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Does the channel for nascent peptide exist inside the ribosome?

Immune electron microscopy study

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MS2 phage RNA-directed synthesis of an N-terminal polypeptide of the phage coat protein on *Escherichia coli* 70 S ribosomes was initiated in a cell-free system with the *N*-dinitrophenyl derivative of methionyl-$tRNA_F^{Met}$ and performed in the absence of tyrosine, lysine, cysteine and methionine. As a result, the translating ribosomes carried peptides up to 42 amino acid residues in length with the dinitrophenyl hapten at the N-ends. Using the immune electron microscopy technique the positions of the nascent peptide N-ends on the 70 S ribosomes have been visualized. It has been found that (i) the N-ends of nascent peptides of these lengths are accessible to antibodies, (ii) the exit site of a nascent peptide is the pocket between the base of the central protuberance and the L1 ridge on the 50 S subunit, i.e. presumably its peptidyl transferase center, and (iii) the further pathway of a nascent peptide seems to proceed along the groove on the external surface of the 50 S subunit.

Ribosome; MS2 phage; RNA; Nascent polypeptide; Immune electron microscopy

1. INTRODUCTION

Using the immune electron microscopy technique, Bernabeu et al. [1,2] were able to localize the site of a nascent protein on the 70 S and 80 S ribosomes; this site was found on the 50 S or 60 S subunit body, 150 ± 30 Å distant from the central protuberance and 60–70 Å from the subunit interface. Taking into account such a long distance of the site from the peptidyl transferase center (at the base of the central protuberance), as well as the position of the site, it was proposed that a long channel filled with a nascent peptide proceeds within the large ribosomal subunit and that the site discovered is that of the exit of the peptide outside.

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Indeed, the ribosome was reported to protect 30–40 C-terminal amino acid residues of a nascent peptide from proteases [3–5]; the length of such a section in the fully extended conformation would be about 150 Å [1]. Recently, an elongated zone of low electron density (a 'channel' of about 100–120 Å in length) inside the 50 S ribosomal subunit has been demonstrated by the application of a three-dimensional reconstruction procedure to electron microscopic images of the two-dimensionally ordered particles [6]; the relation of this zone to a nascent peptide, however, has not been established.

At the same time, there are several reports contradicting the hypothesis of such a long intraribosomal channel for a nascent peptide. A growing peptide 15–20 amino acid residues in length proved to be already attacked by the aminopeptidase cleaving off the N-terminal methionine residues [7–9]. The N-terminal acetyla-

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tion can proceed at a nascent peptide length of about 25 amino acid residues [10]. From theoretical considerations it is the α-helix that seems to be the most favorable conformation for a nascent peptide within the ribosome [11]; generally, an extended flexible peptide chain conformation can scarcely be imagined to be pushed through the ribosome during elongation.

From all this evidence we have concluded that the question of the site of exiting of the nascent peptide on the ribosome requires further investigation. In particular, it is important to localize not only an entire synthesized protein on the ribosome surface, as has been done previously [1,2], but specifically the N-terminus of a nascent peptide, the shortest possible. Here, we report the first immune electron microscopy experiments on the localization of the hapten-labeled N-terminal methionine of MS2 coat polypeptide grown as far as the 42nd amino acid residue.

2. MATERIALS AND METHODS

[^{14}C]Methionine (60 mCi/mmol) and [^{3}H]arginine (28 Ci/mmol) were from Amersham. Anti-dinitrophenyl antibodies (anti-DNP) were purchased from Miles. Total tRNA, $tRNA_F^{Met}$ from *E. coli* and phage MS2 were obtained from Biolar (USSR). MS2 RNA was prepared by phenol deproteinization of phage MS2 in the presence of 1% SDS. 70 S ribosomes were isolated from *E. coli* MRE 600 cells and washed with 0.5 M NH_4Cl.

[^{14}C]Met-$tRNA_F^{Met}$ was prepared by enzymatic charging of $tRNA_F^{Met}$ with [^{14}C]methionine using *E. coli* S100 protein extract freed from ribosomes and tRNA. It was then treated with the *N*-hydroxysuccinimide ester of 2,4-dinitrophenyl to produce the *N*-substituted derivative (DNP-[^{14}C]Met-$tRNA_F^{Met}$).

