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Nascent chains: folding and chaperone interaction during elongation on ribosomes

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SUMMARY

Monoclonal antibodies that detect folding intermediates *in vitro* were used to monitor the appearance of folded polypeptide chains during their synthesis on the ribosomes. Nascent immunoreactive chains of the bacteriophage P22 tail-spike protein and of the *Escherichia coli* β_2 subunit of tryptophan-synthase were thus identified, suggesting that they can fold on the ribosomes. Moreover, the immunoreactivity of ribosome-bound tryptophan-synthase β-chains of intermediate lengths was shown to appear with no detectable delay compared to their synthesis. This suggested that β-chains start folding during their elongation on the ribosomes.

However, newly synthesized incomplete β-chains were shown to interact with chaperones while still bound to the ribosome. Because of the peculiar properties of the epitope recognized by the anti-tryptophan-synthase monoclonal antibody used, it could not be concluded whether the immunoreactivity of the nascent β-chains resulted from their ability to fold cotranslationally or from their association with chaperones which might maintain them in an unfolded, immunoreactive state.

1. INTRODUCTION

Our current understanding of the mechanisms by which a protein acquires its native conformation is based on *in vitro* studies of the folding of pure, complete polypeptide chains. *In vivo*, the polypeptide chain grows progressively, starting at its N-terminal end (attached to the ribosome) which is in contact with the complex intracellular medium. Whether or not the observations made *in vitro* are relevant to the process that occurs *in vivo* is still poorly documented, essentially because of the considerable difficulties encountered when characterizing the conformation of nascent chains. This problem is further complicated by the fact that these chains can only be produced in very small quantities and in the presence of numerous other cellular components. It seemed possible to us that these difficulties could be overcome by taking advantage of the remarkable specificity of antibodies and the high sensitivity of immunodetection methods. We therefore used monoclonal antibodies (MAbs) to characterize the conformation of ribosome-bound nascent chains from the bacteriophage P22 tailspike protein and of the β_2 subunit of *E. coli* tryptophane synthase.

Each of these antibodies was shown, through *in vitro* refolding experiments, to recognize an epitope carried by the corresponding polypeptide chains only if they have undergone at least some folding steps (Murry-Brelier & Goldberg 1988; Blond-Elguindi & Goldberg 1990; Friguet *et al.* 1994). Attempts were made to use these antibodies for detecting the presence of folded, ribosome-bound nascent chains during biosynthesis of P22 tailspike protein and of tryptophan-synthase β-chains. Here we describe the results that were obtained by using this approach, the experimental difficulties that were overcome and the uncertainties that remain in their interpretation.

2. EVIDENCE FOR THE EXISTENCE OF FOLDED RIBOSOME-BOUND P22 TAILSPIKE NASCENT CHAINS

Seckler *et al.* (1989) identified several intermediates during the *in vitro* folding of the guanidine unfolded tailspike protein of bacteriophage P22 and characterized the kinetics of their appearance. These intermediates closely resemble those identified during the *in vivo* maturation of the tailspike (Goldenberg & King 1982; Haase-Pettingell & King 1988). We prepared and characterized a panel of mAbs which recognize the native tailspike protein (Friguet *et al.* 1990) and used them to monitor the kinetics of regain of immunoreactivity during the refolding of the tailspike protein (Friguet *et al.* 1994). Three mAbs were thus found to

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detect an antigenic determinant, different for each mAb, that reappears with kinetics corresponding to a specific folding step as identified by Seckler *et al.* (1989). The antigenic site recognized by mAb 236-3 is formed simultaneously with a folded monomer competent for protrimer formation; mAb 155-3 recognizes the protein from the moment it forms a heat labile protrimer; mAb 33-2 recognizes only the heat-stable native trimer (Friguet *et al.* 1994). Because these three antibodies could recognize three intermediates at different stages of the folding pathway, we used them to investigate whether such folded intermediates were present on ribosome-bound nascent chains. Cells infected with deficient phages and expressing high levels of the tailspike protein were lysed, the debris removed by centrifugation, and the ribosomal fraction obtained by centrifugation on a sucrose gradient. To test for the presence of ribosome-bound immunoreactive nascent chains, the ribosomal fraction was immunoprecipitated with each of the mAbs and assayed for the presence of ribosomal proteins in the immunoprecipitate: this was done by the following method.

