$

27.5 μl of H_2O containing the MF-mRNA

27.5 μl of H_2O with the fMet[^{3}H]tRNA^{fMet}

55 μl of ribosome buffer $H_{20}M_6N_{30}SH_4$ containing 110 pmol of ribosomes and incubating 10 min at 37°.

For nonenzymatic A-site occupation prepare a mix containing 27.5 μl of mix II $H_{100}M_{30}N_{750}SH_{20}Sp_{0.25}Spd_{10}$ and 110 μl of H_2O with [^{14}C]Phe-tRNA^{Phe}, and add to the ribosome mixture. For enzymatic A-site occupation prepare a mix containing 55 μl of mix IIE $H_{40}M_{12}N_{300}SH_8Sp_{0.125}Spd_5$, 33 μl of H_2O with [^{14}C]Phe-tRNA^{Phe}, 27.5 μl of HMK buffer containing EF-Tu, and 22 μl of a GTP solution (2.5 mM, pH 6). Incubate at 37° for 2 min and add to the ribosome mixture.

Incubate either of these mixes for 20 min at 37° and add 55 μl composed of 16.5 μl of mix III $H_{66.7}M_{20}N_{500}SH_{13.3}Sp_{0.25}Spd_{10}$, 22 μl H_2O, and 16.5 μl of a GTP solution (2.5 m*M*, pH 6 with KOH). Divide into 10 aliquots of 30 μl each.

The stability of the ternary complex can be checked by cold TCA precipitation. Samples of the preparation of the ternary complex are precipitated at various times (up to 20 min) and compared to samples of aminoacyl-tRNA without the factor under the same conditions. More than 90% of the initial radioactivity is precipitated after 10 min when EF-Tu is present, whereas a significant reduction ($\geq$40%) is detected when the factor is not added. The precipitable material indicates intact aminoacyl-tRNA.

Translocation: Construction of POST Complex

POST complexes are constructed *via* an EF-G dependent translocation of the PRE complex. Add 2.5 μl of HMK buffer $H_{20}M_6K_{150}SH_4$ containing EF-G (0.3 pmol/pmol 70S) to five aliquots and incubate for 10 min at 37°. The other five samples are incubated with identical amounts of HMK buffer lacking EF-G.

Filter Binding Assay

Take two samples (30 μl each = 9.23 pmol ribosomes) with and without EF-G to measure the binding of tRNAs by nitrocellulose filtration. Mix the complexes with 2 ml ice-cold binding buffer $H_{20}M_6N_{150}SH_4Sp_{0.05}Spd_2$ and filtrate immediately through nitrocellulose filters that have been preincubated with binding buffer for 30 min. Wash twice with ice-cold binding buffer. The radioactivity retained on the filter is determined by liquid scintillation counting.

Include binding assays without ribosomes as standard controls in order

to determine the filter background. This background is normally low (below 10% of the binding signal) and directly proportional to the concentration of the radioactive component in the assay.

Puromycin Reaction

The six remaining samples are used in the puromycin reaction: Add 2.5 μl of binding buffer to two control samples (*i.e.,* without puromycin and ± EF-G) as background for the puromycin reaction, and 2.5 μl of puromycin stock solution (10 m*M* in binding buffer $H_{20}M_6N_{150}SH_4Sp_{0,05}Spd_2$, final concentration 0.7 m*M*) to four samples (i.e., two with and two without EF-G) in order to determine the amount of A-site occupation. Incubate at 0° for about 12 hr and stop the reaction by adding 32.5 μl of 0.3 *M* sodium acetate, pH 5.5, saturated with $MgSO_4$. Extract with 1 ml of ethyl acetate: 1 min mixing, incubate 10 min at 0°, centrifuge briefly, and measure the radioactivity contained in 700 μl of the ethyl acetate phase by liquid scintillation counting (corresponds to 7-pmol ribosomes).