The initiation complex (70 S·MS2 RNA·DNP-[^{14}C]Met-$tRNA_F^{Met}$) was formed by mixing 1 nmol 70 S ribosomes, 1 nmol MS2 RNA, 1 nmol DNP-[^{14}C]Met-$tRNA_F^{Met}$, 2 mg S100 protein and 0.1 μmol GTP in 1 ml of 20 mM Tris-HCl, pH 7.4 at 37°C, with 7 mM $MgCl_2$, 100 mM NH_4Cl, 1 mM β-mercaptoethanol, 0.1 mM EDTA, and was incubated for 30 min at 37°C.

To perform further translation 1 ml of the suspension of the initiation complex was mixed with 1 ml of a solution containing amino acids (10 nmol each) except Arg, Tyr, Lys, Cys and Met, 2 mg tRNA, 2 μmol ATP, 0.1 μmol GTP, 6 μmol phospho*enol*pyruvate, 10 μg pyruvate kinase, 5 nmol [^{3}H]Arg in 20 mM Tris-HCl, pH 7.4 at 37°C, with 13 mM $MgCl_2$, 100 mM NH_4Cl, 1 mM β-mercaptoethanol, 0.1 mM EDTA and was incubated for 20 min at 37°C. The radioactive polypeptides synthesized were analyzed by gel electrophoresis in SDS [12].

The initiation complexes or the translating ribosomes were purified by centrifugation in a 5–30% sucrose gradient prepared in 20 mM Tris-HCl, pH 7.4 at 37°C, with 10 mM $MgCl_2$, 100 mM NH_4Cl, 1 mM β-mercaptoethanol, 0.1 mM EDTA (standard buffer). Ribosomes were precipitated using 10% (w/v) polyethylene glycol 6000 and suspended in standard buffer adjusting the ribosomal concentration to about 5 mg/ml.

The immune reaction was performed in standard buffer by adding 19 μg anti-DNP to the ribosome suspension containing 1 pmol DNP-peptidyl-tRNA. After incubation for 10 min at 37°C the mixture was cooled and centrifuged through a sucrose gradient (5–30%) in standard buffer using a Beckman SW41 rotor (19000 rpm, 15 h, 4°C). The monomer and dimer fractions were dialyzed vs the standard buffer, negatively stained with uranyl acetate using the single-layer carbon technique [13] and examined under a JEM-100C electron microscope.

3. RESULTS

Here, the initiator formylmethionyl-$tRNA_F^{Met}$ (F-Met-$tRNA_F^{Met}$) was replaced by its analogue DNP-Met-$tRNA_F^{Met}$ in the cell-free system of MS2 phage RNA translation. Incorporation of DNP-Met-$tRNA_F^{Met}$ into the N-terminal position of peptides was shown to be as effective as in the case of the natural initiator F-Met-$tRNA_F^{Met}$. In both cases the fraction of active (peptide-synthesizing) ribosomes was about 20% of the total 70 S particles. The only protein synthesized at the beginning was the MS2 coat protein.

In order to stop the elongation of MS2 coat protein at a definite point, some amino acids were omitted from the cell-free translation system. In the case of omission of Tyr, Lys and Cys, the polypeptide was unable to grow beyond a length of

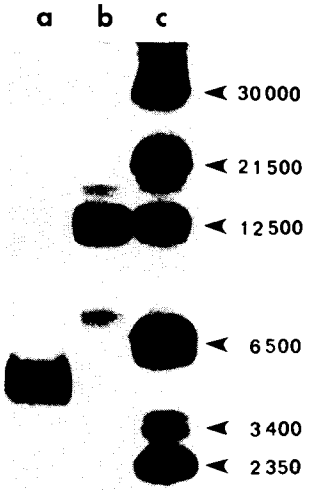

Fig.1. Fluorogram of the electrophoretic distribution of [^{3}H]Arg-labeled peptides from the translation system with the omission of four amino acids, namely Tyr, Lys, Cys and Met (a), and containing the full set of amino acids (b); the standard set of ^{14}C-labeled peptides from Amersham is also given (c).