The ribosomal fraction, prepared as outlined above, was incubated with the various mAbs and subjected to immunoadsorption on Sepharose beads coupled to Protein G. After low speed centrifugation to pellet the beads, the precipitate was washed several times. The immunoadsorbed proteins were dissolved in sodium dodecyl-sulphate (SDS) and submitted to SDS-polyacylamide gel electrophoresis (SDS-PAGE) and Western blotting. The blot was developed with a rabbit immune serum directed against the ribosomal protein L20 and with anti-rabbit immunoglobulins coupled to alkaline phosphatase. When mAb 236-3 was used, a band was clearly visible at a position corresponding to approximately 13.5 kDa, the molecular mass of the L20 protein. This band could not be detected when the anti-tailspike mAb 155-3 or when a control (non-specific) mAb were used. This unambiguously showed that some ribosome-bound chains can indeed react with mAb 236-3, and are therefore likely to have undergone the folding steps that generate the epitope recognized by this mAb.

These experiments were only preliminary, however, and provided no information on the nature (in particular on the length and amount) of the immunoreactive chains. Nor did they provide any information on the conformation of the nascent chains as no attempt was made to investigate the affinity of the mAb for the antigenic structure they carry. To reach a better understanding of the nature and conformation of the ribosome-bound immunoreactive chains quantitative experiments were therefore necessary. This was carried out using a different protein/antibody system, investigated in our laboratory for several years, which seemed better adapted to such studies.

3. QUANTITATIVE ANALYSIS OF RIBOSOME-BOUND IMMUNOREACTIVE TRYPTOPHANE-SYNTHASE β-CHAINS

Extensive investigation of β-chain immunoreactivity during refolding *in vitro* showed that the monoclonal antibody mAb 19 was able to react with the refolding chains only after they had undergone some early folding steps (Murry-Brelier & Goldberg 1988; Blond-Elguindi & Goldberg 1990). Using this antibody, we investigated the immunoreactivity of ribosome-bound nascent β-chains obtained *in vitro* in a cell-free protein biosynthesis system; the general strategy was as follows (Fedorov *et al.* 1992).

Protein synthesis was achieved using either a mRNA carrying the entire *trpB* gene or a truncated mRNA that terminates, without a stop codon, at position 309 of that gene. ^{35}S-methionine was used to radiolabel the newly synthesized polypeptide chains. The ribosomal fraction was purified by centrifugation on a sucrose or glycerol gradient, and was incubated with Sepharose beads coupled to mAb19. The beads were pelleted by low speed centrifugation and washed; the immunoadsorbed material was submitted to SDS-PAGE. Autoradiography revealed the presence of radioactive material specifically immunoadsorbed by mAb 19. The shortest nascent chains that could be seen on the gel had a molecular mass of approximately 11.5 kDa. Moreover, using a newly developed radioimmunoassay (RIA) based method (Friguet *et al.* 1993), the affinity of the ribosome-bound 11.5 kDa chains for mAb 19 could be measured. It was also found to be similar to that of the native protein for mAb19. It was therefore concluded that ribosome-bound polypeptides can fold, even before the synthesis of the complete N-terminal domain of the β-chain, into a conformation that already exhibits some local structural features of the native protein and of folding intermediates observed *in vitro* (Fedorov *et al.* 1992).

4. QUANTITATIVE PULSED IMMUNOLABELLING OF GROWING POLYPEPTIDE CHAINS

In the previously described experiments, the conformation of the nascent chains was probed with the mAb well after the arrest of their synthesis. It was, therefore, impossible to ascertain whether the folded intermediate appeared rapidly while the chains were growing, or slowly after their synthesis. To overcome this difficulty, we developed a procedure to pulse-label the protein rapidly with mAb 19 during its synthesis, and quantitatively analyse the labelled, ribosome-bound, nascent chains.

While developing this procedure, several unexpected observations were made; since they had to be accounted for while setting up a correct pulsed immunolabelling protocol, we shall discuss them here. ^{35}S-Methionine labelled nascent chains were produced *in vitro* with a wheat germ translation system using a truncated mRNA that terminates, without a stop codon, at position 1104 of the *trpB* gene. This mRNA encodes the 368 N-terminal residues of the β-chain.

