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Transit of tRNA through the *Escherichia coli* ribosome: cross-linking of the 3′ end of tRNA to ribosomal proteins at the P and E sites

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Abstract Photoreactive derivatives of yeast tRNA^{Phe} containing 2-azidoadenosine at their 3′ termini were used to trace the movement of tRNA across the 50S subunit during its transit from the P site to the E site of the 70S ribosome. When bound to the P site of poly(U)-programmed ribosomes, deacylated tRNA^{Phe}, Phe-tRNA^{Phe} and *N*-acetyl-Phe-tRNA^{Phe} probes labeled protein L27 and two main sites within domain V of the 23S RNA. In contrast, deacylated tRNA^{Phe} bound to the E site in the presence of poly(U) labeled protein L33 and a single site within domain V of the 23S rRNA. In the absence of poly(U), the deacylated tRNA^{Phe} probe also labeled protein L1. Cross-linking experiments with vacant 70S ribosomes revealed that deacylated tRNA enters the P site through the E site, progressively labeling proteins L1, L33 and, finally, L27. In the course of this process, tRNA passes through the intermediate P/E binding state. These findings suggest that the transit of tRNA from the P site to the E site involves the same interactions, but in reverse order. Moreover, our results indicate that the final release of deacylated tRNA from the ribosome is mediated by the F site, for which protein L1 serves as a marker. The results also show that the precise placement of the acceptor end of tRNA on the 50S subunit at the P and E sites is influenced in subtle ways both by the presence of aminoacyl or peptidyl moieties and, more surprisingly, by the environment of the anticodon on the 30S subunit.

Key words: Photoaffinity labeling; 2-Azidoadenosine; Proteins L1, L27, L33; Protein synthesis

1. Introduction

Protein synthesis on the *Escherichia coli* ribosome entails the unidirectional transit of tRNA through distinct A (aminoacyl), P (peptidyl), E (exit) and F (final) sites (for reviews, see [1–3]). During each cycle of polypeptide chain elongation, the A site-bound aminoacyl-tRNA accepts a peptidyl group from the P site-bound peptidyl-tRNA and the newly formed peptidyl-tRNA is translocated from the A site to the P site. At the same time, the deacylated tRNA is transferred from the P site first to the E site and then to the F site from which it is released into the cytoplasm. Although both 30S and 50S ribosomal subunits contribute to the formation of the A and P sites, most of the free energy of tRNA binding results from interactions between the anticodon arm of tRNA and the 30S ribosomal subunit [4]. In contrast, most of the free energy of tRNA binding to the ribosomal E site is provided by interactions between tRNA and the 50S subunit [5,6]. At least 30% of the free energy of binding can be attributed to the association of the 3′ terminal -CCA of E-site tRNA with the 50S ribosomal particle [7]. Nonetheless, contacts between E site-bound tRNA and the 30S subunit are well documented [8–11].

Although the topography of tRNA binding sites on the ribosome has been elucidated by a variety of experimental approaches, and site-specific tRNA–ribosome complexes have been visualized by cryo-electron microscopy and X-ray crystallography [10,12,13], our understanding of the molecular mechanism of tRNA movement from site to site remains limited. Chemical footprinting of tRNA–ribosome complexes at different stages of protein synthesis led to the formulation of the hybrid-site model for the transit of tRNA across the ribosome [14]. This model suggests that tRNA adopts two intermediate binding states, designated A/P and P/E, as it proceeds from the A site to the P site and from the P site to the E site, respectively. The hybrid-state hypothesis indicates that the site at which the anticodon arm of the tRNA interacts with the 30S subunit (first letter) can differ from the site at which the aminoacyl acceptor end of tRNA binds to the 50S subunit (second letter). The disposition of tRNA–ribosome contacts involved in formation of the intermediate states may underlie the mechanism that ensures unidirectional movement of tRNA on the ribosome.

A number of ribosomal components in proximity to the A and P site-bound tRNAs have been identified by chemical protection and photoaffinity labeling [3,15,16]. Over the past several years, our laboratory has used photoreactive tRNA probes containing 2-azidoadenosine ($2N_3A$) at specific positions to investigate the topography of the tRNA binding sites on the *E. coli* ribosome [1]. In these studies, we identified both ribosomal proteins and sites within the rRNAs that are in proximity to the 3′ end and the anticodon of tRNA molecules bound to the A, P and E sites [1,3,9,17]. These data provided the basis for a low-resolution model depicting the arrangement of the A-, P- and E-site tRNAs on the ribosome [16,18], which corresponds quite well with the high-resolution models of tRNA–ribosome complexes derived from cryo-electron microscopy and X-ray crystallography [3,10,12]. In the

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present report, we have used our photoaffinity labeling approach to identify 50S ribosomal subunit proteins in contact with the 3′ terminus of tRNA from the time it leaves the P site to the time it is ejected into the cytoplasm. Our results show that three proteins, L27, L33 and L1, are labeled during this transit. The labeling of L27 and L33 most likely arises from tRNA in the P/E and E sites, respectively, whereas the labeling of protein L1 is consistent with the location of tRNA at the E2 or F site which has been visualized by cryo-electron microscopy [19,20].

2. Materials and methods

2.1. Materials

Yeast $tRNA^{Phe}$ (specific amino acid acceptance 1000 pmol/A_{260}), poly(U) and poly(A) were purchased from Sigma. The sources of other enzymes, radioactively labeled compounds and biological materials were as described [3,21].

2.2. Preparation of tRNA

Published procedures were used for the preparation of 5′-^{32}P-labeled $p2N_3Ap$ [22] and yeast $tRNA^{Phe}$ derivatives containing $2N_3A$ at position 76 [17,23]. Aminoacylation of [5′-^{32}P](2N$_3$A76)$tRNA^{Phe}$ with [^{14}C]Phe and acetylation of aminoacyl-tRNA were carried out according to Wower et al. [23] and Rappoport and Lapidot [24], respectively. All photoreactive $tRNA^{Phe}$ derivatives were purified by chromatography on BD-cellulose columns (1×5 cm) using salt–ethanol gradients [25].

