Effect of ribosome binding and translocation on the anticodon of tRNA^{Phe} as studied by wybutine fluorescence

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Received 23 February 1982; Accepted 30 March 1982

ABSTRACT

The complexes of N-AcPhe-tRNA^{Phe} (or non-aminoacylated tRNA^{Phe}) from yeast with 70S ribosomes from *E. coli* have been studied fluorimetrically utilizing wybutine, the fluorophor naturally occurring next to the 3' side of the anticodon, as a probe for conformational changes of the anticodon loop. The fluorescence parameters are very similar for tRNA bound to both ribosomal sites, thus excluding an appreciable conformational change of the anticodon loop upon translocation. The spectral change observed upon binding of tRNA^{Phe} to the P site even in the absence of poly(U) is similar to the one brought about by binding of poly(U) alone to the tRNA. This effect may be due to a hydrophobic binding site of the anticodon loop or to a conformational change of the loop induced by binding interactions of various tRNA sites including the anticodon.

INTRODUCTION

There are indications from a variety of experimental approaches suggesting that on the ribosome, the tRNA molecule undergoes conformational changes rather than behaving as a rigid entity (1-3). Owing to the importance of codon-anticodon interaction for the specificity of tRNA-ribosome complex formation, the anticodon loop has received particular attention. A number of studies on tRNA in solution, applying various biophysical methods (4-6), have established that the conformation of the anticodon loop changes upon binding of the proper codon. Moreover, it has been proposed on the basis of oligonucleotide binding data that such a change is transmitted to distant parts of the tRNA molecule (7). A switch of the anticodon loop from a conformation in which the five bases on the 3' side of the loop are stacked on top of the anticodon stem into the alternative 5' stacked one has been proposed to be the molecular basis for

mRNA translocation on the ribosome (8).

For the investigation of the significance of conformational changes for the functions of the tRNA on the ribosome, fluorescence spectroscopy has proven particularly useful. We have previously reported fluorescence measurements on ribosomal complexes of fluorescent derivatives of yeast tRNA^{Phe} carrying covalently inserted ethidium or proflavine in either the D or the anticodon loop (9,10). With respect to the anticodon loop, these and related experiments (11,12) have indicated that upon tRNA-ribosome complex formation, the anticodon loop becomes immobilized in a hydrophobic environment and is effectively shielded against solvent access. Similar results were obtained in experiments in which the fluorescence of wybutine, the weakly fluorescent base 3' to the anticodon of yeast tRNA^{Phe} was monitored (13,14).

As to the question, whether the translocation step is accompanied by a substantial conformational change of the anticodon loop, we have observed no difference between the spectroscopic properties of ribosome complexes carrying proflavine-labeled tRNA^{Phe} in either the A or the P site, making such a change rather unlikely (10). In the present study, we have extended the investigation of the effect of translocation and of codon-anticodon interaction on the anticodon loop by measuring the fluorescence of wybutine in unmodified tRNA^{Phe} from yeast.

MATERIALS AND METHODS

Biochemicals. tRNA^{Phe} (charging capacity 1.5-1.7 nmol/A₂₆₀ unit) was isolated from brewer's yeast tRNA (Boehringer Mannheim, GFR) as described (10). N-AcPhe-tRNA^{Phe} was routinely prepared by aminoacylation with [¹⁴C]phenylalanine (521 Ci/mol) and subsequent acetylation with acetic anhydride (15); the final product after isolation by BD-cellulose chromatography contained 1.6 to 1.7 nmol N-acetyl-phenylalanine/A₂₆₀ unit of tRNA (10). For comparative experiments, N-AcPhe-tRNA^{Phe} was prepared by acetylation with N-acetoxysuccinimide (16). Since the same fluorescence spectra were observed for free and ribosome-bound N-AcPhe-tRNA^{Phe} prepared by the two acetylation procedures, only data for the acetic anhydride product are reported.