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The absence of a stop codon at its end was supposed to ensure a stable association of these chains with the ribosomes and one therefore expected the accumulation of nascent chains of about 40 kDa which should not be released from the ribosomes. To test this prediction, the synthesis mixture was submitted to SDS-PAGE, followed by scanning the ^{35}S radioactivity in the gels with a recently developed bi-dimensional radioactivity scanner that detects the weak electrons emitted by ^{35}S or ^{14}C with very high sensitivity and very low background (β-Imager: Biospace Instruments, Thoiry, France). The results of such scans showed the accumulation of chains of the predicted length (approximately 40 kDa), but also the accumulation of shorter chains that gave rise to a distinct band pattern on the gels. Such bands are usually interpreted as reflecting transient intermediates that correspond to translation pauses at specific sites, often thought to be rare codons. The persistence of radioactive chains of intermediate lengths after a chase experiment, the demonstration that no post-translational degradation of the polypeptide chains had occurred and a quantitative analysis of the kinetics of accumulation of these bands and of the full length products allowed us to rule out this generally accepted interpretation unambiguously. It was then demonstrated that the bands of intermediate lengths in fact correspond to abortive translation products resulting from degradation of the mRNA at preferential sites.

To verify the prediction that the absence of a stop codon at the end of the mRNA should prevent the release of the nascent chains from the ribosomes, the synthesis mixture was submitted to ultracentrifugation through a glycerol cushion (Fedorov *et al.* 1992), and the resulting pellet (ribosomal fraction) and supernatant (free chains) were submitted to SDS-PAGE. Quantitative scanning of the gels with the β-Imager showed that only about half of the chains remained attached to the ribosomes, whereas the other half was released in the solution. An even lower proportion of the nascent chains (about 30%) was found bound to the ribosomal fraction when an *E. coli* translation system was used in similar experiments. This demonstrated that the commonly used method to produce ribosome-bound nascent chains, i.e. the use of a mRNA devoid of a stop codon, does not ascertain that all nascent chains remain associated to the ribosome.

To eliminate the free chains when probing the immunoreactivity of ribosome-bound nascent chains, a new separation method, rapid and compatible with the small sample volumes used (40 μl) had therefore to be devised. This was achieved by centrifugation of the sample of synthesis mixture through a 100 μl cushion of 40% glycerol in buffer A (20 mM Hepes pH 7.6, 100 mM K-acetate, 10 mM Mg-acetate) in an air-driven bench centrifuge (Airfuge, Beckman) kept at 4 °C in the cold room. The air pressure was adjusted at 30 psi, which corresponded to 149000 *g* at the bottom of the centrifugation tube: 30 min of centrifugation was deemed sufficient to pellet all the ribosomal fraction and leave the free chains in solution. It was then observed that the chains thus obtained in the ribosomal fraction remain bound to the ribosomes after further incubation at 4 °C. This separation protocol, which will probably become of general use in studies on nascent proteins, was used throughout the work that is now described.

A pulse-immunolabelling protocol, compatible with the additional constraints imposed by the separation procedure just described, was set up. Aliquots of the translation mixture were supplemented with mAb 19 and incubated for 30 s. To prevent subsequent association of the antibody with nascent chains not yet folded at the time of the 30 s pulse but that would fold at a later stage, an excess of pure native non radioactive β_2 was quickly added to block the unreacted mAb. The sample was incubated with gentle shaking for 30 s, and the temperature kept at 4 °C to minimize dissociation of bound mAb 19. Immunolabelled chains were then precipitated with protein G-Sepharose beads, solubilized, submitted to SDS-PAGE and analyzed quantitatively with the β-Imager. The specificity of the immunoprecipitation had been demonstrated earlier, using a non specific mAb instead of mAb 19 (Fedorov *et al.* 1992); its linearity was verified by immunoprecipitation and quantification of the nascent chains contained in aliquots of different volumes of an arrested synthesis mixture. It was observed that, under these pulse and immunoprecipitation conditions, the fraction of chains immunoprecipitated was indeed constant (9 ± 2%, determined from four independent experiments).

This pulse-labelling and immunoprecipitation protocol was applied to aliquots of a synthesis mixture at different times after the start of synthesis. The results of such experiments are shown in figure 1*a*. Visual inspection of the screen image suggested that the appearance of immunoprecipitated (i.e. folded) chains strictly paralleled protein synthesis; a quantitative analysis of the radioactivity contained in each band was easily made using the β-Imager software. Such analysis provided the kinetics of appearance of newly synthesized (folded or not folded) chains of a given length and thus, chains of about 11, 30 and 40 kDa were shown to appear linearly with time (i.e. each at a constant rate) after a lag that increased with the chain length (see figure 1*b*). For a given chain length, this lag probably represents the time it takes for a ribosome to progress on the mRNA from the point of initiation of translation to the codon corresponding to the end of the polypeptide chain.

