Effect of Buffer Conditions on the Position of tRNA on the 70 S Ribosome As Visualized by Cryoelectron Microscopy*

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The effect of buffer conditions on the binding position of tRNA on the Escherichia coli 70 S ribosome has been studied by means of three-dimensional (3D) cryoelectron microscopy. Either deacylated tRNA_{Met} or fMet-tRNA_{Met} were bound to the 70 S ribosomes, which were programmed with a 46-nucleotide mRNA having AUG codon in the middle, under two different buffer conditions (conventional buffer: containing Tris and higher Mg^{2+} concentration [10–15 mM]; and polyamine buffer: containing Hepes, lower Mg^{2+} concentration [6 mM], and polyamines). Difference maps, obtained by subtracting 3D maps of naked control ribosome in the corresponding buffer from the 3D maps of tRNAribosome complexes, reveal the distinct locations of tRNA on the ribosome. The position of deacylated tRNA_{Met} depends on the buffer condition used, whereas that of fMet-tRNA_{Met} remains the same in both buffer conditions. The acylated tRNA binds in the classical P site, whereas deacylated tRNA binds mostly in an intermediate P/E position under the conventional buffer condition and mostly in the position corresponding to the classical P site, i.e. in the P/P state, under the polyamine buffer conditions.

The ribosome coordinates and facilitates the biosynthesis of the polypeptide chain by the addition of new amino acids, which are brought to the ribosome by transfer RNAs (tRNAs), according to genetic instructions stored on the messenger RNA (mRNA). tRNA is known to occupy successive positions in the intersubunit space (see Ref. 1) of the ribosome in the course of the elongation cycle. There are mainly three such biochemically characterized positions that are referred to as A (for incoming aminoacyl tRNA), P (for peptidyl tRNA), and E (for deacylated exiting tRNA) sites. In addition to mRNA and tRNA, ribosomes also interact with various protein factors, e.g. initiation, elongation, and release factors, which facilitate different steps of the protein biosynthesis. Knowledge of 3D interaction sites of all these ligands with the ribosome is crucial for understanding the mechanism of protein synthesis. Cryoelectron microscopy of the ribosome (2, 3) has offered the first opportunity to investigate various ligand binding positions, in three dimensions, by visualizing tRNA (4, 5), elongation factor Tu (6), and elongation factor-G (7) in different ligand-ribosome complexes. Of the various tRNA binding positions on the ribosome, the position of P-site tRNA has been unambiguously defined in a 3D map of the fMet-tRNA_{Met} ribosome complex at 15 Å resolution (8). Because this position differs from the position of P-site tRNA inferred from an earlier experiment in our laboratory (4), a clarification of the cause of the discrepancy was needed. As in these two studies (4, 8), buffer conditions (conventional versus polyamine), state of the tRNA (deacylated versus acylated), as well as the mRNA (poly(U) versus MF-mRNA, an mRNA with a defined codon) were different, additional experiments were required to answer this question conclusively. To this end, we prepared tRNA-ribosome complexes using both acylated and deacylated tRNAs, each under two different buffer conditions, and analyzed them using the 3D cryoelectron microscopy technique.

Different laboratories use somewhat different buffer conditions for tRNA binding experiments. However, many of the laboratories generally ignore the possible influence of the buffer conditions on the binding properties of tRNA. Three principally different buffer systems are used nowadays for in vitro analysis of tRNA-ribosome complexes: (i) the conventional buffer systems, containing essentially only Mg^{2+} (5 to 25 mM) and monovalent ions (K^{+} and NH_{4}^{+} in the range of 50 to 200 mM), which fails to match the relevant parameters of protein synthesis, viz. rate and accuracy, by more than one order of magnitude; (ii) the polymix system (9, 10); and (iii) the much simpler polyamine system (11, 12). Both the polymix and the polyamine systems contain ions and polyamines in concentrations comparable with those observed in vivo; in both systems the ribosomal activities, in terms of rate and accuracy, are near those observed in vivo. Furthermore, the tRNA binding sites can be occupied up to 100% and the fraction of ribosomes participating in protein synthesis is up to 70% in the polyamine system, whereas the corresponding values in the polymix system are lower by a factor of two (for more details, see Ref. 13). This article presents the cryoelectron microscopy results of experiments in which two buffer conditions (conventional and polyamine) and two states of tRNAs (aminoacylated versus deacylated) were used to investigate the properties of tRNA at the ribosomal P site.

