THE CONFORMATION AND PATH OF NASCENT PROTEINS IN RIBOSOMES

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I. INTRODUCTION

Recent advances have suggested that nascent proteins are extended into a tunnel or cavity within the large ribosomal subunit as they are formed by the successive addition of specific amino acids to their N-terminus. This process appears to be associated with the acquisition of secondary and tertiary structure that is important for folding into the native conformation or transport of the newly formed protein into membranes or other subcellular structures. Our purpose here is to briefly review those aspects of the structure and function of ribosomes that contribute to these processes.

Davis and his co-workers (Smith et al., 1978) postulated that the nascent peptide provides the basis for the intimate relationship between active ribosomes and membranes in bacteria. Their conclusion, based on the observation that treatment with the aminoacyl-tRNA analog, puromycin, caused the release of the ribosomes from the membrane, was that the nascent peptide itself provides the primary point of attachment of the ribosome to the membrane. Such observations prompted the question, treated in other chapters, of how ribosomes bearing the nascent peptides of certain proteins are recognized, bound to membranes, and folded into their native conformation in prokaryotic and eukaryotic cells. Parallel questions involve the conformation of nascent peptides as they are formed on ribosomes and the role of the ribosome in folding nascent peptides into their native conformations. Chantrenne (1961) appears to have been the first to clearly enunciate the proposition that specific folding of a peptide into its native conformation may occur vectorially as it is formed on a ribosome. His statement, remarkable for 1961, is appropriate here as background.

> Complete uncoiling is usually irreversible, for there are too many possibilities of formation of bonds between different parts of the chain or between chains. If the polypeptides were released from the assembly line as randomly coiled molecules, their remolding into the right configuration would be difficult. On the contrary, if the end of the polypeptide is allowed to fold spontaneously as it comes off the template, then folding might go on in a regular and unique manner as the

chain grows. The tertiary structure would then be strictly determined at each step of its formation, by the nature of the amino acid residues and by the shape and position of the already formed parts of the molecule. Folding might conceivably be influenced by other substances present in the vicinity of the template.

This hypothesis has received some limited experimental attention over the years; however, the major effort, prompted by Anfinsen's seminal observations that denatured ribonuclease A would refold spontaneously *in vitro* into the conformation of the native enzyme has focused on renaturation and folding of protein from their denatured state (Matthews, 1993). Our objective here is to consider reports dealing with the conformation of nascent peptides on ribosomes and the role of the ribosome in translocation and in folding of proteins into their native conformation. Special emphasis will be given to the mechanism and structural features of ribosomes that generate the nascent peptide and then facilitate its folding or translocation into and through membranes.

II. RIBOSOME STRUCTURE AND FUNCTIONS

A. General Features

The synthesis of proteins in all living cells is carried out by ribosomes that are composed of two structurally different subunits which associate upon initiation of protein biosynthesis. Ribosomes are massive entities comparable in size to large multienzyme complexes. They have unique structures composed of RNA and proteins of specific primary sequence. These remarkable structures function as molecular machines to carry out the chemical–mechanical steps of movement of the mRNA that is strictly coupled with decoding and binding of tRNA, synthesis of peptide bonds, and protection of the nascent peptide during the early stages of its folding. Although the detailed structure of ribosomes from different organisms differ considerably, the basic features of structure and function are highly conserved throughout all living systems. A typical prokaryotic ribosome contains about one-quarter million atoms and is of a molecular weight of approximately 2.3 million, about one-third of which is made up of 56 different proteins. The remainder is composed of three RNA chains, two of which occur in the large subunit.

The results of intensive biochemical, biophysical, and genetic analyses led to a low resolution, consensus model for the overall shape and quaternary structure of the ribosome, for the spatial proximities of various ribosomal components, and for the approximate positions of some of the reaction sites (Stöffler and Stöffler-Meilicke, 1986). Binding and decoding of the mRNA through codon–anticodon interaction with tRNA takes place on the small subunit, whereas peptide bond formation takes place in a specific domain of the large ribosomal subunit called the peptidyl transferase center. This center, which provides the attachment site for the 3'-end of the tRNA and the nascent peptide, is located on the interfacing surface of the large

subunit below a physically distinct structure known as the central protuberance. The attachment site for an amino acid on ribose at the 3′-end of a tRNA is in the order of 85 Å from the anticodon at the opposite end of the L-shaped tRNA molecule (Kim et al., 1974; Robertus et al., 1974). This is reflected in the distance between the decoding site on the small subunit and the peptidyl transferase center at the base of the central protuberance on the large subunit.

An understanding of the molecular mechanism of protein biosynthesis is still hampered by the lack of high resolution structural information and corresponding molecular models. Development of these models and the mechanism they represent are strictly limited by the techniques and procedures that are available. X-ray diffraction holds great promise for the future. To this end, three-dimensional crystals, diffracting in the best cases to 2.9 Å resolution, have been grown and are being investigated (von Boehlen et al., 1991; Franceschi et al., 1993). Much of the information considered below is derived from two types of procedures that have been extensively utilized by the authors, namely, image reconstruction from electron photomicrographs of crystalline arrays and fluorescence techniques. These are briefly described and considered below.

B. Three-Dimensional Image Reconstruction from Crystalline Arrays

Traditional electron microscopy, which used to be the choice method for viewing isolated ribosomal particles, suffers from several inherent shortcomings originating from its subjective nature, from the limited information emerged from observing surfaces in projection, and from shape distortions introduced by the probable flattening on the microscopical grids or/and from the microscope vacuum. To avoid a significant part of these shortcomings and to enhance the level of objectivity, we performed three-dimensional reconstruction from cystalline arrays. In this procedure, diffraction patterns rather than subjective imaging provide the raw data for the reconstruction, and the reliability of the resulting models is determined by well-established crystallographic criteria, rather than by decisions based on visual inspections. Furthermore, the distortions introduced by the flat electron microscope grids are reduced or eliminated, as the two-dimensional arrays are held by crystalline forces.

A three-dimensional model for crystalline 50S subunits was elucidated at 26–28 Å resolution from tilt series (±60° with intervals of 2–5°) of negatively stained crystalline arrays observed at ambient temperature (Figure 1; Yonath et al., 1987a,b) and cryo-temperature (Y. Fujiyoshi, private communication, and Yonath and Berkovitch-Yellin, 1993). These studies have been extended recently using unstained monolayers embedded in vitreous ice that diffract to 15–18 Å (Avila-Sakar et al., 1993).

The high reliability of the so obtained models can be assessed by the remarkable similarities in shape and size of these images and those obtained by the optimized series expansion (Vogel, 1983) or random-conical reconstruction (Radermacher et
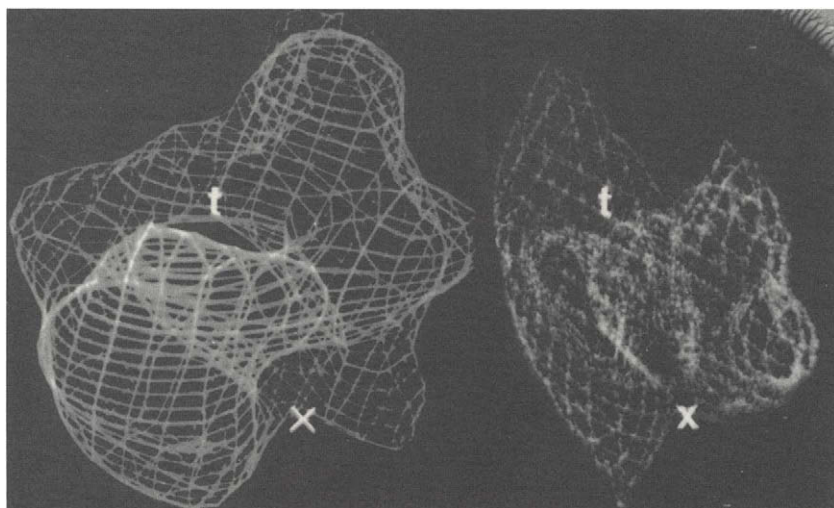
Figure 1. Two views of the model of the 50S subunit, as reconstructed from negatively stained crystalline arrays of these subunits from *B. stearothermophilus* at 28 Å resolution (Yonath et al., 1987). **t** points at the entrance to the internal tunnels and **X** shows the funnel-shape exit from the longest tunnel. This region may provide the site for the initial folding of nascent chains. The *left* views were taken from the peptidyl-transferase center, and the *right* is seen from the side.

al., 1992), both performed on samples embedded in amorphous ice. One of the deviations between our reconstructed models and those observed by traditional electron microscopy, namely a nonstretched rather than extended L7/L12 stalk, indicates that the crystalline arrays or the vitreous ice-embedded particles suffer less distortions than the isolated particles.