2.3. Preparation of ribosomes and poly(U) templates

Tight-couple 70S ribosomes and 50S subunits were isolated from *E. coli* MRE 600 by Dr. V.I. Makhno (Petersburg Nuclear Physics Institute, Russian Academy of Sciences, Gatchina, Russia) as described [26]. In these preparations, 1.8 mol of AcPhe-RNA^{Phe} were bound per mol of 70S ribosomes at 15 mM Mg^{2+}. The poly(U) templates used in binding and cross-linking experiments were hydrolyzed in 50 mM HCl at 25°C for 40 min and the hydrolysate was fractionated on Sephadex G-75 [27].

2.4. Photoaffinity labeling

All tRNA binding and cross-linking experiments were carried out in TNME buffer (20 mM Tris–HCl, pH 7.5, 50 mM NH_4Cl, 10 mM $MgCl_2$, and 0.5 mM EDTA). Cross-linking was accomplished by irradiating non-covalent tRNA–ribosome complexes for 2 min at 0°C in a Rayonet Model RPR-100 photochemical reactor equipped with six RPR-3000-Å lamps [23]. The cross-linked complexes were separated into 30S and 50S subunits by sucrose gradient centrifugation in 0.25 mM Mg^{2+}, which also released non-covalently bound tRNA [23]. The distribution of cross-linked (2N$_3$A76)$tRNA^{Phe}$ between 23S rRNA and 50S subunit proteins was determined by electrophoresis of the 50S subunit fraction in a 5% polyacrylamide gel containing SDS and EDTA [28].

2.5. Identification of cross-linked ribosomal components

50S subunit proteins cross-linked to (2N$_3$A76)$tRNA^{Phe}$ were analyzed by SDS–PAGE and two-dimensional PAGE [23]. The identities of the cross-linked proteins were determined from their characteristic electrophoretic mobilities and were confirmed by immunological analysis [9,29].

3. Results

Poly(U)-programmed *E. coli* ribosomes can bind three molecules of deacylated tRNA which occupy the A, P and E sites [30,31]. In the absence of poly(U), the ribosomes can bind only two molecules of deacylated tRNA which are located in the ribosomal P and E sites [31,32]. Functional analysis of tRNA–ribosome complexes has demonstrated that all three forms of tRNA that participate in protein synthesis — deacylated tRNA, aminoacyl-tRNA and *N*-acetylated aminoacyl-tRNA, an analog of peptidyl — tRNA, can bind to the A and the P sites [31,33]. In contrast, the E site accepts only deacylated tRNA [30,31]. The affinity of deacylated $tRNA^{Phe}$ for the E site of poly(U)-programmed ribosomes is approximately two orders of magnitude less than that for the P site [32,34,35]. Unlike the interaction of tRNA with the A and P sites, the binding of deacylated tRNA to the E site exhibits very high association and dissociation rates [31]. Moreover, the interaction of deacylated tRNA with the E site is only subtly affected by the presence of mRNA [9,32]. We have used these observations to form complexes in which (2N$_3$A76)$tRNA^{Phe}$ derivatives occupy either the P or E site in order to monitor the ribosomal components in contact with the 3′ end of tRNA as it moves from the P site to the E site and is then released from the ribosome.

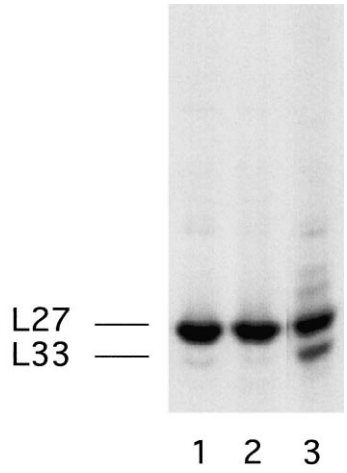

Fig. 1. Affinity labeling of 50S ribosomal proteins by (2N$_3$A76)$tRNA^{Phe}$ derivatives bound to poly(U)-programmed 70S ribosomes under P-site conditions. The UV-irradiated (2N$_3$A76)$tRNA^{Phe}$–ribosome complexes were separated into 30S and 50S ribosomal subunits by centrifugation through a 10–30% sucrose gradient. Aliquots of the 50S ribosomal subunits labeled by Phe- (lane 1), AcPhe- (lane 2) and deacylated (lane 3) (2N$_3$A76)$tRNA^{Phe}$ were digested with RNase T1 and subjected to SDS–PAGE. Positions of (2N$_3$A76)$tRNA^{Phe}$-labeled proteins L27 and L33 are indicated at the left side of the autoradiogram.

3.1. Photoaffinity labeling of ribosomal components by (2N$_3$A76)$tRNA^{Phe}$ derivatives bound to the ribosome under P-site conditions

Ribosomal complexes containing a single photoreactive tRNA derivative were formed by incubating deacylated (2N$_3$A76)$tRNA^{Phe}$, Phe-(2N$_3$A76)$tRNA^{Phe}$ or AcPhe-(2N$_3$A76)$tRNA^{Phe}$ with poly(U)-programmed 70S ribosomes (P-site conditions). Because all three tRNA species are known to have similar, strong affinities for the P site [36], and because Phe- and AcPhe-(2N$_3$A76)$tRNA^{Phe}$ reacted quantitatively with puromycin, we assumed that the tRNAs occupied only the P site in each case. Sucrose gradient centrifugation of UV-irradiated tRNA–ribosome complexes at 0.25 mM Mg^{2+} revealed that the photoreactive tRNA derivatives cross-linked exclusively to 50S ribosomal subunits. About 60% of the cross-linked Phe- and AcPhe-(2N$_3$A76)$tRNA^{Phe}$ labeled 50S subunit protein L27 (Fig. 1, lanes 1 and 2). Deacylated (2N$_3$A76)$tRNA^{Phe}$ primarily labeled protein L27, although some labeling of protein L33 could be observed (Fig. 1, lane 3). Approximately 40% of the P site-bound (2N$_3$A76)$tRNA^{Phe}$