MATERIALS AND METHODS
Preparation of tRNA-Ribosome Complexes—Tight-couple ribosomes and ribosomal subunits were prepared from Escherichia coli MRE-600 strain as described by Agrawal and Burma (14) and Bommer and co-workers (15). A ^32P-labeled tRNA^{32P}_{Met}-ribosome complex was prepared in a conventional buffer (containing 50 mM Tris-HCl (pH 7.8), 15 mM magnesium acetate, 160 mM NH_{4}Cl, and 5 mM β-mercaptoethanol) as

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† The abbreviations used are: 3D, three-dimensional; MF-mRNA, 46-nucleotide-long mRNA with AUG codon in its middle.
described by Agrawal and co-workers (4) using poly(U) as mRNA. The extent of tRNA binding to the ribosome, as determined by filter binding assays, was 2.3 tRNA molecules per molecule of ribosome.

The initiation-like ribosomal complexes were prepared by binding fMet-tRNA<sup>Met</sup> to the P site of ribosomes programmed with a 46-nucleotide mRNA fragment that carries unique AUG and UUC codons in its middle (MF-mRNA) as described by Malhotra and co-workers (8) under low Mg<sup>2+</sup>-containing poly(U) buffer (20 mM Hepes/KOH, 6 mM MgCl<sub>2</sub>-CH<sub>3</sub>COO<sup>-</sup>, 150 mM NH<sub>4</sub>Cl, 4 mM mercaptoethanol, 0.05 mM spermine, 2 mM spermidine) and the conventional buffer conditions. The tRNA binding under polyamine and conventional buffer conditions were 0.92 and 0.87 tRNA molecules per ribosome molecule, respectively, as checked by filter binding assay. All these complexes yielded a homogeneous 70 S peak in the analytical sucrose density gradients, prepared in the corresponding buffers.

Electron Microscopy and Image Processing—In each case, samples were diluted in the corresponding conventional or polyamine low Mg<sup>2+</sup>-containing buffer. In a concentration of 1.5–1.2 mg/ml. Grids were prepared for cryoelectron microscopy according to standard methods (16, 17). Micrographs were recorded using low-dose protocols on a Philips EM420 equipped with low-dose kit and a GATAN (model 626) cryotransfer holder at a magnification of 52,200 × ±2% as checked by a tobacco mosaic virus standard. Each exposure corresponds to an electron dose of about 10 e<sup>−</sup>/Å<sup>2</sup>. Micrographs were checked for drift, astigmatism, and presence of Thon rings by optical diffraction. Scanning was done with a step size of 25 μm, corresponding to 4.78 Å on the object scale, on a Perkin Elmer PDS 1010A microdensitometer. 3D reconstructions were calculated and refined using the 3D projection alignment procedure (18) and CTF-corrected as described earlier (19). The final resolutions, estimated by using the Fourier shell correlation with a value of 0.5 (see Ref 8), for various complexes were: 21 Å, for fMet-tRNA<sup>Met</sup>-ribosome complex in conventional buffer; 15 Å, for fMet-tRNA<sup>Met</sup>-ribosome complex in polyamine buffer (the same reconstruction as shown in Ref. 8); 17 Å, for tRNA<sup>Met</sup>-ribosome complex in conventional buffer; and 17.1 Å, for tRNA<sup>Met</sup>-ribosome complex in polyamine buffer. The resolution of 3D maps of 70 S tRNAs (naked controls) in both buffer conditions and that of the poly(U)-programmed tRNA-bound ribosome complex was 22 Å in each case. In the case of the fMet-tRNA<sup>Met</sup>-ribosome complex in polyamine buffer condition, a difference map was computed by subtracting the map of the fMet-tRNA<sup>Met</sup>-ribosome complex (resolution 19.5 Å) having 41% tRNA occupancy, and was low-pass filtered to 20.5 Å (Fig. 1; for details, see Ref. 8). Other difference maps (Figs. 2, a–d, and 3a) were computed by subtracting the corresponding control (naked 70 S) 3D map in conventional buffer or polyamine buffer. Difference maps were also computed (Figs. 2, e and f, and 3b), by subtracting the 3D maps of Met-tRNA<sup>Met</sup>-ribosome complex from the 3D maps of tRNA<sup>Met</sup>-ribosome complexes (Fig. 2, e and f) and from the 3D map of poly(U)-programmed tRNA<sup>Met</sup>-ribosome complex (Fig. 3b). The use of the 3D map of fMet-tRNA<sup>Met</sup>-ribosome complex as control appears to offset most of the effects of ribosomal conformational changes associated with the binding of the first tRNA. However, its use as a control results in the elimination of a mass of densities related to the P/P position and the overlapping anticodon portion of the tRNA at the P/E position.