The radioactivity extracted in the controls (minus puromycin) is subtracted from the sample containing puromycin in order to calculate the amount of fMet-puromycin formed.

A successful puromycin reaction depends critically on the way in which the puromycin stock solution is prepared and handled. Two basic rules for the preparation of a puromycin stock solution must be observed to achieve maximal activity: (1) The pH of the solution must be neutral. Since the puromycin is obtained commercially as a hydrochloride, the pH of the solution has to be neutralized by adding 1 *M* KOH (2% of the volume). (2) The puromycin stock solution must be kept at room temperature (otherwise it precipitates, lowering the effective concentration). Under these conditions the stock solution retains its maximum activity for about 1 hr.

For cryoelectron microscopy analysis the complexes are diluted with binding buffer $H_{20}M_6N_{150}SH_4Sp_{0,05}Spd_2$ to 1.4 A_{260}/ml (about 35 pmol/ml), the concentration that is used to produce sample grids. This is done just before the preparation of the grids. Due to the strong dilution of the sample, the ribosomes are well separated from unbound ligands, and therefore purification of the ribosome complexes is usually not necessary. In cases that require further purification suitable methods are described in the next section.

Purification of Ribosomal Complexes and Determination of Stoichiometry of Ligands

Ribosomal complexes can be separated from free ligands by several methods such as centrifugation of the complex through a sucrose cushion,

gel filtration by gravity flow, or spun columns. The determination of the stoichiometry of tRNA binding is particularly easy, since it can be achieved by nitrocellulose filtration if the tRNA is radioactively labeled, as in the examples given in the preceding sections. (Charging of tRNAs with radioactive amino acids is described by Rheinberger *et al.*[26] and Triana-Alonso *et al.*,[26a] this volume.) For cases such as factor binding to ribosomes, separation and evaluation of the binding stoichiometry is described later.

Isolation of Ribosome Complexes via Centrifugation through Sucrose Cushion

A 100-μl sample containing 5–10 A_{260} of ribosomes or ribosomal subunits in binding buffer $H_{20}M_6N_{150}SH_4Sp_{0.05}Spd_2$ is loaded on a 200-μl 15% sucrose cushion in binding buffer (sucrose ultrapure, GibcoBRL, UK). After centrifugation for 3 hr at 4° and 40,000 rpm (about 57,000*g*) in a Beckman TLA 120.1 rotor, the pellet is resuspended in 20 μl of binding buffer (yield 30–40% of input).

Isolation of Ribosomal Complexes via Gel Filtration

The choice of the gel matrix depends on the size of the ligand. Here we describe a procedure using Sephadex G-100 DNA grade (Pharmacia, Uppsala, Sweden) that effectively separates ligands of up to 100 kDa from the ribosome.

The sample (100 μl, 5–6 A_{260} of complexes with ribosomes or ribosomal subunits) is applied to a small column (Pasteur pipette closed with glass wool) containing about 2 ml of gel matrix. The column matrix was preequilibrated with binding buffer $H_{20}M_6N_{150}SH_4Sp_{0.05}Spd_2$. Fractions (100 μl) are collected under gravity flow (ribosomal complexes elute in fractions 4–8).

Isolation of Ribosome Complexes via Spun Column

A 100-μl sample containing 2–6 A_{260} of ribosomal complex is loaded onto a cDNA spun column S300 (Pharmacia, Uppsala, Sweden) that has been preequilibrated with binding buffer $H_{20}M_6N_{150}SH_4Sp_{0.05}Spd_2$. The column is centrifuged for 2 min at 1500 rpm at 4° in a Sorvall HB4 rotor. The flow-through (fraction 1) is collected in a microcentrifuge tube. The

[26] H.-J. Rheinberger, U. Geigenmüller, M. Wedde, and K. H. Nierhaus, *Methods Enzymol.* **164,** 658 (1988).