These reconstructed 50S particles as well as 70S ribosomes, at 47 Å resolution, revealed prominent features which were not observed in previous studies of prokaryotic ribosomes, such as tunnels and hollows (Arad et al., 1987; Yonath et al., 1987b). Some of these features were not resolved in traditional or sophisticated electron microscopy, but were seen in reconstructions from *in situ* arrays of eukaryotic ribosomes (Milligan and Unwin, 1986) and in the random-conical reconstruction obtained recently from noncrystalline 70S ribosomes embedded in amorphous ice (Frank et al., 1991).

Modeling experiments were performed on an Evans and Sutherland PS390 computer-graphics terminal using the program FRODO (Jones, 1978), according to the algorithm described in Berkovitch-Yellin et al. (1990). The ribosomes and their subunits were represented by their reconstructed images, obtained from the diffraction patterns collected from tilt series of negatively stained crystalline arrays.

To account for possible shrinkage during the preparation for electron microscopy and due to the microscope vacuum, these were slightly expanded, as described in Eisenstein et al. (1991).

The coordinates used for representing the tRNA molecules were taken from PDB (Protein Data Bank) (Abola et al., 1987) entry 6TNA (Sussman et al., 1978). Significant flexibility was introduced into the hinge regions of this structure to imitate its bound state as it is being aminoacylated (Moras, 1989). We thank Dr. L. Liljas (Uppsala) and his collaborators for the gift of the coordinates of the MS2 coat protein. As no structure of an entire antibody molecule was found in the PDB, it was approximated by juxtaposing an Fc and two Fab fragments (entries 1FC1 and 3FAB in the PDB) in a manner suggested by the low resolution structure of the T-shape IgG_1 molecule (Silverston et al., 1977). A somewhat different low resolution structure was determined for IgG, namely the Y-shape conformation (Huber et al., 1976). The main difference between these two structures, the angle between the arms of the Fab fragments, was found to be negligible at the low resolution of our current studies.

C. Functional Assignment in the Reconstructed Models

Despite the rather low resolution of the reconstructed images (47 and 28 Å, for 70S and 50S particles, respectively) and the shortcomings of electron microscopy studies, they provide a valuable tool for further understanding of the function of the ribosome. For their interpretation, the significant similarities in specific features of corresponding regions in the reconstructed models of the 50S and 70S particles were exploited. These efforts led to: (1) suggestions for the positioning of the 50S subunit within the associated 70S ribosome and possible conformational changes occurring by the association and dissociation of the ribosomal subunits; (2) the approximations of a model for associated 30S subunit; and (3) tentative assignments of biological functions to some structural features (Yonath and Wittmann, 1987b, 1989; Yonath et al., 1990; Berkovitch-Yellin et al., 1990, 1992; Franceschi et al., 1993; Yonath and Berkovitch-Yellin, 1993).

A free space, estimated as 15–20% of the total volume was detected in all models reconstructed from crystalline 70S ribosomes from *B. stearothermophilus* (Figure 2; in: Arad et al., 1987; Yonath et al., 1987b; Berkovitch-Yellin et al., 1990) and from single 70S ribosomes from *E. coli* by the random-conical procedure (Frank et al., 1991). This free space was interpreted as the separation between the small and the large subunit.

Holes, channels, and elongated internal tunnels were detected within the models of the large subunits from prokaryotic (Vogel, 1983; Yonath et al., 1987a,b; Radermacher et al., 1989, Frank et al., 1991) and eukaryotic (Milligan and Unwin, 1986) sources, obtained by a variety of reconstruction methods (optimized series expansion of single particles, diffraction of crystalline arrays, and the random-conical approach, respectively).
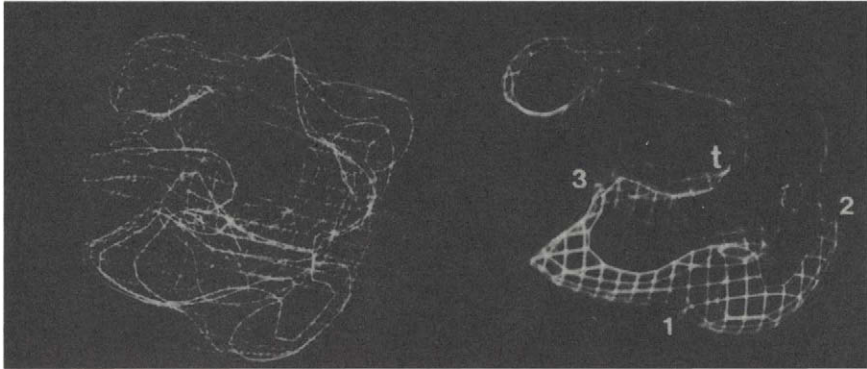
Figure 2. (*Left*) A computer graphics display of the outer contours of the most deviating models of 70S ribosomes reconstructed from negatively stained crystalline arrays of 70S ribosomes from *B. stearothermophilus* at 47 Å resolution (Arad et al., 1987). (*Right*) The net represents a slice of about 50 Å in thickness, through the model shown as solid lines on the left. **t** points at the common entrance to the three internal tunnels, and the numbers 1, 2 and 3 show the exits sites from tunnels t1, t2 and t3, respectively. Superimposed on it is a slice of about the same thickness of the 50S subunit, shown in solid lines.

One of these tunnels (t1 in Figure 2) appears in all the reported reconstructions from crystalline 50S and 70S particles from *Bacillus stearothermophilus* (Yonath et al., 1987a,b, 1990; Yonath and Wittmann, 1989; Berkovitch-Yellin et al., 1990) and *Thermus thermophilus* (Y. Fujiyoshi, personal communication). A feature similar to this tunnel was also detected within the density map obtained at 30 Å resolution from neutron diffraction data, collected from three-dimensional crystals of 50S subunits from *Haloarcula marismortui* (Eisenstein et al., 1991). This tunnel is about 100–120 Å in length and up to 25 Å in diameter, dimensions suitable for accommodating peptide chains of more than 30 amino acids at various conformations. Therefore, based on a large body of evidence derived from biochemical, fluorescence, functional, and immunoelectron microscopy experiments this tunnel was assumed to be the protected path for nascent proteins (reviewed in Eisenstein et al., 1993).

The original and most feasible interpretation of the reconstructed 70S ribosome was based solely on objective structural arguments. The 50S subunit was placed in a position which allows matching of the direction of its main tunnel t1 in the 70S ribosome (Figure 2), and in an orientation allowing the best fit of the external shapes of the 50S subunit and the part of the 70S ribosome assigned to it. This tunnel originates at the intersubunit free space and terminates at the distal, outer surface of the 50S subunit. On the opposite side of the free space, at the interface with the small subunit, a distinct region of crowded rRNA was revealed by preferential
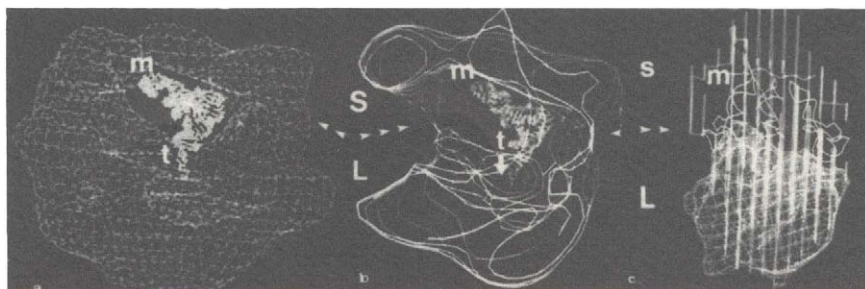
Figure 3. The positioning of the tRNA molecules based on the original interpretation. The arrow heads indicate the approximate directions of the interface between the S (small = 30S) and L (large = 50S) subunits. **t** points at the entrance to the tunnel. The presumed positions of the bound part of the mRNA chain are marked by m. A model built tRNA is shown in the thicker (**a**) and thinner (**b**) models (Figure A2). (**c**) A perpendicular view of (**a**), in which the contours of the 70S ribosome are shown in lines, and the 50S subunit as a net. In the free space at the intersubunit interface we placed three binding sites for tRNA molecules, two with the CCA end very close to the entrance of the tunnel, and the third, somewhat further away, presumably in a position allowing less tight contacts (the three tRNA molecules are bound to the ribosomes simultaneously).

staining. A groove within this region was suggested to be the path occupied by mRNA, in accord with evidence showing that during translation a segment of about 30–40 nucleotides of mRNA is masked by the ribosome (Kang and Cantor, 1985). Although the resolution of the current reconstruction images is too low for the determination of the dimensions of this groove, a rough estimate indicated that it may accommodate a stretch of mRNA comparable in length to that of the masked mRNA segment at a random, U-shaped, or helical conformation.