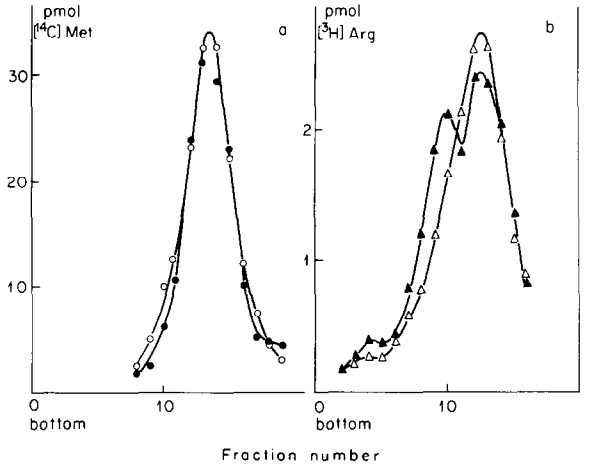

Fig.2. Sedimentation of the initiation complex 70 S·MS2 RNA·DNP-[^{14}C]Met-tRNA$_F^{Met}$ before (○—○) and after (●—●) interaction with anti-DNP antibodies (a), and translating 70 S ribosomes carrying DNP-modified [^{3}H]Arg-labeled peptide before (△—△) and after (▲—▲) interaction with anti-DNP antibodies (b).

42 amino acid residues. Fig.1 shows a fluorogram of the electrophoretic distribution of [^{3}H]Arg-labeled peptides from systems without the four amino acids Tyr, Lys, Cys and Met (a) and with the full set of amino acids (b, control). It can be seen that peptide elongation has in fact been stopped at the proper length. Since Arg as the only significantly labeled amino acid residue (the radioactivity of the terminal DNP-[^{14}C]Met is comparatively negligible) is met for the first time in position 39 of the MS2 coat protein, peptides of shorter lengths may also be present on ribosomes but remain invisible in the fluorogram.

Fig.2 demonstrates that the translating 70 S ribosomes carrying the DNP-labeled peptides form dimers as a result of the interaction with anti-DNP (b). Ribosomes carrying non-elongated DNP-Met-tRNA$_F^{Met}$ are not capable of forming dimers (although, as will be shown below, they also interact with anti-DNP) (a).

Representative electron microscopic images of dimers formed by DNP-labeled peptide-carrying 70 S ribosomes in the presence of anti-DNP are depicted in fig.3. Fig.3A shows examples of particles where the antibodies interact with a site close to that identified earlier as being the exit site of a nascent peptide [1,2]. The images are also seen where the site of the ribosome-antibody interaction is somewhat shifted towards the central protuberance of the 50 S subunit. Unfortunately, only the non-overlap projections of ribosomes show this localization of the antibody, so that the precise position of the 'exit site' on the three-dimensional ribosome model cannot be determined.

In fig.3B particles are displayed where the anti-DNP antibodies interact in an area much closer to the central protuberance. Since both overlap and non-overlap projections of the 70 S ribosomes are found here, the site of antibody interaction can be definitely localized about the base of the central protuberance and the L1 ridge of the 50 S subunit.

Fig.3C shows the monomeric complexes of anti-DNP antibodies with non-translating ribosomes charged with MS2 RNA and DNP-Met-tRNA$_F^{Met}$ (dimers are not found in this case). In this non-overlap projection the antibodies are seen on the 30 S subunit side, in the region of the groove be-