[26a] F. J. Triana-Alonso, C. M. T. Spahn, N. Burkhardt, B. Rhosdamy, and K. H. Nierhaus, *Methods Enzymol.* **317,** [17], 2000.

next fractions are collected by loading 100 μl of binding buffer $H_{20}M_6N_{150}SH_4Sp_{0.05}Spd_2$ and repeating the centrifugation procedure (ribosomal complexes are eluted in fractions 1–3).

Determination of Stoichiometry of Ribosomal Complexes

The amount of ribosomes in a sample is usually assessed via A_{260} measurement. The following equivalence rules might be helpful: 1 A_{260} 70S ribosomes = 24 pmol 70S; 1 A_{260} 50S subunits = 36 pmol 50S; 1 A_{260} 30S subunit = 72 pmol 30S.

Radioactively Labeled Ligand. If the specific activity of the ligand is known, the molar ratio of ligand to ribosome can be determined easily. The A_{260} reflects the amount of ribosomes while the radioactivity indicates the amount of the ligand in the same volume element. If the ligand is a protein, the main challenge is to isolate the protein in a radioactively labeled form; this can be done *in vivo* and *in vitro.*

In vivo incorporation of radioactive labeled amino acids can be achieved via supplying a labeled amino acid to the medium of a strain that is auxotrophe with respect to the same amino acid or, alternatively, the biosynthesis of the corresponding amino acid can be inhibited.[27] An easy and established method for *in vitro* labeling is the reductive methylation or alkylation of ε-NH_2 groups on the lysine side chains of a protein.[28,29]

If a ligand interacts with a secondary ligand (e.g., GTP) the ratio (ligand : ribosome) can be determined via this secondary ligand if it is labeled. In the case of Tet(O), a protein conferring resistance to tetracycline, [^{35}S]GTPγS was used as a secondary ligand.[30]

Identification of Ligand Binding via Staining Methods. Additionally, determination of the protein–ribosome stoichiometry is possible by using SDS–PAGE following by Coomassie staining or Western blotting and digitalization of the stained bands including the ligand–protein and some ribosomal proteins such as L2. This method requires that standards with different and known ratios of protein : ribosome be applied to the same SDS gel. A well-tried procedure for the standard calibration curve is done as follows: After digitalization of the bands the ratio (density of the band of the protein ligand : density of the L2 band) is plotted against the respective ratio (input of the protein ligand : input of ribosomes). The ratio of the density of the band from the bound ligand to that of L2 of the binding ribosomes reliably gives the stochiometry. L2 is the largest ribosomal protein of the 50S subunit

[27] S. Doublié, *Methods Enzymol.* **276,** 523 (1997).
[28] G. M. Wystup and K. H. Nierhaus, *Methods Enzymol.* **59,** 776 (1979).
[29] N. Jentoft and D. G. Dearborn, *Methods Enzymol.* **91,** 570 (1983).
[30] C. A. Trieber, N. Burkhardt, K. H. Nierhaus, and D. E. Taylor, *Biol. Chem.* **379,** 847 (1998).

and the second largest of 70S ribosomes; L2 is clearly separated from the other ribosomal proteins. It is not advisable to use the large ribosomal protein S1 of the small ribosomal subunit for this method, since S1 can be easily sheared from the ribosome. S4 is a good candidate for small ribosomal subunits to be used as a reference protein.

The stoichiometry of ligands bound to ribosomes can also be estimated by cryoelectron microscopy: The average density of the ligand is compared with a distinct part of the ribosome, for example, the L1 protuberance.[12]

In the rest of this article, we present and discuss results of 3-D cryo-EM reconstructions that illustrate the importance of buffer conditions in determining the positions of tRNAs on the ribosomes.