The kinetics of accumulation of all the ribosome-bound chains of a given size, whether immunoreactive or not, were then compared with the kinetics of accumulation of only those ribosome-bound chains of that size that were immunoreactive at the time of the pulse. These kinetics could not be distinguished from one another within the experimental conditions. The radioactivity detected in each band after pulse-labelling and immunoprecipitation represented in all cases about 10% of the total radioactivity initially contained in that band (i.e. before immunoprecipitation). This value was close to that obtained when testing the linearity and efficiency of the immunolabelling/precipitation protocol, well after the arrest of synthesis.

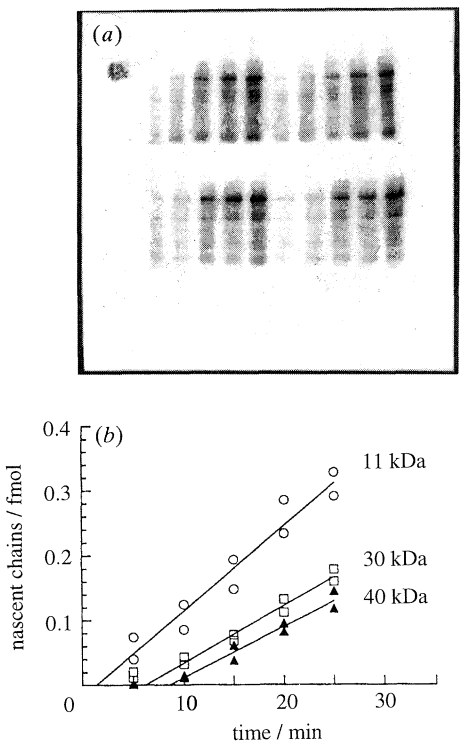

Figure 1. Kinetics of appearance of ribosome-bound ^{35}S-methionine labelled chains. Protein synthesis was performed in a wheat germ translation system, using as a template a mRNA that ends, without a stop codon, at base 1104 of the *trpB* gene. Aliquots were withdrawn after various times and synthesis was arrested by dilution with cold buffer. The ribosomal fraction was separated by Airfuge centrifugation through a glycerol gradient (see text). The pellet was resuspended in sample SDS buffer, heated, and each sample was subjected to SDS-PAGE. (*a*) Screen image of the radioactivity distribution in the gel for the chains present before (top) or after (bottom) immunoprecipitation. Samples used to measure the total synthesis before immunoprecipitation (15 µl, top half) correspond to 1/10 of those used for the immunoprecipitation (bottom half). Assays were performed in duplicate, after 5 min (lanes 1, 6), 10 min (lanes 2, 7), 15 min (lanes 3, 8), 20 min (lanes 4, 9), and 25 min (lanes 5, 10) of incubation of the translation mixture at 25 °C, and were repeated in three independent experiments. A ^{14}C sample of known radioactivity (500 d.p.m.) served as an internal standard (top left hand corner). (*b*) In each migration lane (i.e. for each incubation time) the radioactivity contained in the region corresponding to polypeptide chains of about 11, 30 and 40 kDa was determined by means of the β-Imager software and was converted into fmoles of the corresponding polypeptide chains, taking into account the internal standard, the specific radioactivity of the ^{35}S-methionine used, and the number of Met residues present in each fragment. Based on the sequence of the *E. coli trpB* gene, there are 15, 10 and 4 Met residues in the 40, 30 and 11 kDa fragments respectively. The lags observed for the biosynthesis of the β-chain fragments were 1.5 min (11 kDa fragment, open circles), 6 min (30 kDa fragment, open squares), and 8.5 min (40 kDa fragment, filled triangles). Data were fitted by least squares linear regression, taking into consideration measurements done after 10 min for the 30 and 40 kDa fragments, and including those for 5 min for the 11 kDa fragment.

This result suggested that the antigenic site recognized by mAb 19 was completely immunoreactive at the moment of the 30 s immunopulse; moreover, the lags observed for the accumulation of total and immunoreactive 40 kDa ribosome-bound chains were very similar, the difference not exceeding the 30 s duration of the pulse. This observation strongly supported the conclusion that the appearance of the antigenic site on the 40 kDa nascent chains was completed at the time of the pulse. The same conclusion could be reached with the 11 and 30 kDa fragments which also showed a linear accumulation of immunoreactive ribosome-bound chains with a lag not significantly different from that observed for the synthesis. Because, under the experimental conditions we used, it took the ribosome about five minutes to elongate a nascent chain from 11–40 kDa (as judged from the lags observed in figure 1*b*), these results unambiguously showed that the antigenic site recognized by mAb 19 appears cotranslationally.