Ribosomes were prepared from *E. coli* MRE 600, and 70S couples were isolated by zonal centrifugation; at least 80 % of the particles were active in the binding assay with N-AcPhe-tRNA^{Phe} (10). Elongation factor G from *E. coli* was a gift from A. Parmeggiani (17).

tRNA-ribosome complexes. The preparation and characterization of the ribosome complexes carrying N-AcPhe-tRNA^{Phe} in either A or P sites have been described in detail (10). In brief, the A-site complex was prepared by incubating N-AcPhe-tRNA^{Phe} with an equimolar amount of poly(U)-programmed ribosomes, the P site of which had been filled with nonfluorescent *E. coli* tRNA^{Phe}. In order to remove unbound N-AcPhe-tRNA^{Phe}, the complex was isolated by ultracentrifugation. After resuspension of the pellets, around 95 % of the N-AcPhe-tRNA^{Phe} present determined by TCA precipitation (usually between 0.2 and 0.5 μ M) was bound to ribosomes, as measured by the nitrocellulose filter binding assay. Judging from the puromycin assays before and after incubation with EF-G and GTP, at least 90 % of the bound material was located in the A site. Translocation of the N-AcPhe-tRNA^{Phe} to the P site was accomplished in nearly quantitative yield. Alternatively, N-AcPhe-tRNA^{Phe} was bound to the P site directly by incubation with a two-fold excess of ribosomes. The various types of N-AcPhe-tRNA^{Phe}-ribosome complexes have been assayed after the spectroscopic measurements; the results are given below along with the respective spectroscopic data.

In the case of non-aminoacylated tRNA^{Phe}, the P-site complexes have been prepared by direct binding, as described above. The location of the tRNA in the P site can be assayed indirectly by the inhibition of subsequent P-site binding of N-AcPhe-tRNA^{Phe}, as measured by the puromycin assay (10). Complete inhibition was observed after the addition of an equimolar amount of tRNA^{Phe}, indicating stoichiometric binding to the P site. Although the binding of tRNA^{Phe} to the A site of P site-blocked ribosomes cannot be assayed directly, it is indicated by the fact that a second molecule of ³²P-labeled tRNA^{Phe} is bound after the P site has been filled (18). For the present experiments, the presumed A-site complexes of tRNA^{Phe} have been isolated by a centrifugation procedure analogous to the one

described above.

If not stated otherwise, all experiments have been performed at 25°C in a buffer containing 50 mM Tris-HCl (pH 7.6), 90 mM ammonium chloride, 40 mM KCl, 20 mM magnesium acetate, and 1 mM dithioerythritol.

Spectroscopic measurements. The fluorescence measurements were performed using a Schoeffel RRS 1000 spectrofluorimeter equipped with a double monochromator for excitation and two emission monochromators. Wybutine fluorescence was excited at 320 nm (bandwidth 3 nm) and measured after passing a cut-off filter (Schott KV 335) at a bandwidth of 3 nm. In order to avoid contamination with extraneous fluorescent material, all solutions were made up from quartz-distilled water and contact with plasticware was strictly avoided. The measurement of fluorescence anisotropy and the iodide quenching experiments were performed as described previously (10).

RESULTS AND DISCUSSION

The environment of wybutine in tRNA^{Phe} is slightly different in A and P sites. The fluorescence properties of wybutine in the anticodon loop of tRNA^{Phe} are strongly influenced by the environment, the most important factors being the hydrophobicity of the surrounding medium (19) and the stacking interactions with the neighboring bases (4,20). In view of these properties of wybutine, the 8 nm blue-shift of the emission spectrum, which is observed upon binding of N-AcPhe-tRNA^{Phe} to the A site of poly(U)-programmed ribosomes (Fig. 1), indicates a more hydrophobic environment of wybutine in the ribosomal complex as compared to the unbound tRNA. The substantial fluorescence increase which accompanies A-site binding is largely due to shielding against solvent access, as shown below. Upon translocation to the P site, both effects are partially reversed, the fluorescence intensity dropping by about 15 % and the emission maximum exhibiting a small red-shift by about 3 nm (Fig. 1). Obviously, wybutine, other than proflavine at the same position (10), senses a slight difference between the two ribosomal sites, which may be attributed to either one or both of the environmental factors mentioned above.