The dimensions of the tRNA allow its placement in the intersubunit space, so that its anticodon loop associates with the mRNA, and its CCA-terminus is positioned so that the newly formed peptidyl group extends into the entrance of the tunnel. In this orientation the tRNA molecule may also form several noncognate interactions (Figure 3). At the current resolution of the reconstructions, the two crystallographically determined orientations of tRNA—the native-closed and bound-open one (for review see Moras, 1989)—are indistinguishable.

The assignment of the intersubunit free space as the location of the various enzymatic activities of protein biosynthesis and the positioning of the tRNA molecules in it, between the two subunits, is in agreement with a large volume of circumstantial evidence accumulated during the last two decades (reviewed in Yonath and Berkovitch-Yellin, 1993, and discussed in Spirin et al., 1993). It is further supported by recent findings which show that, upon binding to 70S ribo-

somes, the entire P-site tRNA molecule is inaccessible even to hydroxyl radicals (Huettenhofer and Noller, 1992).

Steric considerations showed that the intersubunit void is spacious enough to provide up to three tRNA binding sites, along with other nonribosomal components that participate in peptide synthesis. It is noteworthy that these tRNA molecules may assume various relative orientations, ranging from parallel, the lowest space-requiring arrangement, to perpendicular (Figure 3), the most spacious one.

The intersubunit free space revealed in the random-conical model was also suggested to host the peptidyl transferase activity (Mitchell et al., 1992), although the assignments of the two subunits (Frank et al., 1991) differ significantly from that used in the original interpretation of the reconstructed model, and no effort was made for the localizations of the paths of the nascent chain. Subsequent effort for alternative interpretations (Franceschi et al., 1992; Yonath and Berkovitch-Yellin, 1993) indicated the considerable uncertainties in the current models, which due to their limited resolution do not possess the level of details required for unequivocal positioning of the two subunits. However, as the intersubunit free space is of dimensions which permit hosting the components participating in protein biosynthesis in several sterically reasonable arrangements, the assignment of the intersubunit void as the site of peptide synthesis does not critically depend on the accurate location of the small and the large subunits.

III. TECHNIQUES TO STUDY SYNTHESIS OF NASCENT POLYPEPTIDE CHAINS AND THEIR FOLDING

A. Fluorescence Techniques

Many of the investigations in the Hardesty laboratory have involved fluorescence measurements. Fluorescence techniques have several advantages for such studies. (1) They can be carried out at very low concentrations. With highly sensitive probes such as coumarin or fluorescein, measurements can be made easily at a probe concentration of 10 nM or below. (2) They can be performed in solution on active systems. For ribosomes, peptide elongation can be blocked at different points and analyzed at different steps in the reaction cycle.

Five different phenomena involving fluorescence can be used to monitor the interaction of labeled components (Hardesty et al., 1992). These are: quantum yield or fluorescence intensity, emission maximum, fluorescence anisotropy, nonradiative energy transfer, and fluorescence quenching by solute molecules.

Quantum yield (Q_f) is equal to the ratio of photons emitted to photons absorbed by the system. The interaction of a fluorophore with its environment (solvent or solute molecules such as proteins and nucleic acids) may have a large effect on Q_f. A change in Q_f is a direct indication of a change in the environment of the probe

and has been used, for instance, to monitor binding of a component to ribosomes or movement of the component during a reaction step of protein synthesis.

Like the quantum yield, the emission maximum (λ_{max}) of most probes is sensitive to the local environment and a shift in the emission maximum can be used to quantitate interaction of one component with another. Generally, emitted light is more sensitive to perturbation by environmental changes than absorption. Coumarins are particularly sensitive and are the probes that we have used most frequently for this purpose. Generally λ_{max} for coumarin and pyrene probes shift towards the blue as the polarity of the solvent decreases, whereas the opposite effect is observed with fluorescein derivatives. Antibodies that we have prepared against coumarin maleimide (CPM) derivatives of proteins and nucleic acids cause a large decrease in λ_{max} and an increase in Q_f to near 1.0, whereas antifluorescein IgG decreases the Q_f of fluorescein probes to which it binds to near zero.

Fluorescence anisotropy (A) or fluorescence polarization (P) can be used as a measure of movement of a fluorophore. If plane-polarized light is used to excite a randomly oriented fluorescent probe held rigidly as in a glass and if the same electronic transitions are involved in absorption and emission (the absorption and emission transition moments are parallel), then the emitted light will be polarized. If, however, the fluorophore molecules can reorient randomly in the interval between the time of excitation and emission, then the emitted light will be completely depolarized. The fluorescence lifetime for many of the fluorophores commonly used in biochemistry is in the time range in which smaller proteins and nucleic acids tumble in solution. Thus binding of specifically labeled protein or RNA such as a tRNA to a relatively massive ribosome generally results in an increase in fluorescence anisotropy (A) or fluorescence polarization (P). The limiting value for A is 0.4, while the corresponding value for P is 0.5.

Heavy ions such as iodide or cesium, oxygen in the triplet ground state, and some organic molecules like acrylamide and methyl viologen cause quenching of fluorescence when they come in very close proximity or collide by Brownian movement with excited fluorophores. Changes in the system that alter the accessibility of the fluorophore to a quenching atom or molecule may be detected by a change in fluorescence intensity. Thus the quenching constant, K_q, for a fluorophore buried within a protein or ribosome may be very different than for a fluorophore free in solution. Changes in charge that repel or attract the quenching molecule to the fluorophore may also cause changes in K_q. We have used this technique to estimate the accessibility of probes attached to the N-termini of nascent peptide chains within ribosomes.

Distance between probes can be measured by non-radiative energy transfer. If a fluorophore in an excited state is adequately close and properly oriented to the electronic transition axis of a molecule at a lower energy state with which it can interact, there may be a direct transfer of energy without emission of a photon. The emission spectrum of the donor fluorophore must overlap the absorption spectrum of the acceptor. If the latter is fluorescent, energy transfer will result in decrease

and increase, respectively, of fluorescence from the donor and acceptor probes. Stryer and his co-workers tested the validity of Förster's basic theory and demonstrated the use of the system as a "spectroscopic ruler" (Stryer and Hoagland, 1967). Distances in the range of 20 to 80 Å can be measured with appropriate choice of commonly used fluorophores.

B. Preparation of Fluorescent Materials

In most of the studies considered here, nascent peptides were fluorescently labeled at their N-terminal amino acid by initiating protein synthesis with a fluorescent derivative of *N*-mercaptoacetylaminoacyl-tRNA. The procedure for synthesizing the labeled tRNA derivative was derived in the Hardesty laboratory (Odom et al., 1990) and involves enzymatic aminoacylation of the desired tRNA, mercaptoacetylation with the succinimidyl monoester of dithiodiglycolic acid (the disulfide of mercaptoacetic acid), reduction of the disulfide with dithioerythritol, and reaction of the resulting sulfhydryl group with an activated fluorophore, most often CPM (3-[4-malemidophenyl]-7-diethylamino-4-methylcoumarin). The CPM-SAc-aminoacyl-tRNA is purified by reversed-phase high pressure liquid chromatography on a C_3 column. In a few cases Lys-tRNA labeled at its ε-amino group (Johnson et al., 1976) or Cys-tRNA (Hardesty et al., 1993) labeled at its sulfhydryl group have been prepared and incorporated internally into nascent peptides.

