Effect of codon shortening and the antibiotics viomycin and sparsomycin upon the behaviour of bound aminoacyl-tRNA

Decoding at the ribosomal A site

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70 S ribosomes were programmed with initiator tRNA and messenger oligonucleotides $AUG(U)_n$ and $AUG(C)_n$, where $n = 1, 2$ or 3. The binding of the ternary complexes [Phe-tRNA·EF-Tu·GTP] and [Pro-tRNA·EF-Tu·GTP] to the programmed ribosomes was studied. If codon–anticodon interaction is restricted to only one basepair, the ternary complex leaves the ribosome before GTP hydrolysis. Two basepairs allow hydrolysis of GTP, but the aminoacyl-tRNA dissociates and is recycled, resulting in wastage of GTP. Three basepairs result in apparently stable binding of aminoacyl-tRNA to the ribosome. The antibiotic sparsomycin weakens the binding by an amount roughly equivalent to one messenger base, while viomycin has the reverse effect.

Ribosome   A site   Translocation   Protein biosynthesis   Fidelity   Antibiotic

1. INTRODUCTION

In the elongation cycle of protein biosynthesis in procaryotes a ternary complex [aa-tRNA·EF-Tu·GTP] binds to the ribosome, GTP is hydrolysed and a new peptide bond is formed (review [1]). The rejection of some non-cognate aminoacyl(aa)-tRNA species has been shown to accompany a GTPase reaction [2] and this was interpreted in terms of a 'proof-reading' model proposed in [3]. The very high turnover of ternary complexes accompanying the 'erroneous' incorporation of non-cognate amino acids into growing polypeptide chains has also been shown to be consistent with the functioning of such a mechanism [4].

In the above, ribosomes were programmed with polyuridylic acid and the binding of different tRNA species was studied. Conversely, we have investigated the binding of a single tRNA species to

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ribosomes pre-programmed with initiator tRNA in the P site and a cognate codon in the A site. This has the advantage that the A site is exactly defined and that by shortening or altering the messenger, or by introducing antibiotics, the strength of the tRNA–ribosome interaction can be progressively altered in a controlled way.

2. EXPERIMENTAL

2.1. Enzymic binding of aa-tRNA to 70 S ribosomes

75 μl of a mixture containing 20 mM Tris–HCl (pH 7.5), 50 mM NH_4Cl, 10 mM 2-mercaptoethanol, 10 mM magnesium acetate, 25 pmol ribosomes [5], 1 nmol oligoribonucleotide messenger [6] and 50 pmol pure deacylated $tRNA_f^{Met}$ was pre-incubated for 2 min at 37°C and cooled on ice. For enzymic Phe-tRNA and Pro-tRNA binding the sample was then supplemented with 25 μl of a solution containing the same [salt]

as above, together with 25 pmol [^{3}H]Phe-tRNA^{Phe} (2 Ci/mmol) or 25 pmol [^{3}H]Pro-tRNA (10 Ci/mmol), 100 pmol EF-Tu and 1 nmol GTP. After timed incubation of the mixtures, ribosomal binding of aa-tRNA was measured by the Millipore filter technique [7].

2.2. GTP hydrolysis

Ternary complexes were allowed to react with oligoribonucleotide-programmed ribosomes as above; incubation mixtures contained in addition [γ-^{32}P]GTP (0.5 Ci/mmol), 2 mM phosphoenolpyruvate and 0.1 mg pyruvate kinase/ml. Reactions were stopped by adding 100 μl 1 M HClO$_4$ containing 1 mM KH$_2$PO$_4$. After 15 min centrifugation at 5000 rev./min, 300 μl of 20 mM Na-molybdate and 400 μl isopropyl acetate were added to 100 μl supernatant. After vigorous mixing for 30 s, low-speed centrifugation separated a clear, yellowish organic phase, an aliquot of which was pipetted onto a paper filter, dried and counted [8]. Each experiment was performed at least 4 times; the standard deviation was <5%. Mixtures containing AUG as messenger were run in parallel and their [γ-^{32}P]GTP hydrolysis, due to residual endogenous GTPase activities contaminating the EF-Tu and ribosome preparations, were subtracted from those of the corresponding complete mixtures.