Figure 1. Wybutine emission spectra of N-AcPhe-tRNA^{Phe}. The A-site complex was prepared by binding N-AcPhe-tRNA^{Phe} to P site-blocked ribosomes and ultracentrifugation, as described in Materials and Methods; according to the millipore filtration assay, 96 % of the N-AcPhe-tRNA^{Phe} present was bound, of which 5 % were puromycin reactive. Translocation to the P site with 100 % efficiency was accomplished by subsequent incubation with EF-G-GTP. Blank spectra measured with solutions which contained all respective components except the tRNA have been subtracted; a representative blank spectrum of a solution containing ribosomes is indicated by the lowest curve.

The spectral changes depicted in Fig. 1 do not depend upon aminoacylation of the tRNA since identical spectra have been observed for the A or P-site complexes of non-aminoacylated tRNA^{Phe} (not shown). As far as P-site binding is concerned, these results are very similar to spectral data obtained previously (21), whereas the present A-site spectrum is rather different from the one reported by these authors. We feel that our spectrum, which has been measured directly with the isolated A-site complex, is more reliable than the previously reported one, which was obtained by decomposing a mixed spectrum of free, P site-bound, and A site-bound tRNA^{Phe} on the basis of relative concentrations calculated from the binding constants. The argument is supported by the fact that we observe identical spectra for the A-site complexes of both uncharged and charged tRNA^{Phe}, the latter being fully characterized by biochemical analyses (cf. Fig. 1).

The accessibility of wybutine is reduced to a large extent in the two ribosomal sites. The exposure of a fluorophor to the surrounding medium can be assessed by fluorescence quenching experiments, in which the fluorescence intensities measured in the presence of varying concentrations of a quenching compound are compared with the fluorescence measured in the absence of quencher. For the present experiments, potassium iodide was chosen as the quenching compound since it has been shown to be an efficient quencher of wybutine fluorescence (22).

When N-AcPhe-tRNA^{Phe} is bound to the A or P sites of poly(U)-programmed ribosomes, wybutine is considerably more shielded against iodide access as compared to the free tRNA (Fig. 2), the Stern-Volmer constant (see legend of Fig. 2) being reduced by a factor of five. Very similar results are obtained with non-aminoacylated tRNA^{Phe}. The quenching efficiency does not show any difference between the A and P-site complexes within the limits of measuring accuracy. These results are in perfect agreement with the ones obtained previously with tRNA^{Phe} derivatives carrying proflavine in the place of wybutine (10). Also with this label the quenching efficiency was found to decrease by a factor of about five when the N-AcPhe-tRNA^{Phe} derivative was bound to either the A or the P site.

Thus, in accordance with the previous results, the quenching experiments with the unmodified N-AcPhe-tRNA^{Phe} suggest that the environment of the base 3' to the anticodon does not change appreciably when the tRNA is translocated from the A to the P site.

The mobility of wybutine is equally restricted in both ribosomal sites. Owing to the slow rotation of the ribosomes, which occurs in the μ sec time range (23), the fluorescence anisotropy of a tRNA-bound fluorophor in the ribosomal complex is influenced only by the local mobility around the fluorophor and not by the rotational motion of the whole complex. Thus, the substantial depolarization of the wybutine fluorescence observed for the ribosomal complexes of N-AcPhe-tRNA^{Phe} (Table 1) indicates that wybutine in the complexes retains a considerable degree of mobility. Similar data have been reported for deacylated tRNA^{Phe} bound to the P site of ribosomes from E. coli (14) or rabbit

Figure 2. Iodide quenching of wybutine fluorescence. The A and P-site complexes were prepared as described in Materials and Methods; more than 92 % of the N-AcPhe-tRNA^{Phe} present was bound in all experiments, of which less than 10 % and more than 90 % was puromycin reactive before and after incubation with EF-G-GTP, respectively. The nonaminoacylated tRNA^{Phe} was directly bound into the P site as described in Materials and Methods. The measurements were performed in buffers containing 50 mM Tris-HCl, pH 7.6, 40 mM ammonium chloride, 20 mM magnesium acetate and varying amounts of potassium iodide and potassium chloride so that [KI]+[KCl] was 90 mM. The fluorescence data are plotted against the concentration of KI according to the Stern-Volmer relationship: $I_0/I = 1 + K_{SV}[KI]$, I and I_0 being the fluorescence intensities measured in the presence and in the absence of iodide, respectively; the slope of the straight line gives the so-called Stern-Volmer quenching constant, K_{SV} . tRNA^{Phe}: □ free, ○ P-site bound; N-AcPhe-tRNA^{Phe}: ■ free, ● P-site bound; ▲ A-site bound.