Ribosomes containing several species of amino acid homopolymers as well as some natural peptides have been prepared. Homopolymers which have been synthesized include polylysine, polyphenylalanine, polyserine, and polyalanine. Natural *E. coli* tRNA was used with poly(adenylic acid) for the synthesis of polylysine, but the other homopolymers all utilize poly(U), polyuridylic acid. This was made possible in the case of polyalanine and polyserine by construction of $tRNA^{Ala}$ and $tRNA^{Ser}$ containing AAA anticodons, which could be enzymatically acylated with alanine or serine, respectively, but which recognized polyuridylic acid as a template (Picking et al., 1991a; 1991b). Synthesis was initiated with CPM-SAc-AcLys-tRNA for polylysine, CPM-SAcPhe-tRNA for polyphenylalanine, and modified CPM-SAcAla-tRNA and CPM-SAcSer-tRNA, respectively, for polyalanine and polyserine. In the case of natural polypeptides, such as phage MS2 coat protein, initiation was obtained with *N*-(CPM-SAc)Met-tRNA$_f$.

To obtain ribosomes with different lengths of nascent peptides, synthesis was arrested in various ways. For the homopolymers the synthesis was allowed to proceed for increasing amount of time at 20 °C and was stopped at the desired time by lowering the temperature to 0 °C. The average chain length was determined by dividing the pmol of the radioactive amino acid incorporated into the body of the peptide by the pmol of fluorescently labeled, radioactive amino acid incorporated at the N-terminus during initiation of the peptide. The lengths of natural peptides were controlled by deleting certain amino acids occurring at known positions or by

hybridization of a complementary oligodeoxynucleotide to a specific sequence of the mRNA. In all cases after translational arrest, material not bound to ribosomes was removed by gel filtration on Sephacryl S300.

C. *In Vitro* System for Coupled Transcription/Translation

A number of proteins with enzymatic activity have been synthesized *in vitro* by coupled transcription/translation. Generally, an *E. coli* S30 extract was prepared according to Zubay (1974), then centrifuged to isolate the ribosomes. The ribosomal pellets contain all protein components necessary for efficient protein synthesis (Kudlicki et al., 1992). For *in vitro* expression of DNA sequences under the T7 or SP6 promoter, the respective plasmid in nonlinearized form was added to the ribosome fraction together with the required RNA polymerase, rifampicin to inhibit *E. coli* RNA polymerase, total *E. coli* tRNA, and all necessary low molecular weight components.

After completion of protein synthesis, the isotopically labeled product was analyzed by SDS-PAGE (polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate), the amount formed was quantitated by TCA (trichlo-roacetic acid) precipitation, and the product was assayed for enzymatic activity. By use of a labeled amino acid of known specific radioactivity, the molar amount of the amino acid that is incorporated can be calculated. In turn, the molar amount of product peptide that is formed can be calculated if a single polypeptide species of known amino acid composition is formed. With rifampicin and a gene that can be transcribed by SP6 or T7 RNA polymerase, the transcription system can be constituted so that only one mRNA species is synthesized; however, the total peptide product that is formed includes some proportion of incomplete chains either as peptidyl-tRNA attached to the ribosomes or those that have been prematurely aborted and released into the soluble phase. Typically, the incomplete chains in the soluble fraction constitute less than 10% of the total. Thus, with this relatively small correction, the amount of full-length enzyme protein in the soluble phase can be calculated. In turn, its specific enzymatic activity can be calculated and compared with the specific enzymatic activity of the native enzyme as a measure of correct folding of the synthesized polypeptide.

IV. THE PATH OF NASCENT PEPTIDES

A. Peptidyl Transferase Reaction

A nascent peptide is bound as peptidyl tRNA in the peptidyl transferase center in such a way that it can be chemically transferred to the free amino group of an L-amino acid attached to the next incoming tRNA. By this peptidyl transferase reaction, the peptide is extended by one amino acid. The chemical reaction involves
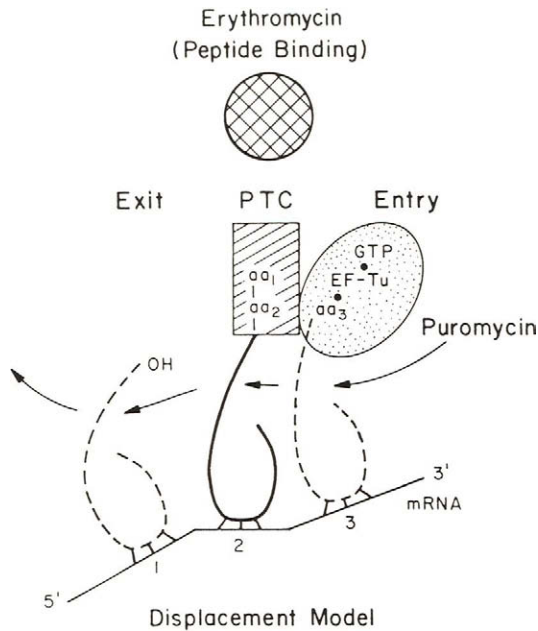
Figure 4. The displacement model for peptide elongation.

the transfer of the carboxyl group of the nascent peptide form an ester linkage with the 3′ hydroxyl of the terminal ribose of the tRNA to the α-carbon amino group of an incoming aminoacyl-tRNA. An amide or peptide bond is formed in what appears to be an SN2-type displacement reaction with nucleophilic substitution through a tetrahedral intermediate. A molecule of deacylated tRNA is generated as the peptide is elongated by one amino acid and the incoming tRNA becomes the new peptidyl-tRNA (Figure 4). This chemical reaction is associated with physical movement of the 3′-end of the newly formed deacylated tRNA of about 20 Å, relative to the ribosome, whereas the nascent peptide itself remains in essentially the same position in the peptidyl transferase center (Hardesty et al., 1986, 1990). These observations led to the postulation of the "displacement model" of ribosome function and peptide synthesis. Thus in this sense it might be more appropriate to refer to the tRNA transferase reaction rather than the peptidyl transferase reaction.

Originally, Watson (1964) postulated that the peptidyl transfer reaction must involve two sites or positions to which tRNA can be bound in the large subunit. These were the peptidyl or P site to which peptidyl-tRNA was bound before transfer of the peptide and an acceptor or A site to which incoming aminoacyl tRNA was bound. However, the mechanism described above requires that there should be three tRNA binding sites (Figure 4): (1) the peptidyl transferase center to which peptidyl

tRNA is always bound; (2) a site into which deacylated tRNA is moved after the peptidyl transferase reaction is completed; and (3) a site to which an incoming aminoacyl-tRNA is bound before a new peptide bond is formed. The number of tRNA binding sites has been a matter of considerable controversy. Noller and his co-workers reported results that indicated only two tRNA molecules can bind through codon–anticodon interaction with the mRNA on the small subunit (Moazed & Noller, 1989a). However, fluorescence techniques using an *N*-acylcoumarin derivative of Phe-tRNA, showed unequivocally that this peptidyl-tRNA analog, a molecule of deacylated tRNA, and a molecule of puromycin could be simultaneously bound to the same ribosome (Odom et al., 1990). Puromycin is an analog of the 3′ end of aminoacyl-tRNA and presumably binds to the site to which aminoacyl-tRNA is bound on the large subunit. Together, these considerations indicated that there are three sites with which tRNA can interact on the large ribosomal subunit but only two sites on the small subunit. Noller and his co-workers proposed a hybrid model of ribosomal function (Moazed and Noller, 1989b) that accommodates these observations.

An essential feature of the peptidyl transferase reaction is that it does not involve a catalytic center or functional group that participates directly in the chemical reaction as an intermediate, as occurs during catalysis by most enzymes. Rather, it is the ribosome itself as a functional unit that mediates the alignment of the two tRNAs in such a way that the displacement reaction takes place. Indeed, a remarkable range of covalent derivatives other than peptides or amides can be formed in the peptidyl transferase center. These include esters (Fahnestock et al., 1970), thioesters (Gooch and Hawtrey, 1975), thioamides (Victorova et al., 1976), and phosphinoamides (Taressova et al., 1981). It is noteworthy that D-amino acid esters of tRNA cannot function in the peptidyl transferase reaction.