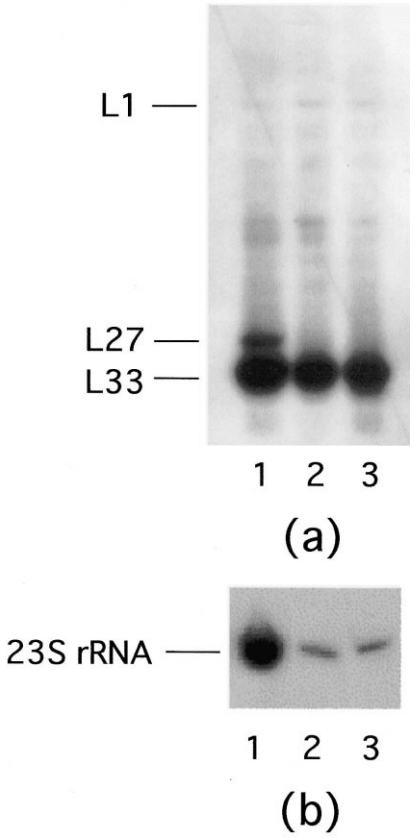

Fig. 2. Affinity labeling of 50S ribosomal subunits by deacylated $(2N_3A76)tRNA^{Phe}$ bound under E-site conditions. a: Proteins labeled by $(2N_3A76)tRNA^{Phe}$. Lane 1: deacylated $(2N_3A76)tRNA^{Phe}$ was bound to poly(U)-programmed 70S ribosomes whose A and P sites were blocked with AcPhe-$tRNA^{Phe}$. Lane 2: deacylated $(2N_3A76)tRNA^{Phe}$ was bound to 50S ribosomal subunits. Lane 3: same as lane 2 but in the presence of AcPhe-tRNA. After UV irradiation, covalent $(2N_3A76)tRNA^{Phe}$–50S ribosomal subunit complexes were isolated, digested with RNase T1 and analyzed by SDS–PAGE. Positions of $(2N_3A76)tRNA^{Phe}$-labeled proteins L1, L27 and L33 are indicated at the left side of the autoradiogram. b: Labeling of tRNA was assessed by electrophoresis of covalent $(2N_3A76)tRNA^{Phe}$–50S ribosomal subunit complexes in a 5% polyacrylamide gel containing SDS and EDTA [28]. Lanes 1, 2 and 3 are as in Fig. 2a.

derivatives became cross-linked to domain V of the 23S rRNA [3]. As previously shown, each of the tRNA derivatives displayed a distinctive cross-linking pattern: deacylated $(2N_3A76)tRNA^{Phe}$ labeled nucleotides U2506, U2585 and C2422, Phe-$(2N_3A76)tRNA^{Phe}$ labeled U2506 and U2585, and AcPhe-$(2N_3A76)tRNA^{Phe}$ labeled U2506, U2585 and G2069.

3.2. *Photoaffinity labeling of ribosomal components by deacylated $(2N_3A76)tRNA^{Phe}$ bound to the ribosome under E-site conditions*

While the affinity of deacylated tRNA for the P site is higher than its affinity for the E site, its affinity for the A and E sites is similar [32,34,35]. To minimize the labeling of A- and P-site components in these experiments, we took advantage of the following observations: AcPhe-tRNA cannot bind to the E site [30,37,38]; the interaction of tRNA with the P site is inhibited by the antibiotic edeine [32,39] and the binding of tRNA to the A site is strictly mRNA-dependent and takes place only in the presence of P site-bound tRNA [40].

When $(2N_3A76)tRNA^{Phe}$ was bound to poly(U)-programmed 70S ribosomes in which the A and P sites were filled with AcPhe-$tRNA^{Phe}$ (E-site conditions), and the tRNA–ribosome complexes were irradiated with near-UV light, $(2N_3A76)tRNA^{Phe}$ mainly labeled protein L33 and 23S rRNA (Fig. 2a, lane 1; Fig. 2b, lane 1). As reported earlier, the principal site of cross-linking in the 23S rRNA was nucleotide C2422 [3]. The small amount of label that was incorporated into protein L27 most likely originated from the binding of a minor fraction of the deacylated tRNA to the P site since L27 was not labeled when edeine was added before binding and cross-linking the photoreactive tRNA (Fig. 3a). This inference was confirmed by experiments carried out in the absence of poly(U). When $(2N_3A76)tRNA^{Phe}$ was bound to vacant 70S ribosomes and the complexes irradiated, the photoreactive tRNA labeled proteins L1, L33, and L27 (Fig. 3b, lane 1). In the presence of edeine, the cross-link to L27 was eliminated (Fig. 3b, lane 2). We therefore conclude that proteins L1 and L33, together with nucleotide C2422 of the 23S rRNA [3], are in close proximity to the 3′ terminus of deacylated tRNA bound to the 70S ribosome under E-site conditions. Interestingly, we note that the labeling of L1, scarcely detectable in the presence of poly(U), is strongly enhanced in the absence of the polynucleotide (compare Fig. 3a, lane 1 with Fig. 3b, lane 1).