3. RESULTS

3.1. Effect of codon shortening

70 S ribosomes were programmed by incubation with tRNA$_f^{Met}$ and messenger. At time zero the ternary complex and excess GTP were added. Fig.1A shows the Phe-tRNA binding and GTP hydrolysis induced by the messengers AUGUUU, AUGUU and AUGU. With AUGUUU the binding of Phe-tRNA reaches a plateau. Likewise the GTP hydrolysis stops, with a plateau level of about 2 GTP hydrolysed/Phe-tRNA bound. AUGUU, in contrast, induces very weak binding but much greater GTP consumption. Even after 10 min, no plateau has been reached. The ratio of GTP hydrolysed to Phe-tRNA bound is >200:1. AUGU results in an almost negligible degree of binding and a low but not negligible GTP turnover.
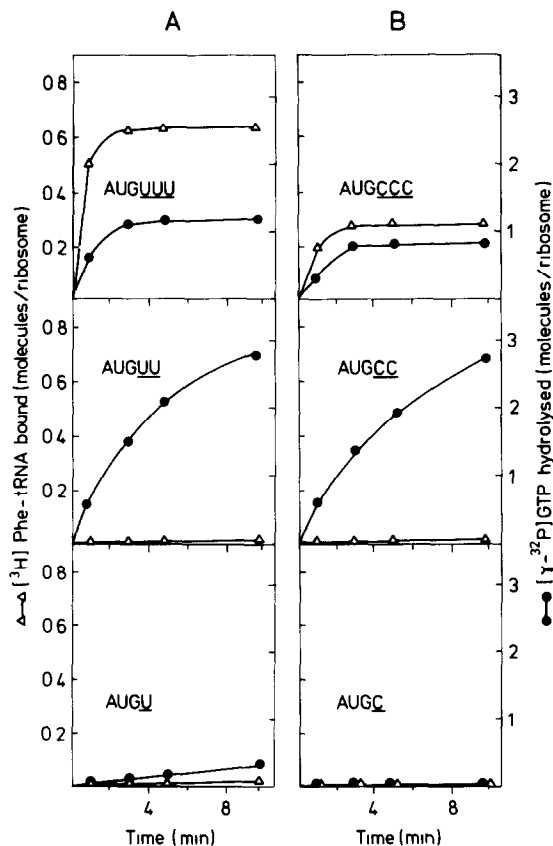

Fig.1. Reaction of ternary complexes with 70 S ribosomes programmed with oligonucleotides AUG(N)$_n$. Deacylated tRNA$_f^{Met}$ was present at the P site in each case: (A) reaction of [Phe-tRNA·EF-Tu·GTP] complexes with 70 S ribosomes and AUG(U)$_n$; (B) reaction of [Pro-tRNA·EF-Tu·GTP] complexes with 70 S ribosomes and AUG(C)$_n$.

For AUGCCC, AUGCC and AUGC the qualitative picture is the same (fig.1B); although the degrees of binding and hydrolysis by the hexanucleotide are somewhat lower, the phosphate turnover per Pro-tRNA bound is roughly the same. This suggests that fig.1 may represent a general behaviour pattern.

Peptide bond formation was also investigated with messengers of different lengths, as it is a criterion for 'correct' occupation of the A site by aa-tRNA [6,9]. In a typical experiment, 25 pmol ribosomes bound 17 pmol Phe-tRNA and produced 14 pmol dipeptide, while 32 pmol GTP were hydrolysed. Replacement of the AUGUUU messenger by AUGUU led to the binding of only

0.25 pmol Phe-tRNA and to the formation of 0.4 pmol dipeptide, while the GTP consumption rose to >75 pmol. The time course of the latter experiment is shown in fig.2: the dipeptide accumulates gradually while the degree of binding shows a plateau. For comparison, the time course of accumulation of dipeptide using AUGUUU as messenger was also determined. A plateau similar to that of the binding experiment (fig.1A, top) was reached.

These observations are explained qualitatively by the assertion that:

(i) The very weak interaction between the anticodon and the single-base codon does not result in any significant recognition;

(ii) The stronger interaction between the anticodon and the two-base codon leads to a degree of recognition sufficient to allow GTP hydrolysis but not sufficient to ensure the continued residence of the charged tRNA on the ribosome or the formation of a peptide bond, so that the charged tRNA is recycled; while

(iii) The strong interaction with the full triplet codon results not only in recognition of the ternary complex and GTP hydrolysis but also in firm binding of the tRNA in the A site.