reticulocytes (13). Whether the mobility of wybutine in ribosome-bound tRNA^{Phe} is due to some rotational freedom of the fluorophor itself or to a flexibility of the anticodon loop cannot be decided on the basis of the present data. Anyway, the comparison between A and P site-bound N-AcPhe-tRNA^{Phe} (Table 1) clearly shows that there is no significant difference in the mobility of wybutine in the two sites. The same conclusion was reached previously on the basis of anisotropy data obtained with the proflavine-substituted tRNA^{Phe} (10).

In summary, the emission spectra, the results of the quench-

Table 1. Anisotropies of free and ribosome-bound N-AcPhe-tRNA^{Phe}

	bound N-AcPhe-tRNA (% of total amount)	puromycin reactive (% of bound N-AcPhe-tRNA)	anisotropy ($\pm$ 0.005)
free	-	-	0.114 (0.220)
A site	94	14	0.154
P site	97	97	0.151

The complexes have been prepared and the anisotropies measured as described in Materials and Methods. The biochemical assays have been performed after the fluorescence measurements. The number in brackets represents the limiting anisotropy, A_0 , obtained by extrapolation from Perrin plots measured in 95 % glycerol upon varying the temperature down to -20°C as described previously (9).

ing experiments, and the anisotropy data lead to the conclusion that the conformation of the anticodon loop is rather similar in both A and P sites. In particular, the finding that the latter two parameters of wybutine fluorescence do not differ at all for A and P site-bound tRNA^{Phe} strongly disfavors the Woese model, which implies a fully stacked and a fully exposed wybutine, respectively, for the two states of the tRNA molecule on the ribosome. Such a large difference would certainly be expected to show up in the two fluorescence parameters. The same conclusion was reached on the basis of photo-crosslinking data which also argue against extensive conformational changes of the anticodon loop during translocation (24). On the other hand, small changes are not excluded by the available data. In fact, the small spectral difference observed between the A and P-site complexes of N-AcPhe-tRNA^{Phe} (Fig. 1) may indicate somewhat less extensive stacking interactions of wybutine in the anticodon loop of the P site-bound tRNA. Possible reasons for such a change include structural alterations which are restricted to the anticodon loop, e.g. the one found in the P site-specific initiator tRNA (25,26), as well as those being induced at distant parts of the tRNA molecule and transmitted to the anticodon loop, as discussed below.

The fluorescence spectrum of wybutine is not influenced by codon-anticodon interaction in the P site. Previous studies (4) showed (we have repeated the experiments and obtained the same result) that upon binding of poly(U) to yeast tRNA^{Phe} free in solution, the emission spectrum of Wybutine is blue-shifted by about 6 nm. This indication of a codon-induced conformational change of the anticodon loop involving the base stacking around the wybutine has been further substantiated by circular dichroism and temperature jump experiments (5,6). In order to see whether codon-anticodon interaction on the ribosome also leads to such an effect, we set out to compare the emission spectra of N-AcPhe-tRNA^{Phe} bound to non-programmed, poly(C)-misprogrammed, and poly(U)-programmed ribosomes.