Previous studies have shown that 50S subunits, unlike 70S ribosomes, bind only one deacylated tRNA molecule and that this ligand exhibits the properties of E site-bound tRNA [6,41]. When $(2N_3A76)tRNA^{Phe}$–50S subunit complexes were irradiated with near-UV light, the tRNA labeled protein L33 and, to a much lesser extent, protein L1 (Fig. 2a, lane 2). However, cross-linking to 23S rRNA decreased more than

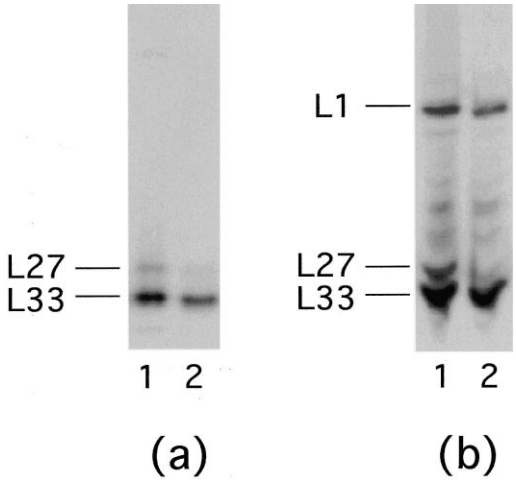

Fig. 3. Influence of poly(U) and the antibiotic edeine on the affinity labeling of 50S ribosomal proteins by $(2N_3A76)tRNA^{Phe}$. a: Affinity labeling of 50S ribosomal subunits by deacylated $(2N_3A76)tRNA^{Phe}$ bound to poly(U)-programmed 70 ribosomes with the P site occupied by AcPhe-$tRNA^{Phe}$ in the absence (lane 1) and in the presence (lane 2) of the antibiotic edeine. b: Affinity labeling of 50S ribosomal subunits by deacylated $(2N_3A76)tRNA^{Phe}$ bound to vacant 70S ribosomes in the absence (lane 1) and in the presence (lane 2) of the antibiotic edeine. Positions of $(2N_3A76)tRNA^{Phe}$-labeled proteins L1, L27 and L33 are indicated at the left side of autoradiograms.

20-fold relative to the level obtained when 70S ribosomes were used (compare lanes 1 and 2, Fig. 2b). This cross-linking pattern was not affected by the presence of AcPhe-tRNA^{Phe} (Fig. 2a, lane 3; Fig. 2b, lane 3), indicating that the labeling of L1 and L33 was specific to isolated 50S subunits and that it derived exclusively from the E site. The low level of (2N$_3$A76)tRNA^{Phe}–23S rRNA cross-links under these conditions suggests that the position of the 3′ end of the E-site tRNA is influenced by contacts with the 30S ribosomal subunit. This inference correlates well with our earlier finding that E site-bound tRNA^{Phe} containing 2N$_3$A in its anticodon loop cross-links to protein S11 and the 3′ end of 16S rRNA [9]. The proximity of the E-site tRNA anticodon to these 30S subunit markers has recently been confirmed by crystallographic studies of the 30S subunit [11,42,43].

3.3. Kinetics of (2N$_3$A76)tRNA^{Phe} binding to the P and E sites of vacant ribosomes

When deacylated (2N$_3$A76)tRNA^{Phe} was incubated with vacant 70S ribosomes for 2 min at 0°C in the absence of poly(U) and then irradiated, L33 was the major cross-linking target. When an excess of tRNA^{Phe} was added to the mixture immediately before irradiation, most of the E site-bound (2N$_3$A76)tRNA^{Phe} was displaced by the non-photoreactive tRNA. As a consequence, labeling of L33 was minimal and labeling of L1 was undetectable (Fig. 4a). These observations are consistent with the lability of tRNA binding at the E site [31] and serve as an additional criterion for differentiating (2N$_3$A76)tRNA^{Phe} cross-links at the E site from those which originated at the A or P sites. The cross-linking pattern was also affected strongly by temperature. Incubation of

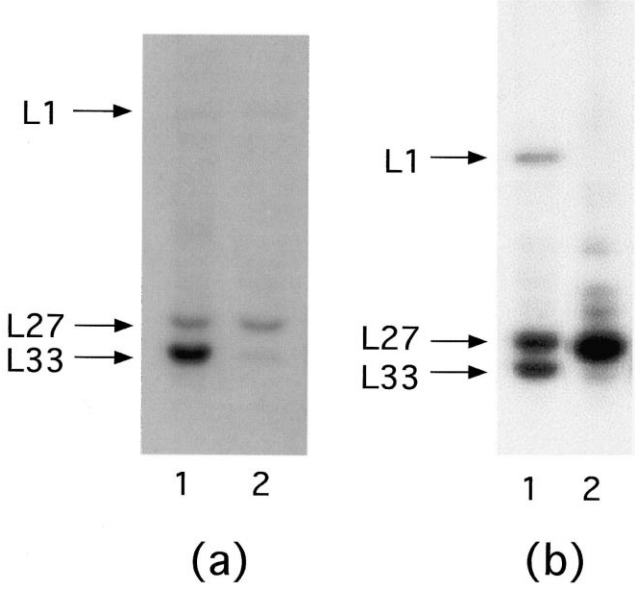

Fig. 4. Identification of protein L33 as a marker for the E site. a: Lability of tRNA binding at the E site. Lane 1: deacylated (2N$_3$A76)tRNA^{Phe} was bound to 70S ribosomes at 0°C in the absence of poly(U) and then irradiated with UV light for 2 min. Lane 2: the same, except that a 16-fold molar excess of tRNA^{Phe} was added immediately before UV irradiation. b: Influence of poly(U) on the cross-linking pattern. Deacylated (2N$_3$A76)tRNA^{Phe} was bound to 70S ribosomes at 37°C in the absence (lane 1) and in the presence (lane 2) of poly(U) and then irradiated with UV light for 2 min. Cross-linked proteins were analyzed by SDS–PAGE as described in the legend to Fig. 1.