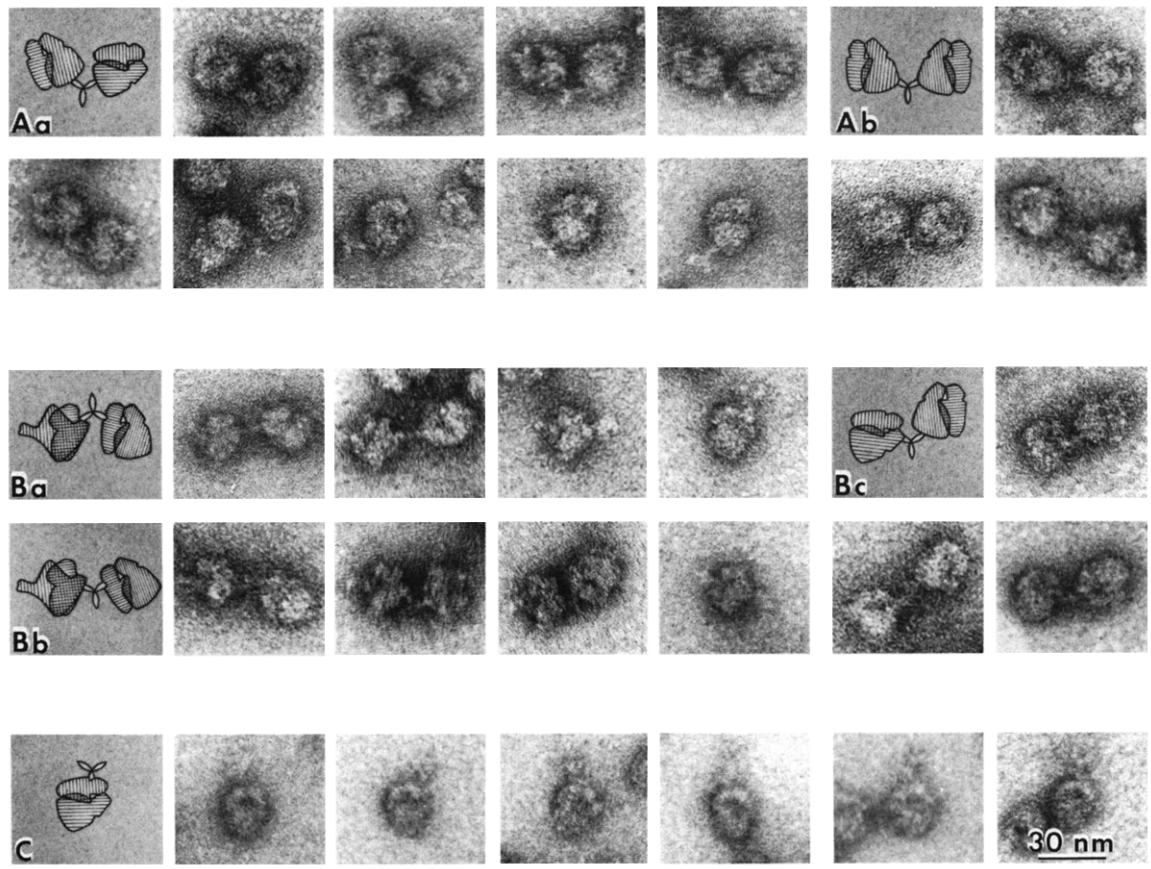

Fig.3. Electron microscopy images of translating DNP-labeled peptide-carrying 70 S ribosomes and the initiation 70 S·MS2 RNA·DNP-Met-tRNA$_F^{Met}$ complexes treated with anti-DNP antibodies. Interpretative drawings of the observed image types are given in the left-hand frames. (A) Antibody-linked ribosome dimers and single immunocomplexes showing the binding of antibodies near the 'exit site' [1,2]. The antibody-binding site is either in the exit site (a) or in the 'apex' of the 50 S subunit, i.e. shifted towards its central protuberance (b). Only non-overlap views of the 70 S ribosome are observed. A total of 100 immunocomplexes have been analyzed. (B) Dimers and single immunocomplexes showing the binding of antibodies in the region between the central protuberance and L1 ridge of the 50 S subunit. Both overlap and non-overlap projections of 70 S ribosomes are observed. In the non-overlap projection the antibody-binding site is either in the region of the groove between the head and body of the 30 S subunit (a,b) or of the central protuberance of the 50 S subunit (c). In the overlap projection the antibody-binding site is located in the region between the central protuberance and the L1 ridge of the 50 S subunit (a) or is shifted somewhat towards its notch [23] (b). A total of 130 immunocomplexes of such a type have been analyzed. (C) Single 70 S ribosome·MS2 RNA·DNP-Met-tRNA$_F^{Met}$·antibody complexes. Ribosomes are in the non-overlap projection only. No dimers were observed.

