INTERACTION OF Ac-Phe-tRNA WITH *E. COLI* RIBOSOMAL SUBUNITS. 2. RESISTANCE OF THE SPARSOMYCIN-INDUCED COMPLEX TO HYDROXYLAMINE ACTION

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1. Introduction

Sparsomycin stimulates interaction between N'-Ac-Phe-tRNA and *E. coli* ribosomes to form a complex containing Ac-Phe-tRNA, the 50 S and the 30 S ribosomal subunit, and probably sparsomycin [1–3]. The evidence indicates that sparsomycin and the CCA-Phe-Ac part of the substrate interact with the 50 S subunit (with CCA-Phe-Ac probably at the P-site on the peptidyl transferase centre) [4], while the anti-codon part of the substrate interacts with the 30 S subunit [1–3]. Poly U hastens complex formation but it is not obligatory [2, 3]. The present paper shows that formation of the complex stabilizes Ac-Phe-tRNA against attack by hydroxylamine. Poly U-directed binding in absence of sparsomycin also stabilizes the substrate but to a lesser extent. Implications of these observations are discussed.

2. Materials and methods

2.1. *Materials*

Ribosomes and Ac-^{14}C-Phe-tRNA preparations were the same as in the accompanying paper [2]. Salt-free hydroxylamine was prepared from NH_2OH-HCl (Merck) as described by Davie [5], and was stored at $-20°$ after adjusting the pH value to about 7.2 with HCl.

2.2. *Assay of reaction with hydroxylamine*

The reaction of free Ac-^{14}C-Phe-tRNA with hydroxylamine was estimated from the fall in acid-precipitable radioactivity. tRNA (1 mg/ml) was included as carrier in incubations which did not contain ribosomes. At the indicated times, cold 5% trichloroacetic acid was added and, after 15 min at $0°$, the samples were filtered on glass fibre papers (Whatman GF-81B). The filters were washed with cold 5% trichloroacetic acid and with 1% acetic acid, then dried, immersed in scintillation fluid, and the radioactivity estimated in a scintillation spectrometer. Ac-Phe-tRNA was quantitatively recovered while Ac-Phe-hydroxamate was not retained by the filters.

The reaction of ribosome-bound Ac-^{14}C-Phe-tRNA with hydroxylamine was estimated from the fall in ribosome-bound radioactivity. The Ac-Phe-tRNA complexes were first formed by a preincubation, and then hydroxylamine was added and incubation continued. The ribosome-bound radioactivity was estimated by adsorption to Millipore filters and determinations of radioactivity as in the accompanying paper [2]. Ribosomes and ribosome-bound Ac-Phe-tRNA are quantitatively adsorbed by the filters, while free Ac-Phe-tRNA and Ac-Phe-hydroxamate are not retained.

3. Results

Effect of hydroxylamine on free and ribosome-bound Ac-Phe-tRNA were examined. In the latter case, the ribosome...Ac-Phe-tRNA complexes were formed by a 30 min preincubation (under the same conditions as in the accompanying paper [2]) before addition of hydroxylamine. Fig. 1 shows time courses for the reaction with 0.2 M hydroxylamine at $30°$,
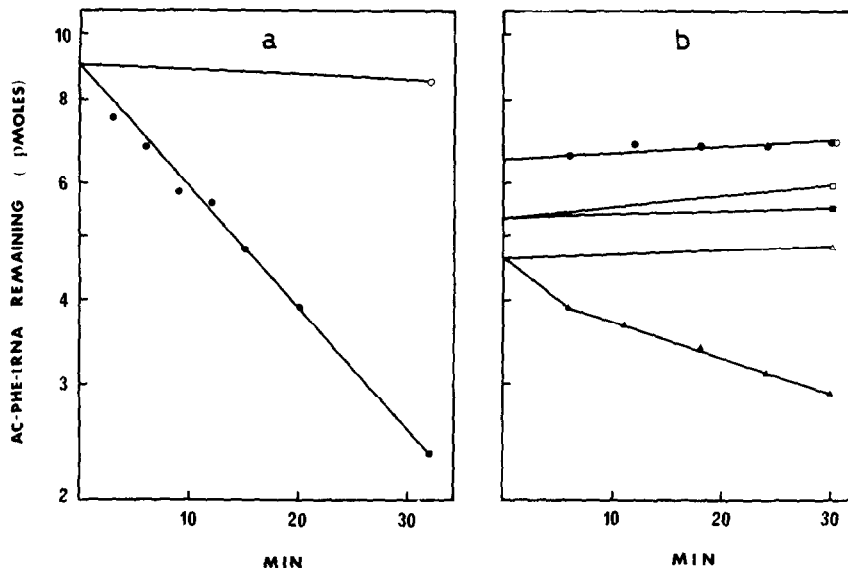
Fig. 1. Reaction of (a) free and (b) ribosome-bound Ac-Phe-tRNA with hydroxylamine. The incubation mixtures (0.1 ml/tube) contained 54 mM tris buffer (pH 7.4), 10 mM Mg acetate, 160 mM NH_4Cl, 10 mM dithiothreitol, and 9 pmoles Ac-^{14}C-Phe-tRNA (about 456 Ci/mole). The mix for b contained, in addition 1 mg/ml ribosomes plus 0.1 mM sparsomycin or 100 μg/ml poly U as indicated, and the ribosome-substrate complexes were first formed by preincubation for 30 min at 30°. The samples in a were pre-incubated for only 30 sec at 30°. The reaction was initiated by addition of 5 μl of salt-free, 4 M hydroxylamine (pH 7.2), and the incubation was continued at 30°. In controls, 5 μl water were added instead of hydroxylamine. The reactions of free Ac-Phe-tRNA (in a) or of ribosome-bound Ac-Phe-tRNA (in b) were estimated as described in Section 2.2. (a) $\circ$, no hydroxylamine; $\bullet$, plus hydroxylamine. (b) $\circ$ or $\bullet$, sparsomycin plus poly U; $\square$ or $\blacksquare$, sparsomycin; $\triangle$ or $\blacktriangle$, poly U. Open symbols, no hydroxylamine; closed symbols, plus hydroxylamine.