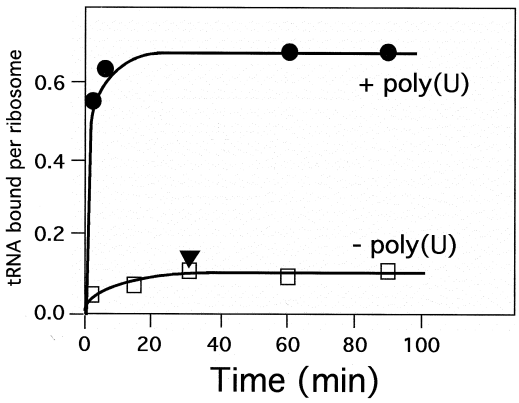

Fig. 5. Kinetics of the binding of (2N$_3$A76)tRNA^{Phe} to 70S ribosomes. Binding of deacylated (2N$_3$A76)tRNA^{Phe} to 70S ribosomes (molar tRNA:ribosome ratio 0.7:1) was carried out in the absence of poly(U) at both 0°C (□) and at 37°C (▼) and in the presence of poly(U) at 0°C (●). Formation of tRNA–ribosome complexes was monitored by membrane filter binding.

(2N$_3$A76)tRNA^{Phe} with vacant 70S ribosomes for 30 min at 37°C prior to irradiation resulted in a distribution of label between proteins L27 and L33 that was noticeably different than that at 0°C (Fig. 4b, lane 1). In particular, L27 was labeled to about the same extent as L33 and the L1 band was clearly visible. Once again the labeling of L33 and L1 was readily eliminated by the addition of an excess of unmodified tRNA while the labeling of L27 was unaffected (see Fig. 6a, lanes 5 and 6). In the presence of poly(U), essentially all of the cross-linked tRNA was attached to L27, even at very short incubation times (Fig. 4b, lane 2). The progressive shift in labeling from L33 to L27 as conditions change to favor more rapid tRNA binding suggests that deacylated tRNA may enter the P site via the E site.

To further investigate this possibility, the kinetics of (2N$_3$A76)tRNA^{Phe}–70S ribosome interaction were measured by a filter binding assay (Fig. 5) and the relative amounts of (2N$_3$A76)tRNA that were cross-linked to L27 and L33 after various times were analyzed by SDS–PAGE (Fig. 6). At 0°C, the labeling of protein L33 was highest within the first few minutes (Fig. 6a, lane 1) and steadily decreased to reach a minimum at 90 min (Fig. 6a, lane 3) despite a rise in the total (2N$_3$A76)tRNA^{Phe} bound from 0.04 to 0.10 mol tRNA/mol ribosomes (Fig. 5). In contrast, cross-linking of protein L27 increased with time and reached a maximum at 90 min (Fig. 6a, lane 3). A similar labeling pattern was observed after 30 min at 37°C (Fig. 6a, lane 5). We conclude from these findings that in the absence of poly(U), deacylated (2N$_3$A76)tRNA^{Phe} is distributed between the E and P sites.

All of the (2N$_3$A76)tRNA^{Phe} incorporated into protein L33 at either 0 or 37°C was judged to be E site-specific since cross-linking could be eliminated by exchange with unlabeled, non-photoreactive tRNA prior to irradiation (Fig. 6a, compare lane 3 with 4 and 5 with 6). On the other hand, the labeling of protein L27 at 37°C was entirely P site-specific as it was not displaced by exchange with non-photoreactive tRNA (Fig. 6a, compare lanes 5 and 6). Even after 90 min at 0°C, however, 60% of the (2N$_3$A76)tRNA^{Phe} which would otherwise be cross-linked to protein L27 was chased by the addition of non-photoreactive tRNA (Fig. 6a, compare lanes 3 and 4). Rapid exchange is characteristic of E site-bound

($2N_3$A76)tRNA^{Phe}, suggesting that this fraction of the tRNA occupies a hybrid site, intermediate between the P and E sites, which we call the P/E site. Thus, deacylated tRNA appears to be able to adopt three distinguishable states, P, P/E and E, when bound to ribosomes in the absence of poly(U).

The rapid binding of tRNA^{Phe} to ribosomes in the presence of poly(U) makes it more difficult to follow the kinetics of the interaction than in the absence of the polynucleotide. When ($2N_3$A76)tRNA^{Phe} was bound to poly(U)-programmed ribosomes, the labeling of protein L27 reached its maximum value after 2 min and did not change substantially upon further incubation (Fig. 6b). At the same time, the cross-link to protein L1 was eliminated and the small amount of labeled L33 could, for the most part, be exchanged with non-photoreactive tRNA^{Phe} (not shown). This suggests that the binding of ($2N_3$A76)tRNA^{Phe} to poly(U)-programmed ribosomes also involves formation of the P/E state of binding. That tRNA binding to the P site of the vacant ribosomes both in the absence and presence of poly(U) involves the formation of an intermediate state of binding excludes the possibility that the observed labeling patterns are the result of direct and mutually independent binding of ($2N_3$A76)tRNA^{Phe} to the P and E sites. The experiments described in this section are therefore consistent with the hypothesis that deacylated tRNA binds to the P site of 70S ribosomes by first passing through the E site. Indeed, this conclusion is almost inevitable given the inaccessibility of the P site to solvent that is evident in the crystal structure of the bacterial 70S particle [10].