As described in greater detail below, analysis of quenching of fluorescence from a fluorophore covalently attached to the N-terminus of a nascent peptide by methyl viologen (MV^{2+}) led to the conclusion that the peptidyl transferase center itself and much of the ribosomal environment encountered by the nascent peptide as it is extended is due to RNA. The results indicated that positively charged MV^{2+} molecules are bound to ionized phosphate in the backbone of the ribosomal RNA within a few angstroms of the N-terminal probe (Picking et al., 1992). They appear to indicate that the peptidyl transferase center and the path encountered by the nascent peptides as it transverses the ribosome is largely or totally comprised of RNA.

The mechanism of the peptidyl transferase reaction described above and the RNA environment found at the peptidyl transferase center as well as the path for the nascent peptide have implications for the early steps of evolution. RNA molecules are capable of catalyzing the formation of certain covalent bonds and are likely candidates as the first self-replicative polymers in the evolution of living systems (Joyce and Orgel, 1993). However, it was the development of the unique reaction system of the peptidyl transferase center and decoding of mRNA with tRNA by

ribosomes that, during early evolution, marked the point at which self-replication of a nucleic acid polymer was coupled with the synthesis of peptides. This transition was essential for the evolution of catalytically versatile proteins and the generation of "living" systems as represented by modern organisms. Indeed, even though the structural details of ribosomes and tRNA from diverse organisms vary considerably, their overall structure and the fundamentals of the mechanism by which they function appear to be essentially comparable in all ribosomes and may be the most evolutionarily conserved features of living systems.

B. The Peptidyl Transferase Center

Changes in the emission spectrum, fluorescence quantum yield, and fluorescence anisotropy that occur upon binding of *N*(fluorophore)-aminoacyl-tRNAs into the peptidyl transferase center of ribosomes indicate that the probes are held rigidly in a relatively hydrophobic environment in which they are inaccessible to immuno-globin G (IgG) (Picking et al., 1992). Upon binding of IgG, fluorescence anisotropy increased to nearly the theoretical maximum, 0.40, indicating that the probe and presumably the amino acid to which it was attached was held rigidly in a way that allows very little movement of the probe independent of the ribosome (Odom et al., 1991). After binding to the peptidyl transferase center, a nascent peptide was formed in which the *N*(fluorophore)-amino acid was in the N-terminal position. Coumarin was used as the fluorescent probe for most of our fluorescence experiments considered below.

C. Homopolymeric Peptides

Coumarin-labeled amino acids were incorporated into the N-terminal positions of various nascent amino acid homopolymers and the coumarin fluorescence parameters were examined as a function of chain length. The first homopolymer to be examined was polyphenylalanine (Odom et al., 1991; Picking et al., 1991b). The emission maximum of coumarin at the N-terminus of polyphenylalanine showed progressive shifts to the blue with increasing chain length, accompanied by corresponding increases in quantum yield. This indicated that the probe encountered an increasingly hydrophobic environment as the peptide was extended. Fluorescence anisotropy remained very high even at peptide lengths approaching 100 phenylalanine residues. Moreover, synthesis of polyphenylalanine was refractory to erythromycin. This antibiotic inhibits the elongation of most peptides beyond a di- or tripeptide but does not inhibit the synthesis of the first peptide bond (Roberts and Rabinowitz, 1989). It binds tightly to 50S subunits near the fluorescent probe of N-coumarin-Phe-tRNA bound in the peptidyl transferase center (see Figure 4). However, it does not bind to ribosomes bearing longer nascent chains of polyphenylalanine or other amino acids. These observations are consistent with the hypothesis

proposed by Arevalo et al. (1988) that erythromycin blocks the entrance to the tunnel through which the nascent peptide must normally pass.

Using erythromycylamine labeled with coumarin, it was shown that prebound erythromycin was not displaced during the synthesis of polyphenylalanine (Odom et al., 1991). Rather, the prebound coumarin–erythromycin became increasingly resistant to exchange with unlabeled erythromycin as the polyphenylalanine chain was extended. Fluorescence from the probe underwent a marked increase in intensity and shifted towards the blue indicating that the environment of the probe became increasingly hydrophobic. These results appear to indicate that the coumarin–erythromycin was buried in or under the nascent polyphenylalanine chain. Polyphenylalanine is very insoluble in nearly all aqueous and nonpolar solvents. Considered together, these results indicate that polyphenylalanine forms an insoluble mass near the peptidyl transferase center as it is formed.

N-terminal coumarin-labeled nascent polylysine exhibited greatly different changes in fluorescence properties with increasing chain length when compared to polyphenylalanine. Fluorescence anisotropy dropped from 0.34 to 0.22 as the nascent peptides were extended an average length of four or five lysine residues and fell somewhat more upon further extension of the peptide. The lower value is near that observed for $\varepsilon(N$-coumarin)Lys-tRNA free in solution and indicated the probe was relatively mobile in solution. This decrease in anisotropy was accompanied by a red shift in emission maximum and a decrease in quantum yield, indicating a relatively polar, probably aqueous environment. This rapid increase in rotational flexibility and shift to a hydrophilic environment prompted the suggestion that polylysine may not enter the internal ribosomal channel but rather exit the ribosome directly into the solvent from the peptidyl transferase center. It should be noted that polylysine free in solution should have high inherent flexibility since it exists mainly as a random coil at neutral pH due to charge repulsion.

Polyamino acids, such as polyalanine that have a propensity to form an α-helix, might be used as rulers of designed length to study the environment of the nascent peptides as it is extended through the ribosome. The construction of alanyl-tRNAs and seryl-tRNA having AAA as their anticodons and therefore recognizing poly(U) as a template (Picking et al., 1991a; 1991b) made it possible to investigate by fluorescence the environment encountered by these homopolymers as they are synthesized on the ribosome. Polyalanine forms a stable α-helix in solution (Chou and Fasman, 1974) and is relatively soluble. Polyserine is very soluble and tends to exist as a random coil in aqueous solution, but with a nucleation site at the peptidyl transferase center it was expected to exhibit α-helical formation that is intermediate between polyalanine and polylysine.

Using synthetic CPM-SAcAla-tRNA or CPM-SAcSer-tRNA to initiate either polyalanine synthesis or polyserine synthesis, fluorescence parameters of the N-terminal coumarin were monitored as a function of chain length (Picking et al., 1992), particularly fluorescence anisotropy and accessibility to four soluble molecules of increasing size: methyl viologen, MV^{2+} ($M_r = 257$), proteinase K ($M_r =$

27,000), Fab fragment of anti-coumarin IgG (M_r = 50,000), and intact anti-coumarin IgG (M_r = 150,000). Initially fluorescence anisotropy was very high, near 0.38, but decreased for both polyalanine and polyserine up to an average chain length of about 80 residues. The rate of decrease was considerably slower for polyalanine than for polyserine. This may reflect a greater tendency of polyserine α-helix to degenerate into a less rigid conformation as elongation continues.

Coumarin at the N-terminus of both polyalanine and polyserine was accessible to quenching by MV^{2+} at all chain lengths. In fact, the quenching was greater at short chain lengths, as described above. This accessibility to MV^{2+} suggests that the interior of the tunnel is accessible to this relatively small organic molecule and presumably to solvent throughout its length.

Probes at the N-terminus of both polyalanine and polyserine were inaccessible to proteinase K at short chain lengths, but became accessible at an average length of about 40 residues with polyalanine and 25–30 residues with polyserine. These lengths are comparable to previously reported protected lengths of natural nascent proteins during limited proteolysis (Malkin and Rich, 1967; Blobel and Sabatini, 1970; Smith et al., 1978; Ryabova et al., 1988).

The N-terminal coumarin on both polyalanine and polyserine was inaccessible to binding of anti-coumarin Fab fragments until chain lengths reached 55 residues and 45 residues, respectively. Finally, accessibility to intact anti-coumarin IgG was only achieved at lengths of about 80 alanine residues or 60 serine residues (Picking et al., 1991b). Binding of anti-coumarin IgG or its Fab to the N-terminal coumarin caused a large increase in quantum yield and a shift in the emission maximum, reflecting the hydrophobic nature of the coumarin binding site in the antibody, as considered above. The difference in accessibility with nascent polyalanine and polyserine apparently reflects differences in the secondary structure of the peptides, whereas the difference between IgG, its Fab fragment, and proteinase K probably reflects differences in their molecular size. If the polyalanine is in the α-helical conformation, 80 residues would correspond to a length of about 120 Å. Yonath and co-workers have shown that the main ribosomal tunnel can accommodate a peptide of this length (Figure 5). In addition, a plausible model for the apparently partial penetration into the tunnel by the Fab fragments was constructed (Eisenstein et al., 1993).