In our earlier study (4), a deacylated tRNA<sup>Met</sup>-ribosome complex was prepared using poly(U)-programmed 70 S ribosomes in a conventional buffer system. Since then the resolution of the 3D map of the same complex has been improved. The overall difference map at improved resolution (Fig. 3a), computed by subtracting the 3D map of naked control 70 S ribosome, remains the same as shown earlier (4) and did not show a strong mass of density that would superimpose with the position of fMet-tRNA<sup>Met</sup> but had a very weak density (marked with an asterisk) in that region (compare with position of difference peaks in Fig. 2, a and b). However, that result was very similar to the result now obtained, except for the absence of a difference mass in the A-site region, with a single deacylated tRNA<sup>Met</sup>, which was bound to the ribosomes containing only one AUG codon in conventional buffer conditions (Fig. 2, c and e). It is apparent that under the conventional buffer conditions, deacylated tRNAs, irrespective of the nature of mRNA (poly(U) or MF-mRNA), acquire a different preferred orientation, in which most of the 50 S side portion of the tRNA is found on the L1 side.

The position of P-site tRNA in the fMet-tRNA<sup>Met</sup>-ribosome complex agrees with the tRNA position observed in the P site in the pre- and post-translational complexes. Although by definition, a peptidyl-tRNA occupies the authentic P site (Fig. 1).
Effect of Buffer Conditions on the Position of tRNA

FIG. 1. Position of the fMet-tRNA<sub>Met</sub> (green) in the P-site of the initiation-like fMet-tRNA<sub>Met</sub>/ribosome complex, as obtained earlier by Malhotra and co-workers (8). As explained by Malhotra and co-workers (8) and also pointed out under “Materials and Methods,” this difference map (green) was obtained by subtracting the 3D map of an fMet-tRNA<sub>Met</sub>/ribosome complex having 41% tRNA occupancy from that obtained for 81% tRNA occupancy. Panel a, view of the ribosome as a transparent surface from the side with the 30 S subunit on the left and the 50 S subunit on the right; panel b, view from the top; panel c, same view as in panel b, but 70 S is not transparent and the top portion, i.e. the central protuberance of the ribosomal 50 S subunit (right) and the head of the 30 S subunit (left), has been cut off to expose the intersubunit space where tRNAs interact with the ribosome. The asterisk (*) in panel c refers to a piece of the cut plane that comes from the cut head of the 30 S subunit and hides the anticodon region of the tRNA mass. This piece is not shown in the subsequent figures. Panel d, diagrammatic representation of the intersubunit space region (highlighted with a dashed line inset in panel c) with the difference map corresponding to fMet-tRNA<sub>Met</sub> on blue background, to be used as reference frame in the subsequent presentation of difference masses in Figs. 2 and 3. Features of the 30 S subunit: ch, channel through the neck; h, head; pt, platform; sp, spur. Features of the 50 S subunit: CP, central protuberance; L1, L1 protein; St, L7/L12 stalk.

and a similar site is occupied by the fMet-tRNA<sub>Met</sub> under both buffer conditions, it is mainly the presence of the peptidyl residue that helps to orient the tRNA in the classical P site. Most of the deacylated tRNA<sub>Met</sub> under the polyamine buffer conditions (Fig. 2d) also acquire the same position. The results of these experiments reveal that under optimal buffer conditions, i.e., when the polyamine system is used, a deacylated tRNA can occupy the P site (Fig. 2d), in contrast to conventional buffer conditions in which only a weak signal is observed in that region (Figs. 2c and 3a).