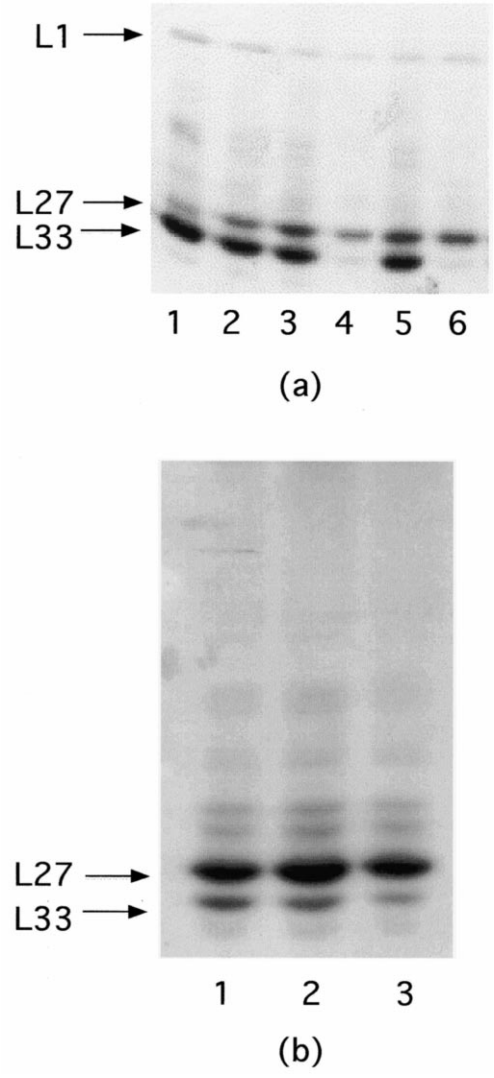

Fig. 6. Monitoring the binding of ($2N_3$A76)tRNA^{Phe} to 70S ribosomes by affinity labeling of 50S subunit proteins. ($2N_3$A76)tRNA^{Phe}–70S ribosome complexes were formed and irradiated, and the cross-linked proteins were analyzed, as described in the legend to Fig. 1. a: Lanes 1, 2 and 3: covalent ($2N_3$A76)tRNA^{Phe}–ribosome complexes formed in the absence of poly(U) after 2, 30 and 90 min incubation at 0°C. Lane 4: the same as lane 3, except that an excess of cold tRNA^{Phe} was added before UV irradiation. Lane 5: covalent ($2N_3$A76)tRNA^{Phe}–ribosome complexes formed after 30 min incubation at 37°C. Lane 6: the same as lane 5, except that an excess of cold tRNA^{Phe} was added before UV irradiation. Positions of ($2N_3$A76)tRNA^{Phe}-labeled proteins L1, L27 and L33 are indicated at the left side of the autoradiogram. b: Lanes 1, 2 and 3: covalent ($2N_3$A76)tRNA^{Phe}–ribosome complexes formed in the presence of poly(U) after 2, 7 and 60 min incubation at 0°C. Positions of ($2N_3$A76)tRNA^{Phe}-labeled proteins L27 and L33 are indicated at the left side of the autoradiogram.

4. Discussion

Prior to peptide bond formation, peptidyl-tRNA is positioned in the ribosomal P (P/P) site where it straddles the peptidyl transferase center (PTC) of the 50S subunit and the decoding site of the 30S subunit. The 3′ end of the P-site tRNA interacts with PTC in a manner that facilitates transfer of the peptidyl moiety to aminoacyl-tRNA in the A (A/A) site, extending the nascent polypeptide by one residue. Recent results from cryo-electron microscopy and crystallography place the 50S subunit portion of the P site below the central protuberance where it is in close proximity to several segments of domain V of the 23S rRNA [10,12,13,44]. Various lines of evidence suggest that nucleotides 2069, 2252, 2451/2, U2505/6, U2584/5 and 2602 participate in structural or functional interactions with the -ACCA end of P-site tRNA [3,10,45–51]. In addition, protein L27 has been implicated in the binding or stabilization of P-site tRNA by short-range cross-links from $2N_3$A at the 3′ terminus of the RNA molecule [1,29,52]. Experiments described in this report show that when tRNA^{Phe} is bound to the ribosomal P site in a poly(U)-dependent fashion, interaction of its 3′ terminal nucleotide is influenced by the presence of aminoacyl or peptidyl moieties. Although deacylated ($2N_3$A)tRNA^{Phe}, Phe-($2N_3$A)tRNA^{Phe} and AcPhe-($2N_3$A)tRNA^{Phe} all cross-link to protein L27, Phe-($2N_3$A)tRNA^{Phe} labels only U2506 and U2585, AcPhe-($2N_3$A)tRNA^{Phe} labels U2506, U2585 and G2609, and deacylated ($2N_3$A)tRNA^{Phe} labels U2506, U2585 and C2422 [3]. As labeling of C2422 is characteristic of E site-bound tRNA, this cross-link may be due to the presence of a small fraction of the deacylated tRNA in either the E site or the hybrid P/E binding state [14].

When bound to poly(U)-programmed 70S ribosomes carrying AcPhe-tRNA^{Phe} at the A and P sites, ($2N_3$A76)tRNA^{Phe} labels protein L33 and C2422 of the 23S rRNA (this work; [3]). In contrast, ($2N_3$A76)tRNA^{Phe} bound to 50S subunits labels only protein L33. Although ($2N_3$A76)tRNA^{Phe} is located at the E site in both cases, as indicated by its high association and dissociation rates and its mRNA independence, differences in the pattern of cross-linking to 70S and 50S particles indicate that the two binding states are not identical.

In 70S complexes programmed with a non-cognate mRNA such as poly(A), $(2N_3A76)tRNA^{Phe}$ labels only L33 [9], much as in the 50S complex, while significant labeling of protein L1 occurs only in the absence of poly(U). The region of the ribosome conventionally referred to as the E site thus appears to encompass multiple binding states. The site with the lowest affinity for tRNA, the highest susceptibility to exchange, and from which the tRNA is probably dissociated or ejected into the cytoplasm, has been called the E2 or F (final) site [3,19,20,52]. The markers for the F site on the 50S subunit include protein L1 and nucleotides 2112, 2116 and 2169 of the 23S rRNA [3,46], whereas those for the E (E/E) site are L33 and C2422. A corollary of this hypothesis is that the E and F sites together define a region of low tRNA affinity within which the tRNA may move or be moved with little hindrance after it leaves the P site but before it is released form the ribosome. This stands in contrast to the strict spatial and stereochemical requirements imposed upon tRNA at the A and P sites.