D. Signal Peptides

A number of laboratories have attempted to define the shortest nascent chain that is capable of binding the signal recognition particle (SRP) and translocation of the peptide into a membrane (Ibrahimi et al., 1986; Siegel and Walter, 1988; Okun et al., 1990). The approach has been to prepare a series of synthetic mRNAs that produce truncated proteins of different lengths, then examine the ability of these peptides to interact with SRP and membranes. The results are somewhat variable but indicate that peptides containing 70–75 amino acids were translocated while
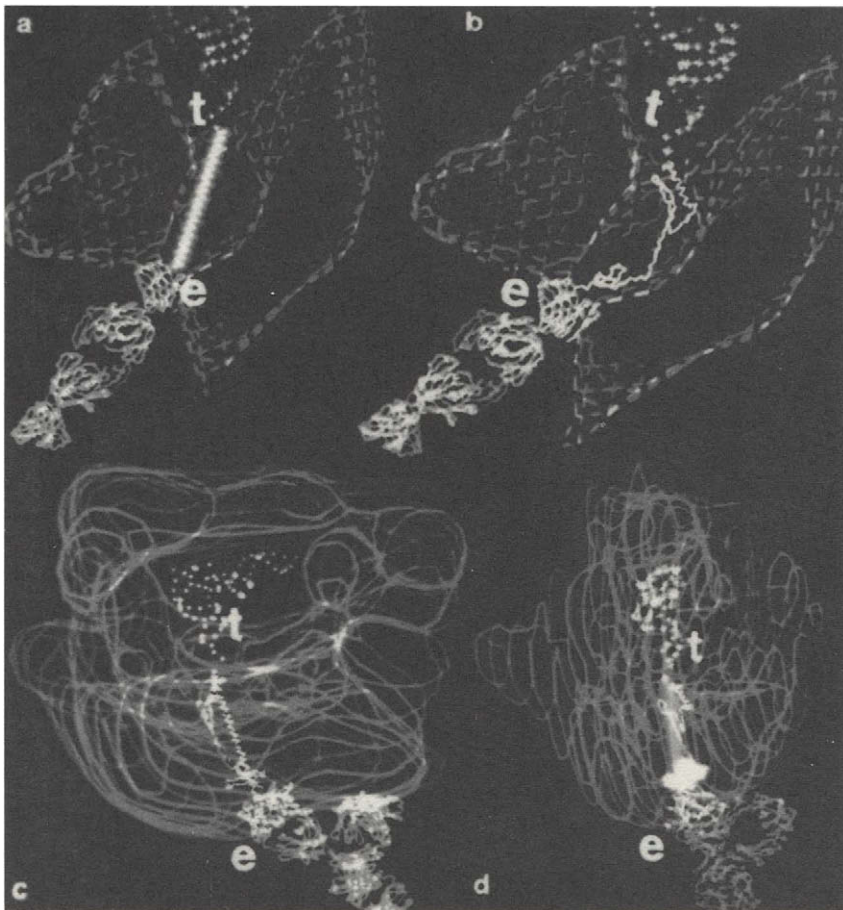
Figure 5. A slice of 50 Å thickness of the 50S subunit. In tunnel t1 (Figure 2) is placed: (a) a theoretical, undeformed stretch of an α-helix of 67 residues; (b) the main chain of the MS2 coat protein in a partially unfolded conformation, maintaining the native fold of its β-stretches and the native (crystallographically determined) conformation of the segment 1–47 (the C-terminus is in the vicinity of the proposed peptidyl transferase center and the N-terminus at the exit domain of the t1 tunnel); (c) and (d) show perpendicular views of the 70S ribosome, with an imaginary composite of (a) and (b) showing in tunnel t1 both the α-helix and the partially folded MS2 protein; (d) the segment 1–47 of the MS2 protein is shown as a space filling model. The partial penetration of the Fab molecule is evident. A molecule of tRNA (*in crosses*) was model built in the intersubunit space, so its anticodon is close to the mRNA binding site and its CCA end is placed in the entrance to the tunnel. A hypothetical IgG molecule (main chain as *solid lines*) was attached at the end of the tunnel, so that it makes the closest possible contacts with the ribosome. **t** shows the entrance to the tunnel and **e**, its exit.

those containing 50–55 amino acids or less were not. It appears likely that the variability of the results with different proteins reflects differences in the folding of these proteins that take place within the ribosomes.

Johnson and co-workers (Crowley et al., 1993) labeled truncated forms of pre-prolactin by incorporation of ϵ-N(NBD)-lysine from the corresponding Lys-tRNA during translation. NBD is a highly fluorescent, environmentally sensitive fluorophore, 6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino hexanoic acid. NBD-lysine was incorporated in place of lysine internally along nascent preprolactin peptides that were blocked at specific points during translation as the ribosomes came to the end of a truncated mRNA that did not contain a termination codon. Analysis of the resulting fluorescence lifetime data was interpreted to indicate that the fluorophore and the nascent peptides to which they were attached were in an aqueous environment as they were extended through the ribosome. Furthermore, the probe was accessible to iodide ions at all peptide lengths as indicated by Stern–Volmer constants for collisional quenching. However, upon addition of an endoplasmic reticulum (ER) membrane fraction to the translational reaction mixture collisional quenching by the iodide ions decreased to very low levels with little or no apparent increased quantum yield. The results indicated that attachment of the ribosomes to the ER membranes blocks access from the aqueous solvent and iodide ions to the nascent chain. They provide strong support to the hypothesis that nascent peptide follows an enclosed tunnel through the ribosomes, rather than an open groove or channel on its surface, to reach the exit domain on the outer surface of the large subunit.

E. MS2 Coat Protein

The homopolymeric peptides considered above are useful to illuminate some basic features of the biosynthetic process, but provide a poor model for following the fate of nascent chains of proteins since they do not fold into the complex tertiary structure of a typical globular protein. MS2 coat protein was chosen for initial experiments involving fluorescence in which the same general strategy described above was used to monitor the extension of nascent homopolymeric peptides. This protein contains 130 amino acids including the N-terminal methionine and is folded into a well-defined globular-like structure containing seven antiparallel β-strand and two short α-helices (Valegard et al., 1990). To label the N-terminus of the nascent protein, a coumarin derivative was covalently attached to the methionine amino group of Met-tRNA$_f$, which was then bound to ribosomes in the presence of MS2 RNA. Synthesis of defined lengths of nascent MS2 coat protein was accomplished by limiting specific tRNAs or amino acids in the polymerization reaction mixture or by truncating the MS2 RNA with ribonuclease H after hybridization with an antisense DNA oligomer. Fluorescence anisotropy was used as a measure of probe mobility as a function of nascent peptide length. The resulting anisotropy profile was more similar to that for polyalanine rather than polyserine, polylysine,

or polyphenylalanine. Anisotropy remained very high (near 0.38) until a peptide length of 30–40 residues was reached, followed by a slow decline to values near those for full-length MS2 coat proteins that had been released from the ribosome (Hardesty et al., 1993). MV^{2+} quenching of fluorescence from N-terminal coumarin–methionine was used to characterize the environment of the probe as the nascent peptide was extended. Although quenching for the ribosome-bound MS2 protein initially declined rapidly as the chains were extended, values remained well above those for the corresponding puromycin-released material to lengths of about 125 residues. This result appears to indicate that fluorescence from the N-terminal coumarin even in nearly full-length nascent MS2 peptides is subject to elevated quenching by MV^{2+} due to binding of the quenching agent to RNA within the ribosome. This is a strikingly different result than was observed for nascent polyalanine and appears to indicate that the N-terminus of the nearly full-length nascent MS2 peptides are within the large ribosomal subunit.

Experiments similar to those described above were carried out with coumarin on the N-terminus of nascent MS2 coat protein and IgG or Fab derived from it. N-terminal coumarin becomes accessible to Fab at an average length of about 45 residues. This is approximately the length at which N-terminal coumarin on nascent polyalanine reacts with the Fab. However, in contrast to the results with nascent polyalanine with which N-terminal coumarin became accessible at an average chain length of about 60 residues, the N-terminus of all lengths of ribosome-bound nascent MS2 peptides were largely shielded from IgG. Both IgG and Fab interacted readily with N-terminal coumarin of all lengths of puromycin-released MS2 peptides (Hardesty et al., 1993). The different accessibility for Fab and IgG is consistent with the hypothesis that the N-terminal coumarin of nearly full-length peptides is shielded from IgG by the ribosome in a conformation or position in which it is accessible only to the smaller Fab molecule.