Location of tRNAs on Ribosome as Seen by 3-D Cryoelectron Microscopy

Three tRNA binding sites are known on the ribosome. Based on the puromycin reaction, two tRNA binding sites are operationally defined; acyl-tRNAs at the A site (A for acceptor) do not react with puromycin. The A site harbors the decoding center that is located on the small ribosomal subunit. Acyl-tRNAs at the P site (P for peptidyl) do react with puromycin. The third tRNA binding site, the E site (E for exit), is specific for deacyl-tRNA. A detailed description and a discussion of the current models of the ribosomal elongation cycle can be found in reviews.[16,31]

In early cryoelectron microscopy studies, three primary tRNA positions were visualized on the ribosome.[9,10] The A site was identified close to the L7/L12 stalk of the ribosome, the P-site tRNA spans the intersubunit space from the neck of the small subunit to the 50S subunit, and the E-site tRNA was observed close to the mushroom-shaped L1 protuberance (Fig. 3a; see color insert). Both studies placed the anticodon of the E-site tRNA far from the anticodon of the P-site tRNA; however, the overall positions of the E-site tRNA derived from the two studies were remarkably different. Subsequent studies[14,32] assigned the E-site tRNA to a position drastically different from those two, namely, close to the P-site tRNA of a POST complex, placing the E-site and P-site tRNAs in a similar mutual arrangement as the P- and A-site tRNAs in a PRE complex (Fig. 3b). Because the latter E site, which was derived by EF-G-dependent translocation of a PRE complex, was considered to be the authentic E site (see later section), the former one derived from the first visualization study[9] was termed E2

[31] K. S. Wilson and H. F. Noller, *Cell* **92,** 337 (1998).

[32] R. K. Agrawal, A. B. Heagle, P. Penczek, R. A. Grassucci, and J. Frank, *Nature Struct. Biol.,* **6,** 643 (1999).

TABLE I
ION CONCENTRATIONS OF POLYAMINE BUFFER AND CONVENTIONAL BUFFER SYSTEMS WITH CORRESPONDING CONCENTRATIONS *In Vivo*[a]

System	Mg^{2+}	K^+, NH_4^+	Polyamines		
			Spermidine	Spermine	Putrescine
Conventional	7–20	100	None	None	None
Polyamine	3–6	150	2	0.05	None
In vivo	~4	~150	1–4	~0.03	20

[a] Concentrations (m*M*) of ions and polyamines are important for ribosomal functions *in vitro* in various buffer systems and *in vivo*. The comparison shows that the polyamine buffer[b] matches *in vivo* conditions[c–f] closely. Conventional buffers do not contain polyamines but higher Mg^{2+} concentrations.[34] K^+ and NH_4^+ are more or less equivalent in *in vitro* systems of protein synthesis due to their similar ionic radii. Putrescine has no effects on protein synthesis *in vitro* (B. Lewicki and K. H. Nierhaus, unpublished).

[b] A. Bartetzko and K. H. Nierhaus, *Methods Enzymol.* **164,** 650 (1988).

[c] J. E. Lusk, R. J. P. Williams, and E. P. Kennedy, *J. Biol. Chem.* **243,** 2618 (1968).

[d] M. Kamekura, K. Hamana, and S. Matsuzaki, *FEMS Microbiol. Lett.* **43,** 301 (1987).

[e] B. Richey, D. S. Cayley, M. C. Mossing, C. Kolka, C. F. Anderson, T. C. Farrar, and M. T. J. Record, *J. Biol. Chem.* **262,** 7157 (1987).

[f] C. W. Tabor, and H. Tabor, *Microbiol. Rev.* **49,** 81 (1985).

site.[16] Furthermore, a deacylated tRNA that should be present at the P site according to general wisdom was found at two different positions when the ribosomes carried only one tRNA.[33] That study showed that an fMet-tRNA binds to the P site in the same position under different buffer conditions (Figs. 4a and 4b; see color insert), whereas the position of a deacylated tRNA varies with the buffer condition; it binds mostly in a "hybrid" P/E position when the conventional buffer (Table I) is used (Fig. 4c), and at the P site, in a position identical with that for fMet-tRNA (Fig. 4d), when polyamine buffer is used.