The cross-linking results reported here and elsewhere demonstrate that contacts between tRNA and the 30S subunit influence the position of the 3′ end of tRNA on the 50S subunit in subtle yet distinctive ways. These effects are clearly seen at the exit sites: in the presence of poly(U), the cognate mRNA, $(2N_3A76)tRNA^{Phe}$, labels L33 and C2422 and in the presence of poly(A), a non-cognate mRNA, it labels only L33 (this work; [9]). In the absence of mRNA, by contrast, $(2N_3A76)tRNA^{Phe}$ becomes cross-linked to L1 and L33 – but not 23S rRNA – when bound to 70S ribosomes, and to L33 alone when bound to 50S subunits. Such effects are not limited to the E site, however, as we have shown previously that $AcPhe\text{-}tRNA^{Phe}$ at the P site labels C2452 in the 23S rRNA when poly(U) is absent in addition to the cross-links normally observed in the presence of mRNA [3]. The precise placement of the acceptor end of tRNA is therefore sensitive both to the group (aminoacyl or peptidyl) attached to the 3′ terminus as well as to the environment of the anticodon and whether or not it is able to pair with a cognate codon at the decoding site.

The changes in cross-linking pattern observed during $(2N_3A76)tRNA^{Phe}$ binding to vacant ribosomes in vitro lead us to propose that tRNA enters the P (P/P) site by first passing through the ribosomal exit sites. This conclusion is consistent with the crystal structure of the bacterial 70S ribosome which shows that the P site is almost completely inaccessible to solvent [10]. During this process, tRNA first binds to the F site where its 3′ end interacts mainly if not exclusively with the 50S subunit. This state is typified by cross-links from $(2N_3A76)tRNA^{Phe}$ to proteins L1 and L33. In the next step, tRNA enters the E (E/E) binding state, in which its 3′ end contacts protein L33 and nucleotide C2422 of the 23S rRNA. The tRNA then adopts the hybrid P/E state of binding in which its 3′ A residue contacts protein L27, but where it remains exchangeable with tRNA in solution. Finally, tRNA binds to the P (P/P) site where its 3′ terminus interacts with protein L27 and nucleotides 2506 and 2585 of the 23S RNA. On the way from the E (E/E) site to the P (P/P) site, the anticodon of the tRNA site makes contact(s) with protein S11 and the 3′ end of 16S rRNA [9]. We believe that the movement of deacylated tRNA from the P/P site to the F site during protein synthesis proceeds through these same intermediate states but in reverse order. Our findings thus extend and modify the current hybrid-site model for the movement of tRNA on the ribosome.

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References

[1] Wower, J., Wower, I.K., Kirillov, S., Rosen, K., Hixson, S.S. and Zimmermann, R.A. (1995) Biochem. Cell Biol. 73, 1041–1047.
[2] Kirillov, S.V., Porse, B.T., Vester, B., Wooley, P. and Garrett, R.A. (1997) FEBS Lett. 406, 223–233.
[3] Wower, J., Kirillov, S.V., Wower, I., Guven, S., Hixson, S.S. and Zimmermann, R.A. (2000) J. Biol. Chem. 275, 37887–37894.
[4] Nekhai, S.A., Parfenov, D.V. and Saminsky, E.M. (1994) Biochim. Biophys. Acta 1218, 481–484.
[5] Lill, R., Robertson, J.M. and Wintermeyer, W. (1984) Biochemistry 23, 6710–6717.
[6] Kirillov, S.V. and Semenkov, Yu.P. (1986) J. Biomol. Struct. Dyn. 4, 263–269.
[7] Lill, R., Lepier, A., Schwagele, F., Sprinzl, M., Vogt, H. and Wintermeyer, W. (1988) J. Mol. Biol. 203, 699–705.
[8] Vladimirov, S.N., Graifer, D.M., Zenkova, M.A., Karpova, G.G., Olenina, L.V., Kirillov, S.V., Makarov, E.M., Makhno, V.I. and Semenkov, Yu.P. (1989) Biopolym. Cell 5, 35–40.
[9] Wower, J., Scheffer, P., Sylvers, L.A., Wintermeyer, W. and Zimmermann, R.A. (1993) EMBO J. 12, 617–623.
[10] Yusupov, M.M., Yusupova, G.Zh., Baucom, A., Lieberman, K., Earnest, T.N., Cate, J.H.D. and Noller, H.F. (2001) Science 292, 883–896.
[11] Yusupova, G.Zh., Yusupov, M.M., Cate, J.H.D. and Noller, H.F. (2001) Cell 106, 233–241.
[12] Agrawal, R.K., Penczek, P., Grassucci, R.A., Li, Y., Leith, A., Nierhaus, K.H. and Frank, J. (1996) Science 271, 1000–1002.
[13] Stark, H., Orlova, E.V., Rinke-Appel, J., Junke, N., Mueller, F., Rodnina, M., Wintermeyer, W., Brimacombe, R. and van Heel, M. (1997) Cell 88, 19–28.
[14] Moazed, D. and Noller, H. (1989) Nature (London) 342, 142–148.
[15] Noller, H.F., Moazed, D., Stern, S., Powers, T., Allen, P.N., Robertson, J.M., Weiser, B. and Triman, K. (1990) in: The Ribosome: Structure, Function and Evolution (Hill, W., Dahlberg, A., Garrett, R.A., Moore, P.B., Schlessinger, D. and Warner, J.R., Eds.), pp. 73–92, American Society for Microbiology, Washington, DC.
[16] Wower, J., Sylvers, L.A., Rosen, K.V., Hixson, S.S. and Zimmermann, R.A. (1993) in: The Translational Apparatus (Nierhaus, K.H., Franceschi, F., Subramanian, A.R., Erdmann, V.A. and Wittmann-Liebold, B., Eds.), pp. 455–464, Plenum, New York.
[17] Sylvers, L.A., Kopylov, A.M., Wower, J., Hixson, S.S. and Zimmermann, R.A. (1992) Biochimie 74, 381–389.
[18] Wower, J. and Zimmermann, R.A. (1991) Biochimie 73, 961–969.
[19] Frank, J. (1998) J. Struct. Biol. 124, 142–150.
[20] Agrawal, R.K., Spahn, C.M.T., Penczek, P., Grassuci, R.A., Nierhaus, K.H. and Frank, J. (2000) J. Cell Biol. 150, 447–459.
[21] Semenkov, Yu.P., Shapkina, T.G. and Kirillov, S.V. (1992) FEBS Lett. 296, 207–210.
[22] Sylvers, L.A., Wower, J., Hixson, S.S. and Zimmermann, R.A. (1989) FEBS Lett. 245, 9–13.
[23] Wower, J., Hixson, S.S. and Zimmermann, R.A. (1988) Biochemistry 27, 8114–8121.
[24] Rappaport, S. and Lapidot, Y. (1974) Methods Enzymol. 29, 685–688.
[25] Litt, M. (1968) Biochem. Biophys. Res. Commun. 32, 507–511.
[26] Makhno, V.I., Peshin, N.N., Semenkov, Yu.P. and Kirillov, S.V. (1988) Mol. Biol. 22, 528–536.
[27] Kirillov, S.V., Makhno, V.I. and Semenkov, Yu.P. (1978) Eur. J. Biochem. 89, 297–304.
[28] Brimacombe, R., Greuer, B., Gulle, H., Kosack, M., Mitchel, P., Osswald, M., Stade, K. and Stiege, W. (1990) in: Ribosomes and Protein Synthesis. A Practical Approach (Spedding, G., Ed.), pp. 131–159, Oxford University Press, Oxford.