Further evidence for the recycling of Phe-tRNA in the presence of AUGUU is provided by fig.2, in which the yield of bound Phe-tRNA quickly reaches a very low plateau while the dipeptide fMet-Phe continues to accumulate, even when the quantity of dipeptide formed exceeds that of bound Phe-tRNA.

In a further experiment, AUGUUU-programmed ribosomes were allowed to bind Phe-tRNA as in fig.1A (top) and, once the plateau had been reached, the GTP pool was suddenly depleted by the addition of a small quantity (10 units) of alkaline phosphatase. The bound Phe-tRNA dissociated slowly from the ribosomes, while in a control (no phosphatase added) it remained bound. This suggests that Phe-tRNA in the presence of AUGUUU may also be recycled, albeit very slowly.
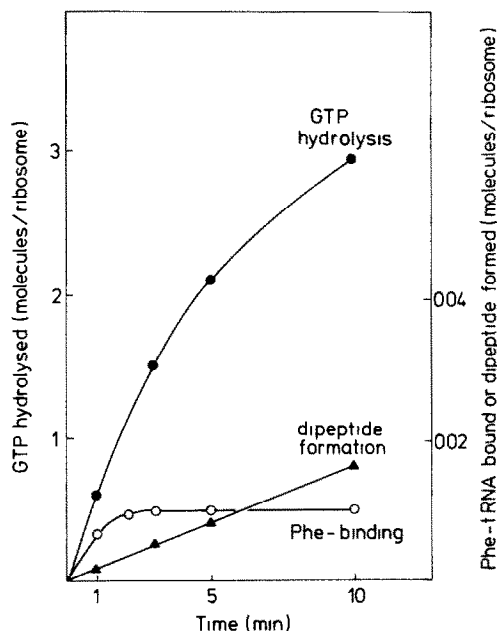
3.2. Effect of viomycin and sparsomycin

Viomycin has been shown to strengthen the binding of aa-tRNA to the A site [6]. The same effect is observed in the experimental system of fig.1, as shown in fig.3 for AUGUUU and AUGUU: while the strong binding of Phe-tRNA^{Phe} by AUGUUU is unaffected, the pattern for AUGUU with viomycin is the same as that for AUGUUU without viomycin. Thus the 'decrement' of binding energy caused by shortening the codon appears to be compensated for by an 'increment' of binding energy produced by the viomycin. Likewise, viomycin confers upon AUGUU the ability of AUGUUU to form dipeptide in high yield [6].

Sparsomycin, in contrast, is known to destabilise aa-tRNA in the A site [10]. This property is reflected in its effect upon the messenger AUGUUU (fig.3); in the presence of sparsomycin, the characteristic behaviour of the hexanucleotide AUGUUU (strong binding, low GTP turnover) changed in the direction of AUGUU (less efficient binding, higher GTP turnover). In contrast to this behaviour, sparsomycin had no apparent effect upon the A-site reaction when the messenger was

Fig.2. Time course of fMet-[³H]Phe dipeptide synthesis with AUGUU-programmed 70 S ribosomes. Phe-tRNA bound and P_i formed were determined as in section 2, except that the deacylated tRNA$_f^{Met}$ was replaced by 50 pmol non-radioactive fMet-tRNA$_f^{Met}$. Specific radioactivity of [³H]Phe-tRNA^{Phe} was 10 Ci/mmol. Dipeptide formation was assayed essentially as in [6]. Background values, measured in the presence of AUG only, were subtracted.
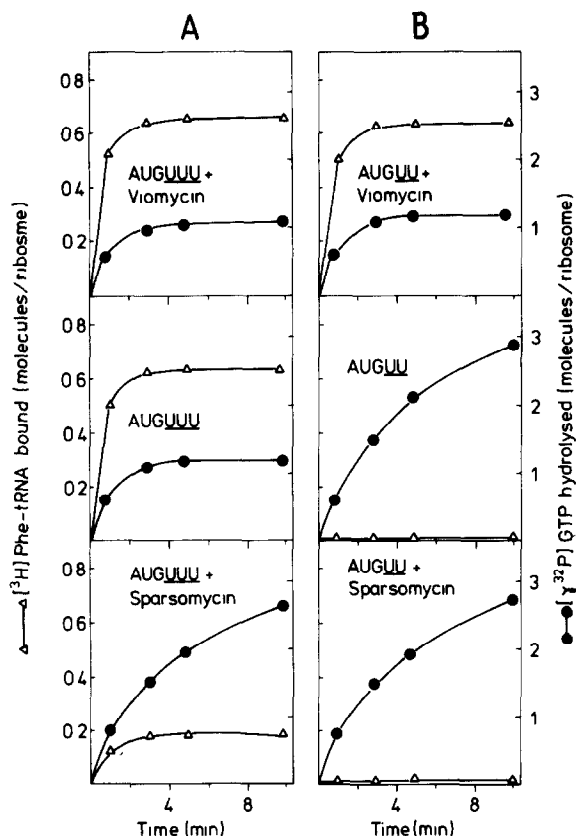
Fig.3. Reaction of Phe ternary complexes with AUGUUU- and AUGUU-programmed 70 S ribosomes in the presence of viomycin or of sparsomycin. Where indicated, 50 μM viomycin or sparsomycin was included in the reaction mixture.