Because the complex prepared using tRNA<sub>Met</sub> contained only a single AUG codon, it was expected that no tRNA would be present at the A or E sites under the conditions of the polyamine system (22). According to the generally accepted view (23), the A site is on the L7/L12 side, whereas the E site is on the L1 side of the P-site tRNA. Although the P site proves to be well occupied (Fig. 2d) in the polyamine buffer, there is indeed no difference mass attributable to tRNA seen at the A site. However, the mass of densities observed on the L1 side (Fig. 2c and d) cannot be explained by an E-site tRNA, as the E-site position, inferred from a separate 3D study of a post-translational complex (2), is different (though partially overlapping) from the additional mass of densities seen here. The anticodon end and the elbow of the E-site tRNA is found between the anticodon ends and elbows of tRNAs at the P/P and E2 (see below) positions. In contrast, in this study, a portion of the mass of densities on the L1 side of the P site is connected to the codon-anticodon region of the P-site tRNA (compare the difference masses shown in Fig. 2, a and b with Fig. 2, c and d, respectively). Thus, the anticodon of the tRNA appears to be still in the place of the P site, whereas elbow and CCA arm point to the E-site region. It is therefore justified to call this a P/E hybrid position.

Another connected mass, not explained by the presence of a deacylated tRNA at the observed P/E position, clings to the L1 protein and is weakly developed in the polyamine buffer conditions (Fig. 2, d and E, as compared with the conventional buffer (Fig. 2, c and E, and Fig. 3). It represents another tRNA that can be more clearly seen in the difference map in Fig. 3b (see Ref. 4, where it was first observed and assigned as the E-site tRNA) and will now be called E2 position. As the E-site position obtained in a well defined post-translational complex (see above) is different from P/E and E2 positions, it appears that the reconstruction of poly(U)-programmed ribosomes shows an average mass in the intersubunit space, which is probably formed by superposition of tRNAs situated in P/E, E, and E2 positions (Fig. 3b). Thus, apart from P and A sites, deacylated tRNAs can bind at three additional positions that are probably not occupied simultaneously for two reasons: (i) the maximal capacity of tRNA binding to programmed ribosomes is three deacylated tRNAs per 70 S ribosome (24, 25); and (ii) the various positions partially overlap (see below).

**Fitting of the Atomic Structure of tRNA into Various Difference Masses**—Difference maps (shown in Fig. 2, a–d) obtained
by subtracting the 3D maps of corresponding naked control 70 S ribosomes from tRNA-ribosome complexes, altogether yield two distinct masses that could be assigned to two different positions of tRNA related to the P site, the P/P and P/E positions. The next step was to find out the exact positions and orientations of the tRNAs by fitting the tRNA$^{\text{Phe}}$ x-ray structure (26). To obtain an accurate fitting of the tRNA x-ray structure into these discrete masses, we applied a three-dimensional spherical mask, large enough to accommodate a tRNA (43 Å radius), around the centers of these masses. This gave us a clear 3D view of the difference mass corresponding to each of the two particular tRNA positions and thus allowed us to obtain an unequivocal fitting of the tRNA x-ray structure.

The P-site tRNA was identified by fitting the x-ray structure of initiator tRNA (27) directly into the 15-Å resolution electron microscopy density map (8) of the ribosome-Met-tRNA$^{\text{Met}}$ complex. However, the difference map obtained by subtracting the two MF-mRNA-programmed ribosomes-Met-tRNA$^{\text{Met}}$ complexes containing the same level of mRNA but different levels of tRNA (see Ref. 8; and shown in Fig. 1 in green) precisely matches and tightly accommodates the x-ray structure of the initiator tRNA (Fig. 4, top panel). The anticodon orientation of tRNA$^{\text{Phe}}$ x-ray structure into these discrete masses, we applied a three-dimensional spherical mask, large enough to accommodate a tRNA (43 Å radius), around the centers of these masses. This gave us a clear 3D view of the difference mass corresponding to each of the two particular tRNA positions and thus allowed us to obtain an unequivocal fitting of the tRNA x-ray structure.

DISCUSSION

A Single Deacylated tRNA Can Bind in Either the P/P or the P/E Position—Deacylated tRNA$^{\text{Met}}$ is observed mainly in the P/P position (the classical P site) and only weakly in the P/E position if the polyamine buffer is used (Fig. 2, d and f), whereas the preference is reversed under the conditions of the conventional buffer.
conventional buffer, containing higher Mg$^{2+}$ and lacking polyamines (Fig. 2, c and e). It follows that a ribosome binding a single tRNA at the operationally defined P site may carry that tRNA at strikingly different locations, namely at the P site corresponding to a peptidyl-tRNA analog (e.g. fMet-tRNA$^{f\text{Met}}$), and also at the P/E position if the tRNA is deacylated. The only feature that the two tRNAs, in the P/P and P/E positions, have in common is that they share the region where the anticodon of the P-site tRNA interacts with the mRNA codon, whereas the rest of the tRNA molecule is located in different ways (Fig. 5).