Because studies on the refolding *in vitro* of unfolded β-chains had demonstrated that the appearance of immunoreactivity requires some early folding steps, one was tempted to conclude that these pulsed immunolabelling experiments solidly demonstrated that nascent β-chains undergo these folding steps cotranslationally. A detailed characterization of the antigenic site showed that such a conclusion would have been premature.

5. CHARACTERIZATION OF THE EPITOPE RECOGNIZED BY ANTIBODY 19

To understand the nature of the early folding steps detected *in vitro* by mAb19, it seemed desirable to define as precisely as possible the residues involved in the corresponding antigenic determinant. This was done by a variety of techniques including DNA sequencing of an epitope library, enzymatic and chemical cleavage of β_2 at specific sites, and chemical peptide synthesis. The epitope recognized by mAb19 has thus been localized in the aminoacid sequence 2–9 of the β-chain. The affinities of mAb 19 for several synthetic peptides of different lengths containing the 2–9 sequence have been determined and were all close to 10^9 M^{-1}, the affinity of mAb19 for the native β_2 protein. Because an isolated peptide of ten residues is very unlikely to adopt a prefered rigid conformation, this suggested mAb 19 may recognize the octapeptide 2–9 even in a flexible disordered state. To account for the fact that mAb19 fails to react with β-chains before they have undergone some folding steps, we propose that, at the very beginning of the *in vitro* folding process, the antigenic N-terminal end of the polypeptide chain (which turns out to be very hydrophobic) would get very rapidly, but transiently buried in the hydrophobic interior of a molten globule. At a later stage of the folding process, upon compaction of the molten globule, the 2–9 sequence would be expelled towards the solvent and only then would its immunoreactivity to mAb19 become apparent. It should be noted that, *in vitro*, the antigenic site recognized by

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mAb 19 was shown to appear after the formation of a molten globule, but before the final compaction of the protein core (Goldberg *et al.* 1990).

Thus, mAb 19 appears capable of recognizing either unfolded β-chains, or chains that have already gone some way along the folding pathway, but not molten globule-like intermediates situated early on the folding pathway.

6. ASSOCIATION OF NEWLY SYNTHESIZED β CHAIN FRAGMENTS WITH CHAPERONES

Because mAb19 can recognize unfolded β-chains, the identification by pulsed immunolabelling of immunoreactive nascent chains with this mAb could no longer be considered as evidence that these chains had undergone some folding steps. Rather, it could be envisaged that, by preventing the nascent chains from undergoing a hydrophobic collapse before their release from the ribosomes, chaperones might maintain the nascent chains in an immunoreactive state. We therefore investigated the possibility that chaperones might bind efficiently to nascent β-chains during *in vitro* biosynthesis.

First, a quantitative detection of GroEL and of DnaK on Western blots was set up using commercially available monoclonal antibodies specific of these two proteins; anti-mouse immunoglobulin antibodies labelled with ^{35}S and the β-imager radioactivity scanner. This allowed us to estimate the concentrations of endogenous GroEL and DnaK as being about 0.25 and 1 mg/ml, respectively in the *E. coli* extract used for cell-free protein synthesis. These concentrations of chaperones are certainly large enough to allow for significant association with proteins in the process of folding.

To detect a possible association of chaperones with newly synthesized polypeptide chains, either bound to, or released from the ribosomes, the following experiments were conducted. Protein synthesis was allowed to proceed *in vitro* for 60 min at 37 °C, using an *E. coli* extract, ^{35}S-methionine, and the truncated mRNA that terminates at position 1104 without a stop codon. The newly synthesized chains, released and ribosome-bound were separated from each other by centrifugation on a glycerol cushion as described above. Chains released in the supernatant were immediately subjected to electrophoresis on a non-denaturing, 6–10% acrylamide-gradient gel according to Lambin & Fine (1979), under conditions where the migration of the proteins depend essentially on their molecular mass. After migration, staining and drying, the radioactivity distribution in the gel was analysed by use of the β-imager; a typical recording is shown in figure 2. The diagram obtained shows that the newly synthesized polypeptide chains are all found in high molecular mass complexes, distributed in two peaks. The first, very sharp peak migrates with an apparent molecular mass of approximately 900 kDa, at a position which exactly coincides with that of GroEL: it was detected using either an anti-GroEL monoclonal antibody to Western blot a lane of the gel that contained the whole translation mixture, or by Coomassie blue staining of a lane of the gel that contained only pure GroEL. The second, much wider, peak which corresponded to apparently heterogeneous species, migrated in a region of the gel where DnaK could be detected by Coomassie blue staining in a lane with the pure protein, and by Western blot with an anti-DnaK monoclonal antibody in a lane containing the nascent chain preparation. Thus, newly synthesized incomplete polypeptide chains released from the ribosomes were found in stable high molecular mass complexes containing either GroEL or DnaK, perhaps associated with other chaperones.