Importance of Charging State of tRNA and the Buffer Conditions for Position of tRNAs on Ribosome

The cryo-EM studies, which revealed five distinct tRNA locations, were done under a variety of conditions differing mainly in the charging state of the tRNA and the ionic conditions. Apparently, both parameters can drastically influence the position of tRNAs.

[33] R. K. Agrawal, P. Penczek, C. M. T. Spahn, R. A. Grassucci, K. H. Nierhaus, and J. Frank, in preparation (1999).

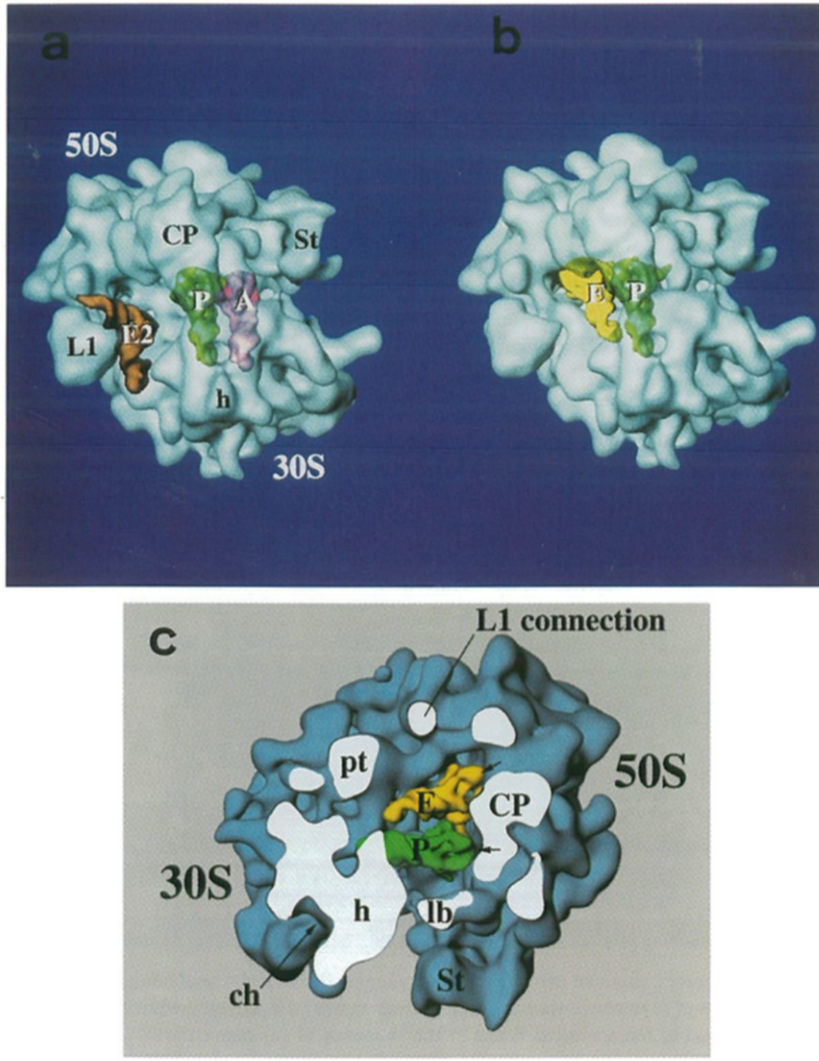

FIG. 3. tRNA positions identified by cryoelectron microscopy when more than one tRNA was bound to programmed ribosomes. (a) The complex was formed under conventional buffer conditions. Occupation of the E site is not observed but rather a tRNA bound to L1 in a presumably labile fashion, in a position where no codon–anticodon interaction is possible. This position is termed "E2" (brown) in order to avoid confusion with the E site. (b) The POST complex was formed under the conditions of the polyamine buffer. Both tRNAs in P (green) and E (yellow) sites can undergo codon–anticodon interactions. We note that acyl-tRNAs are found in the same A (pink) and P (green) positions under both buffer conditions. The view is from the top of the transparent 70S ribosome, with the 30S ribosomal subunit in front (h, head) and the 50S subunit at the back (L1, L1 protuberance; CP, central protuberance; St, L7/L12 stalk). Without transparent display of the ribosome, the 30S head and the central protuberance of the 50S subunit would cover the tRNAs at the A and P sites completely. (Reproduced with permission from Agrawal *et al.*[14]). (c) Cut-open view of a ribosome in the POST state with tRNAs at the P (green) and E (yellow) sites. Note that the tRNA at the E site is surrounded by ribosomal matrix.