Under conventional buffer conditions, the P site is exclusively occupied for peptidyl-tRNA analogs (Fig. 2a), and the P/E position if the tRNA is deacylated. The only feature that the two tRNAs, in the P/P and P/E positions, have in common is that they share the region where the anticodon of the P-site tRNA interacts with the mRNA codon, whereas the rest of the tRNA molecule is located in different ways (Fig. 5).

Comparison with Site Assignments of Earlier Works—The tRNA in the P/E position observed here adopts a similar orientation as reported earlier for the P-site tRNA (4); however, the whole molecule is shifted toward L1 by approximately 30 Å. The different positional assignment for the P-site tRNA in our earlier study (4) was because of two main reasons: (i) as the current study shows, the deacylated tRNA preferably assumes the P/E position for the conventional buffer system used in the earlier study; and (ii) the anticodons and elbows of the A-site and the weakly occupied P-site tRNAs appeared fused. This latter problem was solved in this study (Fig. 3b) where the 3D...
map of fMet-tRNAfMet ribosome complex, visualized in the same conventional buffer (Fig. 2a), was used as control to compute the difference map. This subtraction helped in determining the boundary between the A- and P-site tRNAs and gave distinct and separate masses related to A and E2 (see "Results") positions and partially overlapping P/E and E positions. Because this article is mainly concerned with the P and P/E states, detailed results about other superimposable tRNA positions will be presented elsewhere.

The P-site assignment of Stark and co-workers (5) appears to be similar to the P-site position described here, as far as it can be inferred from the published information, but a detailed comparison of the maps has not been made thus far. Interestingly, a different location for P-site tRNA, similar to P/E position seen here, was also suggested by Mueller and co-workers (31) on the basis of experiments where single deacylated tRNAfMet was bound to the ribosome under the conventional buffer conditions. Furthermore, a cross-linking result between the L1 protein and elbow region (through nucleotide 20) of the deacylated tRNAMet (32), bound at the presumed P site, can only be explained by the P/E position.

The hybrid-site model (29) describes the translocation of tRNA as an alternating movement of the two ends of the molecule on one or the other subunit. Accordingly, the states A/P and P/E might be observable between the pre- and the post-translocational steps of the elongation cycle. We do see a mass attributable to a tRNA in the hybrid P/E position, a position which is occupied by a deacylated tRNA and apparently predominant under conventional buffer conditions (Fig. 2, c and e, and Fig. 3). In the polyamine buffer a strong P site and a weak P/E position are observed (Fig. 2, d and f). It should be pointed out that the hybrid-site model (29) was suggested from experiments done in conventional buffer conditions. Thus, our results are of crucial importance in evaluating the functional relevance of this model.

Results presented here clearly show that under the polyamine buffer conditions, even the deacylated tRNA mostly occupies the P site, whereas the same tRNA moves mostly to the P/E position under the conventional buffer condition. Our observation is also supported by the studies of Lill and Wintermeyer (33), which indicated that the higher concentration of Mg2+, in general, triggers the tRNA movement toward the L1 side. It is possible that under the conventional buffer condition, a change in local conformation or the microenvironment of the ribosome is induced in a way that favors the binding of tRNA in the P/E position. In this context, a detailed cryoelectron micros-

**Fig. 5.** Stereo-view presentation of the relative positions of P/P- and P/E-state tRNAs. Panel a, the crystal structure of tRNA corresponding to P/P position (green) and P/E positions (light blue), as viewed from the top of the ribosome (ribosome not shown). Note the overlapping anticodon-loop regions of the two tRNAs. Three anticodon bases in P/P- and P/E-position tRNAs are shown in red and magenta, respectively. Panel b, two tRNAs situated inside the 15-Å resolution 3D map of the 70 S ribosome (8). Crystal structure of tRNA corresponding to P/P (green) and P/E (blue) positions were filtered to 5 Å resolution. The 70 S ribosome is shown as a transparent blue surface. It should be mentioned that these two tRNAs are not present simultaneously on the ribosome (see text) but have been presented together to show their relative position inside the ribosome.
copy analysis of various tRNA-ribosome complexes should be mentioned, which has shown that the L1-protein conformation is substantially altered by switching the buffer conditions.3

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