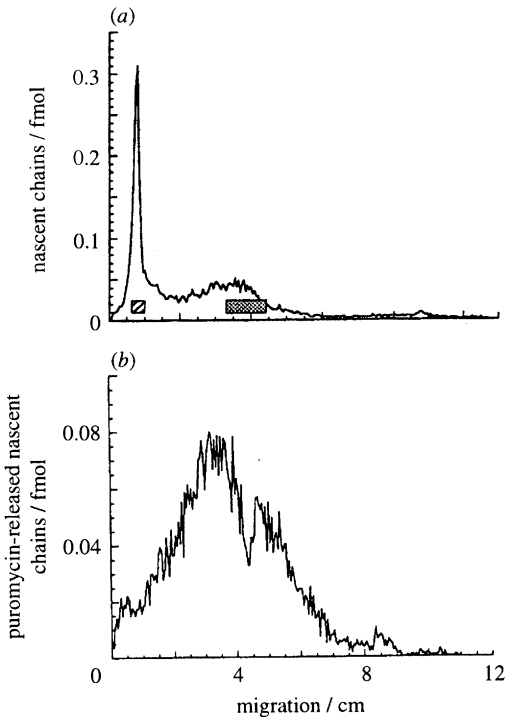

Figure 2. Association of ^{35}S-labelled newly synthesized chains with chaperones revealed by electrophoresis in acrylamide gradient non denaturing gels. ^{35}S-methionine labelled chains were synthesized in an *E. coli* translation system using the same mRNA as in figure 1. (*a*) The ribosomal fraction was removed by Airfuge centrifugation through a glycerol cushion. The ribosome-free fraction was submitted to electrophoresis on a 6–10% acrylamide gradient non-denaturing gel. Radioactivity was recorded in the β-imager, and was plotted (in amount of synthesized nascent chains per 10 μl of translation mixture) as a function of the migration in the gel. The bars indicate the position of a band revealed by anti GroEL (hatched shading) or antiDnaK (stippled shading) antibodies in Western blotting experiments. (*b*) The ribosome-bound nascent chains were separated by centrifugation through a glycerol cushion in the Airfuge and resuspended in 50 μl of buffer (20 mM Hepes pH 7.6, 100 mM K-acetate and 10 mM Mg-acetate) containing 10 mM puromycin and 1 M KCl. After 30 min incubation at 25 °C, the sample was freed of ribosomes by a second Airfuge centrifugation. An aliquot of the supernatant, corresponding to 25 μl of the initial translation mixture and containing the radioactive nascent chains that were released from the ribosomes by the puromycin treatment was submitted to electrophoresis on a nondenaturing 6–10% acrylamide gradient gel. After staining and drying, the gel was analysed in the β-imager and the radioactivity was plotted as a function of the migration in the gel.

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To discover whether these complexes with chaperones were formed before or after the release of the nascent chains from the ribosomes, the same approach (i.e. electrophoresis in a non-denaturing, acrylamide gradient gel) was applied to the ribosomal fraction obtained after the Airfuge centrifugation (see previous paragraph). However, before electrophoresis, the nascent chains had to be released from the ribosomes; to permit their migration in the gel; this was achieved by incubation of the resuspended pellet with 10 mM puromycin and 1 M KCl for 1 h at 37 °C (conditions mild enough to respect the integrity of the ribosomes; Blobel & Sabatini 1971). Under these conditions, about 25 % of the ribosome-bound nascent chains were released in solution and were found in the soluble fraction after a second Airfuge centrifugation.