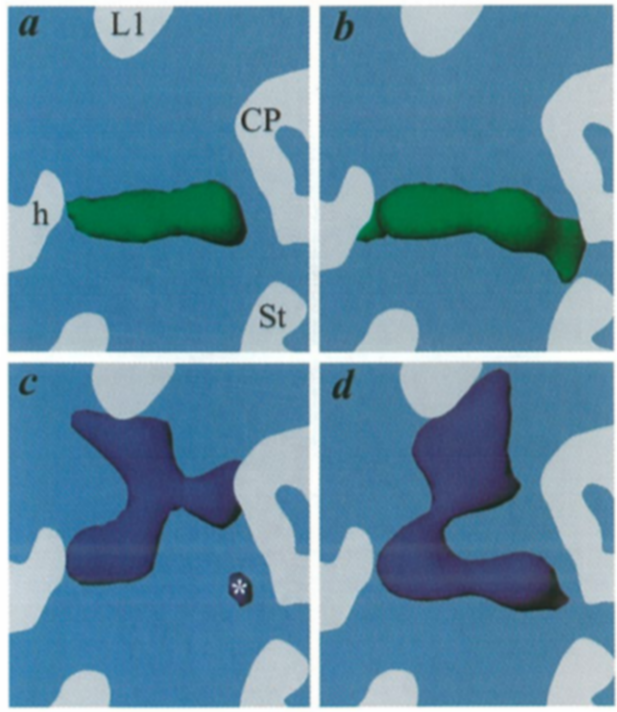

FIG. 4. Difference maps of programmed ribosomes. fMet-tRNA was bound to the P site under conditions of (a) a conventional buffer system and (b) the polyamine buffer. Deacylated tRNA was bound to the so-called P site in the presence of (c) conventional buffer and (d) polyamine buffer.