Both MV^{2+} quenching and the antibody accessibility experiments suggest that the nascent MS2 peptides of about 125 residues are in a folded conformation while they are being extended through the ribosome. This conclusion was tested directly by nonradiative energy transfer between coumarin covalently attached to cysteine at position 47, the first cysteine residue in the MS2 sequence, and fluorescein on the N-terminal methionine (Hardesty et al., 1993). The observed level of energy transfer appears to be a minimum value in that a number of factors might reduce the level of transfer that was observed in the experiments. A high level of energy transfer, probably greater than 95%, would be predicted from the distance between the N-terminus and cysteine[47] in the crystal structure of the native protein in the phage (Valegard et al., 1990). Again, the results appear to indicate that the nascent MS2 peptides are folded into some type of compact tertiary structure before they exit from the ribosomes. The relation of this pre-native state to the conformation of folded globular proteins is not known yet. Using fluorescence techniques, we were unable to detect binding of a 26-residue RNA to the nascent MS2 coat protein (unpublished results). The oligonucleotide had the base sequence of the hairpin

fragment of the MS2 and R17 RNA to which the coat protein binds (Berzin et al., 1978; Romaniuk et al., 1987). These results—though negative—agree with those described below indicating that even full-length polypeptides have no detectable enzymatic activity as long as they are bound to ribosomes.

·V. *IN VITRO* SYNTHESIS AND FOLDING OF PROTEINS INTO BIOLOGICALLY ACTIVE ENZYMES

Specific binding of a ligand to an enzyme protein generally requires at least partial folding of the peptide into the native conformation. Therefore this might be used to monitor folding of nascent proteins into their native form. Similarly, the ultimate test of whether or not a nascent enzyme is folded into its native conformation is expression of its enzymatic activity. Covalent attachment of heme to cytochrome *c* was shown to occur posttranslationally (Dumont et al., 1988); however, other studies suggest that chlorophyll is bound to protein D1 of the membrane-bound chloroplast reaction center as a cotranslational event (Mullet et al., 1990; Kim et al., 1991). Spirin and his co-workers have reported that [^{3}H]-labeled hemin binds to nascent globin chains that are still attached to ribosomes (Komar et al., 1993).

Nascent peptides attached to the ribosome were reported in early publications to acquire the characteristics of the completed protein with a native tertiary structure. β-galactosidase provides a well-known example. In addition to folding of the polypeptide into the correct tertiary structure, its enzymatic activity requires that the four subunits of the native enzyme be united into a defined quaternary structure. It was shown that the nascent β-glactosidase peptide, prior to its completion and when it was still attached to the ribosome, associated with free subunits of the same protein and that this complex exhibited enzymatic activity while it was associated with the ribosome (Zipser and Perrin, 1963; Kiho and Rich, 1964). From these experiments it is not clear whether or not the nascent peptide itself was enzymatically active, but it can be assumed that substantial proper folding of the nascent peptide must have taken place to provide a basis for its interaction with free subunits of the enzyme.

Other results indicate that nascent chains of β-galactosidase acquire a conformation in which they can react with antibodies that recognize specific regions on the surface of the mature enzyme before translation is completed and the protein is released from the ribosome (Hamlin and Zabin, 1972). The antibodies used were concluded to be specific for the tertiary structure of the protein in that denaturation of the ribosome-bound material by heating destroyed its capacity to react with these antibodies. Much of the immunologically reactive peptide was released from the ribosomes by incubation with puromycin.

The results of these and other similar experiments were interpreted to indicate that the polyclonal antibodies that were used did indeed recognize and bind to the nascent peptides. However, the specificity of the antibodies for tertiary conforma-

tion is limited to small surface patches so that some questions remain concerning whether or not the nascent peptides were folded into their native conformation. Goldberg and his co-workers (Fedorov et al., 1992) carried out rather similar experiments with monoclonal antibodies that were shown to be highly specific for determinants formed by the tertiary structure of the β subunit of tryptophan synthetase (M_r~44,000). One monoclonal antibody isolate was found to react with ribosome-bound nascent peptides provided that their size was above 11.5 kDa. Further, it was shown by gel filtration chromatography that the 11.5-kDa fragment formed a condensed structure characteristic of a folded protein. The results were interpreted to indicate that a nascent peptide starts to fold into its native configuration while it is being formed on a ribosome. It is unfortunate that the nascent chains were not shown to be released from the ribosomes by puromycin or to be extended to give enzymatically active full-length polypeptides.

Experiments in our laboratory thus far have failed to demonstrate enzymatic activity of nascent chains of either *E. coli* DHFR (dihydrofolate reductase) or mammalian rhodanese on ribosomes (Kudlicki et al., 1994). The ribosomes were isolated from a bacterial coupled transcription/translation reaction system in which these enzymes were expressed from the respective plasmid. The folding of these enzymes after *in vitro* synthesis is considered in the subsequent sections.

E. coli DHFR cloned into a plasmid from the *fol A* gene was expressed in the *in vitro* coupled transcription/translation system and its activity tested. Mostly full-length DHFR was synthesized (Ma et al., 1993) and over 80% of newly synthesized DHFR was released into the soluble phase after 30 min synthesis at 37 °C (unpublished results). Enzymatic activity was followed by the decrease in absorbance at 340 nm over time due to oxidation of NADPH. One unit of DHFR activity is the amount of enzyme required to reduce 1 μmol DHF/min based on a molar extinction coefficient for NADPH of 12.3×10^3 at 340 nm. *In vitro* synthesized DHFR was calculated to have a specific activity of about 58 units/mg protein (Ma et al., 1993). Isolated native DHFR was reported to have 19 units/mg (Baccanari et al., 1975). The basis for this apparent difference between the specific enzymatic activity of newly formed and isolated DHFR is unknown.

Rhodanese deserves special attention as a model enzyme for studies of protein folding and membrane transport. Mammalian rhodanese is a monomeric 33-kDa sulfur transferase which is coded for by a nuclear gene and synthesized on cytoplasmic ribosomes and then transported into mitochondria where it is found as a soluble enzyme in the matrix. The crystal structure shows the enzyme to be composed of two domains of approximately equal size (Ploegman et al., 1978).

It has been shown that all the information required for transport, targeting, and folding into an enzymatically active conformation is contained in the primary sequence of the enzyme. Rhodanese contains four cysteine residues, yet has no disulfide bonds (Ploegman et al., 1978). Its only posttranslational processing is cleavage of its N-terminal methionine residue (Miller et al., 1978). Its enzymatic activity is easily monitored by a relatively simple and sensitive calorimetric assay

(Sorbo, 1953). This activity is lost in the first stage of denaturation (Tandon and Horowitz, 1986). Refolding from the denatured state has been extensively studied by Horowitz and his co-workers. Under proper conditions the denatured enzyme can refold spontaneously (Mendoza et al., 1991a), but the efficiency of refolding can be enhanced and greatly facilitated by the chaperonins, GroEL plus GroES (Mendoza et al., 1991b), or lipid micelles (Zardeneta and Horowitz, 1993).

Enzymatically active rhodanese has been produced from plasmid DNA containing its coding sequence in cell-free coupled transcription/translation systems (Tsalkova et al., 1993). Two additions when present during its synthesis were found to stabilize the newly synthesized enzyme and increase its specific activity. One component is thiosulfate, one of the enzyme's substrates. Though thiosulfate above 15 mM strongly inhibits protein synthesis (with all plasmids tested), it increased the specific enzymatic activity of rhodanese formed *in vitro* apparently by stabilizing the protein. Secondly, the chaperonins GroEL/ES increased the specific enzymatic activity of *in vitro* synthesized rhodanese, but only when they were included during protein synthesis (Tsalkova et al., 1993).