This fraction was subjected to non-denaturing electrophoresis; the gel was stained, dried and analysed in the β-imager. Unlike what had been observed for chains spontaneously released from the ribosomes, the radioactivity pattern obtained with the puromycin released chains did not show the sharp peak corresponding to a complex with GroEL. On the contrary, the broad peak corresponding to a complex containing DnaK was also present with the puromycin released chains; in addition, no radioactivity was detected in the region of the gel corresponding to free (i.e. not bound to DnaK) polypeptide chains. Because no free chaperones were present in the solution at the time of, or after, the puromycin treatment, these observations demonstrated that DnaK was stably bound to the nascent chains before their release from the ribosomes, and that no nascent chain of significant length was found free of chaperone. But although GroEL was not found in a ribosome-bound complex, it does not exclude the possibility that such complexes exist: dissociation could have occured during the puromycin treatment and centrifugation step. Thus, our results demonstrate that if they exist, complexes of GroEL with ribosome-bound nascent chains (unlike the complexes with chains released from the ribosomes) should be rather unstable.

7. CONCLUSION

The results described in this communication deal with two distinct, though not unrelated, problems: protein synthesis *in vitro* and protein folding during elongation; they can be summarized as follows. Concerning protein synthesis, we showed the following.

1. In the systems we used at least, the pattern of discrete bands observed on SDS gels does not reflect transient translation pauses. Rather, it results from the presence of premature translation termination caused by the degradation of the mRNA at preferential sites.

2. The absence of a stop codon at the end of the mRNA does not ensure that the nascent chains will remain ribosome bound. Indeed, a large fraction of the chains were released in solution in both a wheat germ and an *E. coli* translation system. This lead us to develop a small-scale method for separation of the ribosome-bound from the free chains.

3. The efficiency of the translation system was quantitatively determined by measuring the number of full length chains produced.

4. The concentrations of endogenous GroEL and DnaK in cell-free translation systems were determined.

Concerning the folding problem, we showed the following.

1. For the phage P22 tailspike protein a specific monoclonal antibody detecting structured monomers during the folding *in vitro* can bind to ribosome-bound nascent chains. This suggests that folding steps that take place in solution during *in vitro* refolding also occur in a ribosome-bound state *in vivo*.

2. In the case of the β-chain of *E. coli* tryptophan-synthase, the use of a short immunochemical pulse-labelling method demonstrated that the appearance of the immunoreactivity is cotranslational.

3. Newly synthesized β-chains or β-chain fragments were always found in high molecular complexes. The chains released from the ribosomes were found in complexes containing either GroEL or DnaK. The ribosome-bound nascent chains were found in complexes with DnaK, but not with GroEL.

4. The epitope recognized by the antibody (mAb19) used for the immunochemical labelling was characterized. Its properties suggest that only some collapsed early folding intermediates fail to interact with mAb19. Conversely, unfolded β-chains or β-chain fragments, as well as chains that have already progressed some way along the folding pathway, should bind to the antibody. Thus the immunoreactivity to mAb19 is not a direct proof that folding has occurred.

That all ribosome-bound nascent β-chains were found associated to DnaK suggests the possibility that this chaperone (and perhaps others) may prevent the polypeptide chains from undergoing the initial folding steps that lead to transiently bury residues 2–9, and hence maintain them in a state where they are recognized by mAb19. Therefore, in view of the recently discovered properties of mAb19, our previous conclusion (Fedorov *et al.* 1992) that nascent chains can start their folding on the ribosome was premature, and the question of whether or not folding starts cotranslationally remains unanswered. Nevertheless, using the experimental approach described here together with a mAb that would recognize a discontinuous epitope should bring an answer to this question.

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PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