tween the head and body. The interacting Fab arm appears to be overlapped, partly or completely, with the ribosome. Although the other projection necessary for the unambiguous localization of the antibody-binding site was not found, it seems that the antibody is attached in the region of the base of the central protuberance or between it and the L1 ridge on the 50 S subunit. The absence of dimers suggests, rather, that the hapten is in a groove or cavity of the 70 S ribosome which produces steric hindrance to dimerization.

The results of the localization of the nascent peptide (its N-end) on the 70 S ribosome presented in fig.3A and B, are summarized in fig.4.

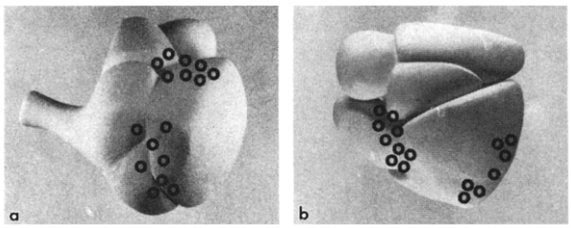

Fig.4. Localization of the N-end of the nascent peptide of the 70 S ribosome. The antibody-binding sites which are observed in the micrographs in fig.3 are denoted by open circles in the two views of the 70 S ribosome model [24].

4. DISCUSSION

It is obvious that the growth of a peptide on the ribosome commences in the peptidyl transferase center. The first amino acid residue to become settled in the peptidyl transferase center is the initiator methionine. Fig.3C demonstrates that this methionine (or, more precisely, the DNP label on the amino group of the methionine) is accessible to antibodies when the initiation ribosome·MS2 RNA·DNP-Met-$tRNA_F^{Met}$ complex is formed. The anti-DNP antibodies in fig.3C seem to indicate the site of the peptidyl transferase on the non-overlap projections of 70 S ribosomes. This appears to be the pocket between the central protuberance and the L1 ridge of the 50 S subunit (see section 3). This location of the peptidyl transferase site coincides with that previously suggested by others [14–21].

Since the initiation ribosome complexes are incapable of forming dimers through anti-DNP antibodies (see fig.3C), all dimers formed from ribosomes of the translation system (fig.3A,B) seem to reflect interaction of the antibodies with the nascent peptides. Hence, the DNP-labeled N-ends of nascent peptides up to 42 amino acid residues in length are exposed on the ribosome surface for the interaction with antibodies. This observation contradicts the concept of an intraribosomal tunnel containing a nascent peptide.

It can be seen from fig.4 that the position of the nascent peptide N-end on the ribosome is not unique but, rather, a particular distribution of antibody-binding sites along the external surface of the 50 S subunit is observed. It is noteworthy that all the antibody-binding sites are concentrated along the groove starting from the pocket between the central protuberance and the L1 ridge and continuing to the external surface of the 50 S subunit towards the opposite notch [22,23]. Taking into account the possible variation of nascent peptide lengths from 2 to 42 amino acid residues, it can be supposed that the pathway of the peptide during its growth is revealed in the present experiments. The growing peptide (its N-end) goes from the peptidyl transferase center through the pocket between the central protuberance and the L1 ridge along the groove on the external surface of the 50 S subunit downwards to the notch. All the way, except for an intermediate section of the groove, it is accessible, at least partly, to the surrounding medium. The so-called exit site localized by Lake and co-workers [1,2] is the extreme point which is reached by a nascent peptide.

In fact Lake et al. [1,2] observed not the exit of a peptide but the formation of antigenic determinants of such large proteins as β-galactosidase [1] and the small subunit of ribulose-1,5-bisphosphate carboxylase [2] on the ribosome. It is likely that they discovered the protein folding site. The exit site of a nascent peptide appears to be the peptidyl transferase center.

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