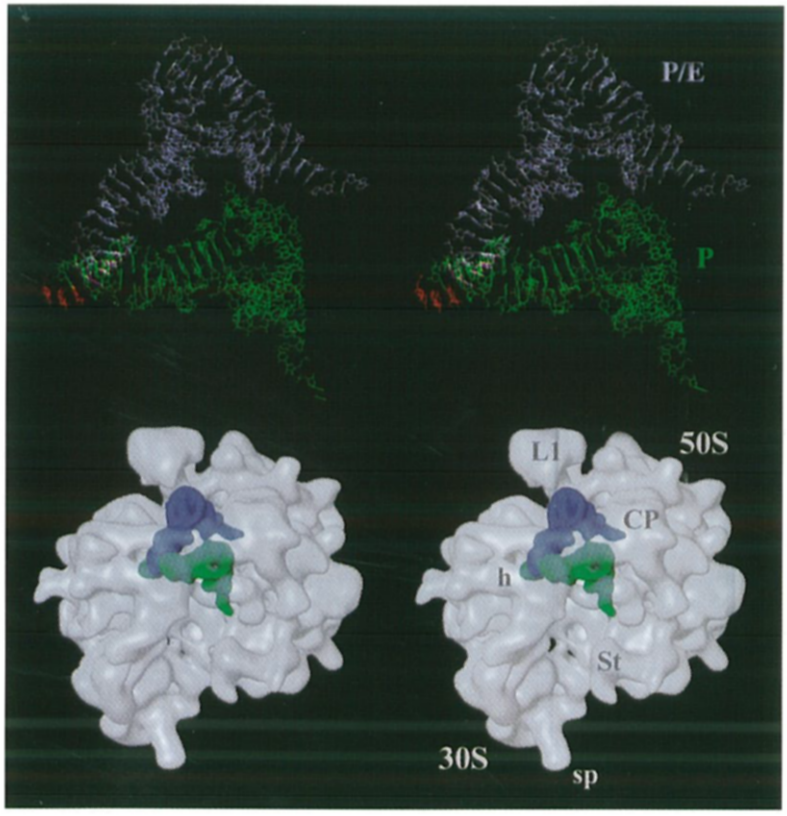

FIG. 5. A comparison of tRNAs observed at the P site and the hybrid site P/E. Note that the anticodon regions (red) of both tRNAs overlap. (Reproduced with permission from Agrawal *et al.*[14])

E and E2 Position. It is known from biochemical data that the features of the E site are strongly dependent on the buffer conditions.[34–36] Deacyl-tRNA is stably bound in the polyamine buffer and it interacts with the mRNA via codon–anticodon interaction after translocation whereas in the conventional buffer system these features are not seen. The different behaviors of a deacyl-tRNA bound to the E site are reflected by the two different positions seen in cryoelectron microscopy reconstructions:

1. In the conventional buffer system the tRNA is found in the E2 site apparently mainly interacting with the L1 stalk.[9] A number of arguments suggest that this tRNA binding site is of secondary importance: (a) L1 is not essential for cell viability[37]; (b) deacyl-tRNA does not form a stable complex with *isolated* ribosomal protein L1 (U. Stelzl, G. Blaha and K. H. Nierhaus, unpublished observations); (c) labile binding to the E site, which was claimed to be characteristic for this site,[34] is not observed in native polysomes where most of the ribosomes carry a deacylated tRNA at the E site in a stable fashion.[18,36]

2. As shown in Fig. 3b, a ribosomal POST state in the polyamine buffer shows the E-site tRNA with the anticodon tips as well as the CCA ends of both P- and E-site tRNAs in close mutual proximity.[14,32] Neutron scattering analysis of a PRE and POST complex reveals a similar mutual arrangement of these tRNAs on the ribosome,[38] in good agreement with the E position. A number of arguments support the view that this site is the authentic E site: (a) A deacylated tRNA at the E site is found to bind into a pocket-like structure in the 50S subunit making intimate contacts with the ribosome (Fig. 3c), in contrast to a tRNA at the E2 site where it has only contacts with the L1 protuberance mainly along the inner bent of its elbow region. In excellent agreement, the iodine-induced cleavage pattern of a deacylated tRNA bound to the P site remains the same during translocation to the E site.[39] These intensive contacts explain the tight binding of tRNA in the E site in native polysomes. (b) The E site, and not the E2 site, is seen in POST complexes when the polyamine buffer is used, although the mass of density representing the E-site tRNA is always weak.

[34] Y. P. Semenkov, M. V. Rodnina and W. Wintermeyer, *Proc. Natl. Acad. Sci. U.S.A.* **93,** 12183 (1996).

[35] H. J. Rheinberger and K. H. Nierhaus, *J. Biomol. Struct. Dyn.* **5,** 435 (1987).

[36] K. H. Nierhaus, R. Jünemann, and C. M. T. Spahn, *Proc. Natl. Acad. Sci. U.S.A.* **94,** 10499 (1997).

[37] A. R. Subramanian and E. R. Dabbs, *Eur. J. Biochem.* **112,** 425 (1980).

[38] K. H. Nierhaus, J. Wadzack, N. Burkhardt, R. Jünemann, W. Meerwinck, R. Willumeit, and H. B. Stuhrmann, *Proc. Natl. Acad. Sci. U.S.A.* **95,** 945 (1998).