plotted on a semi-logarithmic scale. Free Ac-Phe-tRNA reacted rapidly, with a half time of about 16 min, while Ac-Phe-tRNA in the sparsomycin complexes did not react to a detectable extent in 30 min. In the samples with sparsomycin in absence of poly U, the increase of binding in controls without hydroxylamine was the result of complex formation continuing after the preincubation period [2]. The increase was partially inhibited by sparsomycin, presumably as a consequence of reaction with (and removal of) the free Ac-Phe-tRNA. Controls showed that the rate of hydroxylaminolysis was unaffected by sparsomycin or poly U in absence of ribosomes, or by ribosomes in absence of sparso-mycin and poly U. We conclude that Ac-Phe-tRNA in the sparsomycin complex is strongly protected against attack by hydroxylamine.

Fig. 1 also shows that Ac-Phe-tRNA is partially pro-tected when bound to ribosomes with poly U in *ab-sence* of sparsomycin. After the first 6 min, during

which a small more active fraction appears to have reacted, the rate corresponded to a half time of ap-proximately 60 min: i.e. about 27% of the rate with free Ac-Phe-tRNA. Other experiments showed, in agreement with Lucas-Lenard and Lipmann [6], that a high percentage of the bound Ac-Phe-tRNA reacted rapidly with puromycin, thus indicating that the sub-strate was associated with (or had easy access to) the P-site on the peptidyl transferase centre. We conclude that substrate in the P-site, while being highly suscep-tible to nucleophilic attack by specific peptidyl ac-ceptor substrates, may be inactivated for reaction with small, non-specific nucleophiles.

4. Discussion

Ribosomal peptidyl transferase catalyses the trans-fer of peptidyl groups from tRNA or fragments thereof

to peptidyl acceptor substrates ([7] : review) and also, in suitable conditions, to ethanol or methanol ([8] ; and unpublished results). Sparsomycin inhibits both these ribosome-catalysed reactions. The antibiotic induces interaction between the ribosome and the CCA-peptide part of the peptidyl donor substrate in such a manner that the catalytic mechanism cannot operate [4]. The present results show that substrate in the spasomycin complex is not only unable to undergo ribosome-catalysed reactions, but that it is also protected from general nucleophilic attack. It follows that the unreactivity of substrate in the sparsomycin complex is not solely due to blocking of the A-site by sparsomycin (there is some evidence that sparsomycin does block the A-site [9]), but that the peptidyl-adenosine part of the substrate must be intimately associated with the ribosome and/or sparsomycin in such a way as to protect it from nucleophilic attack.

The observation is of considerable interest, that Ac-Phe-tRNA is partially protected from hydroxylaminolysis, when bound to ribosomes in absence of sparsomycin, under the direction of poly U. This is to be distinguished from earlier observations (a) that non-acetylated Phe-tRNA is protected against hydroxylaminolysis [10] and hydrolysis [11] by binding to ribosomes, and (b) that a peptidyl-tRNA...protein complex isolated from ribosomes is less reactive towards hydroxylamine than free peptidyl-tRNA [12]. In the experiments with aminoacyl-tRNA the substrate was presumably bound mainly at the A-site, while in the experiments with peptidyl-tRNA the substrate was associated with proteins in an inactive form. In contrast, the present observations are concerned with substrate bound at the P-site in a form activated for reaction peptidyl acceptor substrates. The possibility

is intriguing that the 2'-hydroxyl group of the peptidyl acceptor substrate in the A-site takes a vital part in the catalysis of peptidyl transfer [13, 8]. It is particularly noteworthy that the transfer of f-Met from tRNA to ethanol is catalysed by ribosomes only in the presence of de-acylated tRNA or CCA [8]. It is quite possible that the reaction of ribosome-bound Ac-Phe-tRNA with hydroxylamine in the present system would have been stimulated rather than inhibited if de-acylated tRNA or CCA had been bound at the A-site.

Acknowledgements

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