AUGUU (fig.3B). Further experiments showed that dipeptide formation was in all cases completely abolished by sparsomycin at the concentration used in the binding tests of fig.3.

4. DISCUSSION

The A site was defined in all experiments by the prior binding of tRNA$_f^{Met}$ and messenger AUG(N)$_n$. Under the conditions used the complex [70S · tRNA$_f^{Met}$ · messenger] is formed stably and stoichiometrically, so that the binding of the oligonucleotide messenger is also guaranteed [6,11]. Therefore, the only variable is the length of the A-site codon. (Analogous experiments in which the identity of the triplet is varied are in progress and their results will be published in due course.)

Our interpretation in terms of progressively increasing binding energy can be made more general by the working hypothesis that the behaviour of a tRNA molecule on the ribosome is at each stage dependent upon the *total* free energy of all its interactions, at all points, with the programmed ribosome, and that positive and negative contributions to this, 'increments' of free energy, can be made by controlled stabilisation or destabilisation of the bound tRNA. In terms of the above discussion, the increasing codon length provides such incrementally increasing binding energy, and the interactions with viomycin and sparsomycin cause an increment and a decrement, respectively. A discontinuous message produces a similar decrement [6].

We note in this connection that sparsomycin has been shown, by oligonucleotide binding studies, to act on the part of the A site which accommodates the aminoacylated 3'-end of aa-tRNA [10]. It is consistent with this that the effect of sparsomycin is clearest when the incoming aa-tRNA is stably bound (by AUGUUU messenger; fig.3A) and is hardly noticeable when the aa-tRNA is largely lost (with AUGUU messenger; fig.3B). This finding supports our assertion that after hydrolysis of GTP a dinucleotide-programmed aa-tRNA leaves the ribosome instead of becoming properly accommodated in the A site, with its 3'-end in a suitable position for peptidyl transfer to take place.

The importance of considering not just the number of cognate basepair interactions or the number of hydrogen bonds but also such factors as stacking energy has already been stressed [12,13]; the results presented here illustrate this clearly for the case of AUG(C)$_n$ compared with AUG(U)$_n$. In [2], the reaction of non-cognate Leu-tRNA$_{II}^{Leu}$ with poly(U)-programmed ribosomes was examined. In this case the A-site anticodon GAG can form two basepairs with the codon UUU. Extensive GTP hydrolysis was seen. Furthermore, Leu-tRNA$_f^{Leu}$, whose anticodon CAG can form only one basepair, did not cause GTP hydrolysis. Both observations agree with our own (cf. fig.1) in respect of the effect of reducing the codon–anticodon complementarity step-by-step.

However, varying the messenger and keeping the tRNA species the same seems to represent a more rigorous approach than varying the tRNA species for a fixed messenger, since the overall energy of interaction between the aa-tRNA molecule and the

A site clearly depends upon further factors besides the codon–anticodon interaction. We therefore believe that our interpretation in terms of 'increments' of binding energy will prove a useful approach for unifying the effects of using different messengers, antibiotics, different tRNA species and interrupted messengers, each one of which may be formalised as incrementing or decrementing the (free) energy of binding of tRNA before and/or after the GTP hydrolysis step.

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