[39] M. Dabrowski, C. M. T. Spahn, M. A. Schäfer, S. Patzke, and K. H. Nierhaus, *J. Biol. Chem.* **273,** 32793 (1998).

P and P/E Positions. The different P-site positions of deacylated tRNA have been systematically examined.[15] In a conventional buffer, a deacyl-tRNA is seen in a position that may be interpreted as the P/E hybrid site postulated by Moazed *et al.*[40] The anticodon mass shows up in the anticodon region of the P-site tRNA, the elbow and CCA end point to the E site (Fig. 5; see color insert). In the polyamine buffer, both the deacyl-tRNA and the charged fMet-tRNA are positioned in the P site,[15] even though a small fraction of deacylated tRNA is always present in the P/E position when decylated tRNA was bound to the so-called P site.

Conclusions

It has been suggested that the buffer conditions (e.g., Mg^{2+} concentration) have a strong influence on the tRNA binding behavior of the ribosome.[35,41] Cryo-EM has now shown that different buffers lead to markedly different observed positions of the ribosome-bound tRNA. In addition to positions that can be attributed to the A, P, and E sites, two new positions, termed P/E and E2, are observed almost exclusively in the conventional buffer system, with E-site occupancy markedly reduced.

These results are interesting in the context of the long-standing controversy regarding the role of the E site in protein synthesis. According to one view,[42] the E site plays a minor role as it is seen to merely facilitate the dissociation of the tRNA from the P site where it is bound too tightly to allow an energetically easy release from the ribosome. According to the other view,[43,44] the role of the E site is essential in protein synthesis, because codon–anticodon interaction at this site is seen to have two important functions: that it helps to maintain the reading frame during the translocation reaction, and that it signals the ribosome to adopt the POST state. The POST state so defined, in turn, is a prerequisite for the accurate selection of the cognate ternary complex during the decoding process[43,45]

The cryo-EM results have a bearing on the controversy in the following way: Whether or not tRNA in a given assay forms codon–anticodon contact at the third tRNA binding site (which is possible according to the position of the anticodon of the observed E-site tRNA) is evidently dependent on which site is predominantly occupied, which in turn (again according to cryo-EM, as we have seen) depends on the buffer conditions. In the conven-

[40] D. Moazed and H. F. Noller, *Nature* **342,** 142 (1989).
[41] R. Lill and W. Wintermeyer, *J. Mol. Biol.* **196,** 137 (1987).
[42] J. M. Robertson and W. Wintermeyer, *J. Mol. Biol.* **196,** 525 (1987).
[43] K. H. Nierhaus, *Biochemistry* **29,** 4997 (1990).
[44] K. H. Nierhaus, *Mol. Microbiol.* **9,** 661 (1993).
[45] U. Geigenmüller and K. H. Nierhaus, *EMBO J.* **9,** 4527 (1990).

tional buffer system, the weak occupancy of the E site yet strong occupancy of other sites termed P/E and E2 would lead to the conclusion that codon–anticodon interaction at the third tRNA binding site plays no essential role. However, a significant influence of codon–anticodon interaction on the occupation of the third tRNA binding site has been observed under assay conditions, where binding to the E2 site is favored,[41] a site where the anticodon is too far away to contact the condon. It is therefore possible that the buffer conditions do not influence the tRNA position per se, but that E and E2 site are in equilibrium with each other, such that, dependent on the buffer conditions, the equilibrium is shifted toward the E site (polyamine buffer) or the E2 site (conventional buffer). Thus, depending on the buffer conditions used, one would come to opposite conclusions regarding the properties of the third tRNA binding site.

As to the significance of the observed P/E and E2 positions, it is quite possible that both represent transitional states that are favored under the conventional buffer conditions, while only transiently occupied under the conditions of the polyamine buffer and probably also in vivo. The P/E position might in fact represent a "frozen" hybrid state,[40] while the E2 position, with the tRNA attached to the L1 protein, might reflect a deacyl-tRNA released from the E site and on its way to leave the ribosome.

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