VI. CHAPERONES

A. Association with Ribosomes

In vitro renaturation of many proteins from their denatured state is facilitated by chaperones as is considered in other chapters. A central issue involves the question of whether or not chaperones play a role in folding of nascent peptides while they are attached to ribosomes. Several recent reports indicate this may be the case. Beckmann et al. (1990) observed that in HeLa cell extracts newly synthesized peptides ranging in size from 20 to 200 kDa were precipitated by monoclonal antibodies that were specific for the 70-kDa heat-shock protein, Hsp 72,73, in HeLa cells. The peptides were associated with polysomes, but could be released by reaction with puromycin. The results indicated that the Hsp 70 was transiently associated with the nascent peptide on ribosomes as they were being extended.

Craig and her co-workers (Nelson et al., 1992) reported intriguing results from a series of experiments with yeast which indicate that two members of the Hsp70 family of heat-shock proteins, Ssb1 and Ssb2, bind directly to nascent peptides as they are formed on polysomes. Puromycin caused the release of Ssb1 and Ssb2 with the nascent peptides. "Knock out" alleles of both Ssb proteins were prepared and studied with respect to their sensitivity to antibiotics that inhibit peptide elongation, but at different points in the peptide elongation cycle. Growth of the mutant strains was hypersensitive to puromycin, hygromycin B, and G418, all of which are amino glycoside antibiotics that act on the 40S ribosomal subunit. However, the mutant strains did not exhibit hypersensitivity to cycloheximide or anisomycin, both of which bind to the 60S subunit and block peptide bond formation. Hypersensitivity

suggests that the mutants affect a component of a reaction step that is inhibited by the antibiotic. The slow-growth phenotype of the Ssb1 and Ssb2 mutant strains could be partially overcome by increasing the copy number the HBS1 gene which encodes a protein that appears to be homologous to peptide elongation factor eEF1-α.

These provocative results were interpreted to indicate that the Hsp70 proteins, Ssb1 and Ssb2, bind to the nascent peptide and cause a direct inhibition of peptide elongation. It was suggested that the absence of these proteins causes the nascent peptide to back up in the channel or tunnel through the large subunit, thereby slowing the rate of peptide elongation.

A corollary of this hypothesis is that movement of the nascent peptide through the large ribosomal subunit to the exit domain is dependent on binding of Hsp70 to a region near its N-terminus. It is difficult to imagine the mechanism by which this might occur, and certainly there is no indication that Hsp70 is required for translation of most peptides. The effect of overcoming such an inhibition by increasing the concentration of eEF1–α•amino acyl–tRNA complex in the cell is even more puzzling. Clearly these provocative observations require further investigation.

The inhibition of peptide elongation described above is reminiscent of inhibition of peptide elongation that is caused by the SRP that binds to the N-terminal signal sequence of proteins destined to be transported into or through membranes (Walter and Blobel, 1981; Wolin and Walter, 1989). The SRP facilitates establishment of a ribosome membrane junction. In the case of SRP, however, inhibition of peptide elongation is inhibited by binding of the SRP, not its absence. Although the mechanism by which the SRP inhibits peptide elongation is not understood, it is likely that it somehow blocks the tunnel or exit site so that the N-terminal domain of the nascent peptide cannot leave the ribosome.

B. Chaperone-Deficient *In Vitro* Translation Systems

At least 30 different protein components are required for translation. The list includes at least 20 aminoacyl·tRNA synthetases as well as the individual factors that are required for the ribosomal steps of peptide initiation, elongation, and termination. Weissbach and his co-workers (Weissbach et al., 1981) carried out the monumental task of isolating each of these components from prokaryotic sources and then reconstituting them into a completely defined translation system in which enzymatically active β-glactosidase was formed on bacterial ribosomes. These are important observations in that they provide very strong support for the hypothesis put forth by Anfinsen in which he proposed that folding of a protein into its tertiary conformation is dependent on the sequence of amino acids that constitute its primary structure, and thus is inherent in the base sequence of the DNA from which it was coded (Anfinsen and Scheraga, 1975). However, the amount of protein product that could be formed in a completely defined system was low and isolation

of all of the required components is far beyond the resources that are available to most laboratories. Furthermore, a number of more recent studies indicate that chaperone proteins may facilitate folding of newly formed protein as well as function by promoting refolding from the denatured state. Studies designed to elucidate the role of chaperones in folding of nascent peptides on ribosomes have been severely hampered by the lack of an adequate translation system in which the effect of the chaperones on folding could be studied directly.

Recently we developed a coupled transcription/translation system from *E. coli* that is deficient in certain chaperones, at least in GroEL and DnaK which we could quantitate by antibodies (Kudlicki et al., 1994). DnaK and GroEL are the representatives of two "classes of molecular chaperone machines" (Georgopoulos, 1992) that have been hypothesized to act sequentially in the folding of newly synthesized proteins (Langer et al., 1992). Even though the amounts of GroEL and DnaK were reduced to 17 and 3%, respectively, of the level determined for the S30 extract, we were able to synthesize DHFR and rhodanese in high yield and at nearly the same specific enzymatic activity as seen in the cell-free system using S30 or crude ribosomes (cf. Section V). Thus it appears that enzymes can be synthesized and folded on ribosomes without the requirement of chaperones.

VII. CONCLUSIONS, PROBLEMS, AND PROSPECTS

Images of ribosomes reconstructed from electron micrographs of crystalline arrays viewed at different tilt angles show an empty space between the ribosomal subunits and a cavity within the large subunit that extends to the exit domain on its outer surface. Although polyphenylalanine and polylysine may be exceptions, most nascent peptides including polyalanine and MS2 coat protein appear to be extended from the peptidyl transferase center into a cavity. Nascent MS2 coat protein and probably most other globular proteins fold into some type of tertiary conformation within this cavity as they are elongated by addition of new amino acids to their C-terminal end. A nascent protein eventually emerges from the cavity at a point on the outer surface of the large subunit called the exit domain that is distal to the peptidyl transferase center. It is this region of the large ribosomal subunit that provides the docking site for ribosomes on the endoplasmic reticulum. The cavity within the large subunit is accessible to the solvent in that small molecules of the size of methyl viologen and iodide ions penetrate into the subunit. However, binding of the ribosome to a membrane appears to cap or block the outer entrance so that access by iodide ions is prevented. These observations led to the conclusion that the cavity is closed in the form of a tunnel rather than an open channel. The cavity in the large subunit of bacterial ribosomes is adequate to accommodate nearly full-length MS2 coat protein and can be penetrated to some depth by relatively small proteins such as trypsin, proteinase K, or a Fab fragment of IgG. However, IgG itself is excluded. These considerations led to the hypothesis that the N-terminal

region of a nascent peptide begins to fold into a tertiary conformation within the ribosomal subunit as the peptide is extended. It follows that the cavity may provide a sheltered environment in which the nascent peptide is at least partially protected from proteolysis and other potentially destructive agents in the cytoplasm surrounding the ribosome. It should be emphasized that the relation of the conformation of the nascent peptide within the large ribosomal subunit to native conformation of the active protein is not known. Thus far, attempts to demonstrate functional activity of nascent peptides bound to ribosomes as peptidyl–tRNA have been negative or inconclusive.

Chaperones facilitate folding of at least some species of newly formed protein into enzymatically active conformation, but in most or all of the cases examined thus far some proportion of newly formed enzyme protein is enzymatically active in their absence, providing strong support for the hypothesis that folding of a protein during its synthesis is an inherent property of its primary structure. The proportion of enzyme protein that is folded into an active conformation in the absence of chaperones varied widely with the protein that is being considered and the conditions of its synthesis. Whether or not vectorial folding from the N-terminus as the protein is formed is a requirement for most proteins to reach their native conformation remains to be established. The requirement for vectorial folding to achieve the native state is likely to be dependent on the protein species and conditions.

A number of lines of evidence strongly suggest but do not conclusively prove that some of the chaperones and other factors can facilitate folding of a nascent peptide while it exists as peptidyl–tRNA on a ribosome. Clearly the signal recognition particle can recognize the signal sequence of nascent peptide that is destined for transport out of the cytoplasm. The mechanism by which elongation of such peptides is blocked in the absence of this interaction is unknown. It appears likely that some newly formed peptides interact with the GroEL/ES complex after they are released from the ribosome but that DnaK or DnaJ may interact with nascent chains. More detailed information about the structure of ribosomes and their function is required to resolve these questions. Generation of a high resolution crystal structure by X-ray diffraction of crystallized ribosomes holds great promise for the former. Fluorescence techniques coupled with procedures by which fluorophores can be incorporated at specific sites in a nascent peptide provide a powerful approach to the latter.

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