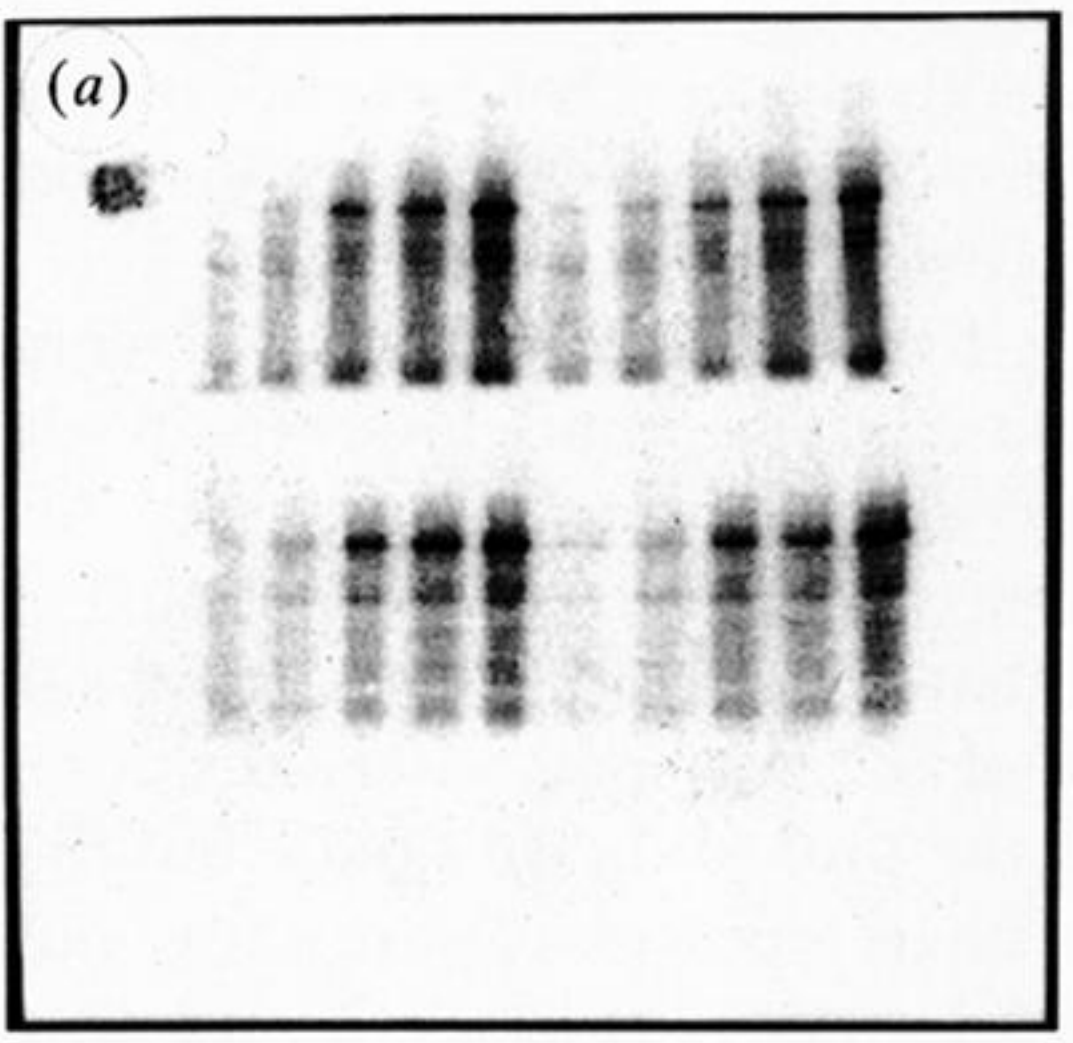

Figure 1. Kinetics of appearance of ribosome-bound ^{35}S-methionine labelled chains. Protein synthesis was performed in a wheat germ translation system, using as a template a mRNA that ends, without a stop codon, at base 1104 of the *trpB* gene. Aliquots were withdrawn after various times and synthesis was arrested by dilution with cold buffer. The ribosomal fraction was separated by Airfuge centrifugation through a glycerol gradient (see text). The pellet was resuspended in sample SDS buffer, heated, and each sample was subjected to SDS-PAGE. (*a*) Screen image of the radioactivity distribution in the gel for the chains present before (top) or after (bottom) immunoprecipitation. Samples used to measure the total synthesis before immunoprecipitation (15 µl, top half) correspond to 1/10 of those used for the immunoprecipitation (bottom half). Assays were performed in duplicate, after 5 min (lanes 1, 6), 10 min (lanes 2, 7), 15 min (lanes 3, 8), 20 min (lanes 4, 9), and 25 min (lanes 5, 10) of incubation of the translation mixture at 30 °C, and were repeated in three independent experiments. A ^{14}C sample of known radioactivity (500 d.p.m.) served as an internal standard (top left hand corner). (*b*) In each migration lane (i.e. for each incubation time) the radioactivity contained in the region corresponding to polypeptide chains of about 11, 30 and 40 kDa was determined by means of the β-Imager software and was converted into fmoles of the corresponding polypeptide chains, taking into account the internal standard, the specific radioactivity of the ^{35}S-methionine used, and the number of Met residues present in each fragment. Based on the sequence of the *E. coli trpB* gene, there are 15, 10 and 4 Met residues in the 40, 30 and 11 kDa fragments respectively. The lags observed for the biosynthesis of the β-chain fragments were 1.5 min (11 kDa fragment, open circles), 6 min (30 kDa fragment, open squares), and 15 min (40 kDa fragment, filled triangles). Data were fitted by least squares linear regression, taking into consideration measurements done after 10 min for the 30 and 40 kDa fragments, and including those for 5 min for the 11 kDa fragment.