[29] Wower, J., Hixson, S.S. and Zimmermann, R.A. (1989) Proc. Natl. Acad. Sci. USA 86, 5232–5236.
[30] Rheinberger, H.-J., Sternbach, H. and Nierhaus, K.H. (1981) Proc. Natl. Acad. Sci. USA 78, 5310–5314.
[31] Grajevskaja, R.A., Ivanov, Yu.V. and Saminsky, E.M. (1982) Eur. J. Biochem. 128, 47–52.
[32] Kirillov, S.V., Makarov, E.M. and Semenkov, Yu.P. (1983) FEBS Lett. 157, 91–94.
[33] Odinzov, V.B. and Kirillov, S.V. (1978) Nucleic Acids Res. 5, 3871–3879.
[34] Lill, R., Robertson, J.M. and Wintermeyer, W. (1986) Biochemistry 25, 3245–3255.
[35] Lill, R. and Wintermeyer, W. (1987) J. Mol. Biol. 196, 137–148.
[36] Makarov, E.M., Katunin, V.I., Odintsov, V.B., Semenkov, Yu.P. and Kirillov, S.V. (1984) Mol. Biol. 18, 11090–11094.
[37] Kirillov, S.V. and Semenkov, Yu.P. (1982) FEBS Lett. 148, 235–238.
[38] Wintermeyer, W., Lill, R. and Robertson, J.M. (1990) in: The Ribosome: Structure, Function and Evolution (Hill, W., Dahlberg, A., Garrett, R.A., Moore, P.B., Schlessinger, D. and Warner, J.R., Eds.), pp. 348–357, American Society for Microbiology, Washington, DC.
[39] Szer, W. and Kurilo-Borovska, Z. (1972) Biochim. Biophys. Acta 259, 357–368.
[40] Watanabe, S. (1972) J. Mol. Biol. 67, 443–449.
[41] Parfenov, D.V. and Saminskii, E.M. (1993) Mol. Biol. 27, 507–510.
[42] Wimberly, B.T., Brodersen, D.E., Clemons Jr., W.M., Morgan-Warren, R.J., Carter, A.P., Vonrhein, C., Hartsch, T. and Ramakrishnan, V. (2000) Nature 407, 327–339.
[43] Schluenzen, F., Tocilj, A., Zarivach, R., Harms, J., Gluehmann, M., Janell, D., Bashan, A., Bartels, H., Agmon, I., Franceschi, F. and Yonath, A. (2000) Cell 102, 615–623.
[44] Ban, N., Nissen, P., Hansen, J., Moore, P.B. and Steitz, T.A. (2000) Science 289, 905–919.
[45] Barta, A., Steiner, G., Brosius, J., Noller, H.F. and Kuechler, E. (1984) Proc. Natl. Acad. Sci. USA 81, 3607–3611.
[46] Moazed, D. and Noller, H. (1989) Cell 57, 585–597.
[47] Lieberman, K.R. and Dahlberg, A.E. (1994) J. Biol. Chem. 269, 16163–16169.
[48] Samaha, R.R., Green, R. and Noller, H.F. (1995) Nature 377, 309–314.
[49] Porse, B.T., Thi-Ngoc, H.P. and Garrett, R.A. (1996) J. Mol. Biol. 264, 472–483.
[50] Kirillov, S.V., Porse, B.T. and Garrett, R.A. (1999) RNA 5, 1003–1013.
[51] Nissen, P., Hansen, J., Ban, N., Moore, P.B. and Steitz, T.A. (2000) Science 289, 878–879.
[52] Wower, J., Wower, I. and Zwieb, C. (1999) Nucleic Acids Symp. 41, 187–191.