Although the omission of the correct mRNA lowers the tRNA affinity of the ribosome, in the presence of 20 mM Mg²⁺ the kinetic stability of the P-site complexes of N-AcPhe-tRNA^{Phe} is sufficiently high to allow their isolation by centrifugation. As shown in Fig. 3, the emission spectrum of P site-bound N-AcPhe-tRNA^{Phe} is not significantly influenced by the presence or absence of the correct codon. In all three cases, by forming the complex, the wybutine emission is blue-shifted and enhanced to the same extent as compared to the unbound N-AcPhe-tRNA^{Phe}. (In the poly(U)-containing complex the fluorescence intensity of wybutine is somewhat higher than in the two other P-site complexes, but we consider this small difference to be not significant.) Similar observations have been made with proflavine as a fluorescent probe of the anticodon loop (10). These results suggest that the hydrophobicity of the local environment of the anticodon loop in the ribosomal P site is not significantly influenced by codon-anticodon interaction. The alternative explanation which assumes that in the P site the anticodon is not bound to the mRNA can be excluded from several lines of evidence including results from fluorescence (9,12), photocrosslinking (24,27), and filter binding (28,29) experiments which demonstrate the occurrence of codon-anticodon interaction in the P site.

An allosteric behavior of tRNA in interactions with the ribosome? There remain two alternative interpretations to ex-

Figure 3. Influence of the mRNA on the emission spectrum of P site-bound N-AcPhe-tRNA^{Phe}. N-AcPhe-tRNA^{Phe} was directly bound to the P site, as described in Materials and Methods, in the absence of mRNA or in the presence of poly(C) or poly(U). Between 89 and 95 % of the total N-AcPhe-tRNA^{Phe} present was bound in various experiments, as assayed by the filtration assay; all bound N-AcPhe-tRNA^{Phe} was reactive towards puromycin, indicating exclusive P-site binding. The spectra have been standardized to 95 % binding. The spectrum of N-AcPhe-tRNA^{Phe} measured in the absence of ribosomes is given for comparison ("free").

plain the spectral shift observed upon binding of tRNA^{Phe} to ribosomes even in the absence of poly(U). First, it may reflect an increased hydrophobicity of the environment of the anticodon loop provided by surrounding ribosomal proteins. As indicated by the quenching data reported above, the anticodon loop in the P site indeed appears to be embedded into the ribosomal structure. On the other hand, the effect of such an environmental change on the fluorescence spectrum of wybutine is not easy to predict. An alternative interpretation is suggested by the similarity of the spectral changes induced by ribosome binding of tRNA^{Phe} to that brought about by binding to poly(U) in the absence of ribosomes (4). As discussed above, the latter effect is due to a conformational transition of the anticodon loop which changes the stacking interactions of wybutine. Thus, the possibility arises that ribosome binding causes a similar conformational change. Since the observed effect does not depend

upon codon-anticodon interaction, this interpretation implies that ribosome contacts of tRNA regions other than the anticodon induce a conformational change of the anticodon loop which is similar to the one induced by codon-anticodon interaction in the absence of ribosomes.

In general support of such a model, solution studies have shown that the tRNA molecule may exist in more than one conformation (30,31). Furthermore, it has been shown that conformational changes of the anticodon loop may be coupled to structural alterations of distant regions of the tRNA molecule (7,32,33). Finally, it should be noted that all the available data concerning the structure of ribosome-bound tRNA, as obtained by fluorescence (9,10), tritium exchange (34), or chemical modification (35) experiments, suggest that ribosome binding induces some change of the tRNA conformation.

To conclude, the present data can be interpreted in terms of an allosteric model of tRNA-ribosome interaction, in which the transition of the tRNA towards a "binding conformation" is promoted by contacts with the ribosome taking place at various sites of the tRNA molecule, including the anticodon, and acting in a synergistic fashion. The latter aspect appears to have important implications for the problem of codon-dependent tRNA selection by the programmed ribosome. Experiments are in progress now in order (i) to verify the correlation of the spectroscopic data, and (ii) to substantiate the assumption of a synergism of different sites of the tRNA molecule with respect to the formation of a particular conformation of ribosome-bound tRNA.

ACKNOWLEDGMENTS

We wish to thank H.G. Zachau for his constant interest and encouragement and A. Parmeggiani for gifts of purified elongation factor. H.P. received a grant from the Studienstiftung des deutschen Volkes, and W.W. a Heisenberg Fellowship from the Deutsche Forschungsgemeinschaft, which also supported the work through the Sonderforschungsbereich 51.

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