

Modeling and Experimental Study of the Progression of Nascent Proteins in Ribosomes

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1. INTRODUCTION

The translation of the genetic code into polypeptide chains is a fundamental cellular process. In rapidly growing bacterial cells the biosynthetic machinery constitutes about half of the dry weight of the cell, and the biosynthetic process consumes up to 80% of the cell's energy (Maaloe, 1979). The process of protein biosynthesis is mediated in all living cells by an organelle called the ribosome, which is a highly complicated ribonucleoprotein particle, operating as a giant multi-functional enzyme with a large range of tasks and constraints. The ribosome is built of two structurally independent subunits of unequal size, which associate upon initiation of protein biosynthesis. A typical bacterial ribosome contains about a quarter of a million atoms and is of a molecular weight of approximately 2.3 million daltons. About one third of its mass comprises some 58-73 different proteins, the rest is built of three chains of rRNA. Interestingly, the catalytic activities of the ribosome are not carried out solely by proteins. A remarkable feature of the ribosome is that the immediate region of the peptidyl transferase center, consists largely or totally of ribosomal RNA (Picking et al., 1992a), and ribosomes from which most of the protein mass has been removed are capable of imitating the production of peptide bonds (Noller et al., 1992). The implications of these observations for the origin of living systems and early evolution are profound.

During normal peptide elongation, the ribosome accommodates an mRNA chain to which one or two tRNA molecules are bound simultaneously

by codon-anticodon interactions. Peptide bond formation takes place in a specific domain of the large subunit, called the peptidyl transferase center. The chemical reaction of forming a new peptide bond includes the transfer of the nascent peptide from an ester linkage of the 3 hydroxyl of the 3' terminal ribose of the peptidyl-tRNA bound in the peptidyl transferase center, to the free amino group of the amino acid on an incoming aminoacyl-tRNA. This chemical reaction is associated with the physical movement of the tRNAs whereas the nascent peptide remains in essentially the same position in the peptidyl transferase center but is elongated by one amino acid (Hardesty et al., 1986; 1990).

Results of intensive biochemical, biophysical and genetic studies illuminated several functional aspects of the translational apparatus and led to suggestions for the overall shape and quaternary structure of the ribosome, for the spatial proximities of various ribosomal components, for the secondary structure of ribosomal RNA chains and for the approximate position of some reaction sites. However, the understanding of the molecular mechanism of protein biosynthesis is still hampered by the lack of molecular models. Such models for the ribosome, its subunits and its functional complexes are currently being determined by X-ray crystallography. For this aim three-dimensional crystals, diffracting best to 2.9 Å resolution, have been grown and are being investigated (von Boehlen et al., 1991; Franceschi et al., 1993), and a monofunctional reagent, prepared from an undecagold cluster, is being used for their quantitative and specific derivatization (Weinstein et al., 1989; 1992).

To aid the structure determination, images of the ribosome and its large subunit were reconstructed from tilt series of crystalline arrays, negatively stained with an inert material, goldthioglucose (Figs. 1 & 2 and in Arad et al., 1987; Yonath et al., 1987). Although the image reconstruction procedure is not free from experimental and conceptual limitations, its superiority over conventional electron microscopy methods was demonstrated by the conclusive and exclusive detection of several key features, associated mainly with internal vacant or partially filled hollows. Thus, the ribosome, which was believed to be a compact particle, is currently assumed as rather porous, containing holes, cavities, tunnels, gaps and voids. In this chapter we discuss the procedures employed for the three-dimensional reconstruction and describe the resulting models and their tentative interpretations, namely the assignment of the intersubunit free space as the site of protein biosynthesis and the ribosomal tunnels as the sheltered paths and folding domains for growing protein or peptide chains. Biochemical and modeling experiments, stimulated by these assignments and designed for shedding light on the progression of peptide chains are being highlighted.

2. THE PROCEDURES

1. Three-dimensional image reconstruction from crystalline arrays

For over three decades electron microscopy has been the choice method for viewing isolated ribosomal particles, despite its inherent shortcomings associated with observing surfaces in projection as well as with the probable shape distortions introduced by the microscope vacuum and the contacts with the flat microscopical grids. These efforts proved to be beneficial albeit somewhat misleading. They stimulated numerous sound functional experiments, but at the same time introduced structural concepts which were frequently based on subjective or incomplete interpretations, and yet became commonly accepted.

To avoid part of these shortcomings and to enhance the level of objectivity, low resolution models were reconstructed from tilt series of crystalline arrays (Arad et al., 1987; Yonath et al., 1987; Yonath and Wittmann, 1989; Berkovitch-Yellin et al., 1990; Yonath and Berkovitch-Yellin, 1993; Y. Fujiyoshi, private communication). This reconstruction procedure is fundamentally different from other electron microscopy methods in its inherent objectivity. Thus, diffraction patterns 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, as the two-dimensional arrays are held by crystalline forces, the distortions introduced by the flat electron microscope grids are reduced or eliminated.

Two different experiments were carried out for the elucidation of the shape of crystalline 50S subunit from *Bacillus stearothermophilus*. In the first, 28 Å resolution images were reconstructed from tilt series of negatively stained crystalline arrays observed at ambient temperature (Fig. 2 and in Yonath et al., 1987). In the second, unstained monolayers, embedded in vitreous ice, gave rise to filtered images at 18-20 Å (Avila-Sakar et al., 1993). Both images are rather similar to each other as well as to the independently reconstructed image (at 26 Å resolution) from crystalline arrays of this subunit from *Thermus thermophilus*, observed at cryo temperature (Y. Fujiyoshi, personal communication and Yonath and Berkovitch-Yellin, 1993). In the three models the extended arm, called also the L7/L12 stalk, which is a prominent feature in the conventional models (Wittmann, 1983), is rather short. Biochemical studies showed that this arm is rather flexible (Carazo et al., 1988), therefore it may either stretch and flatten when the particle is lying in isolation on the microscope grid, or assume a compact configuration when producing the crystalline arrays, in accord with the general assumption that extremely asymmetric conformations are less favorable for crystallization.

In fact, this arm also appears shorter in the random-conical reconstructed model obtained from non-crystalline 50S subunits (from *E. coli*) embedded in amorphous ice, and therefore supposed to be free from contacts with the grid (Radermacher et al., 1992). The advantages of the random-conical reconstruction procedure are noteworthy. This procedure is based on averaging the views of single particles at preferred orientations (Penczek et al., 1992), hence it is independent of the availability of crystalline samples. As particles embedded in amorphous ice may assume many orientations, none, or only a little, tilting is required. Therefore the reconstruction is not limited by the restrictive tilting range or by sample decay, the two major disadvantages of reconstructions from crystalline arrays at ambient temperature. However, as the random conical procedure requires subjective selection of images, its resulting models are only semi-objective. Furthermore, the effective resolution and reliability of such reconstructed models are severely restricted by the inherently low signal-to-noise ratio, as single particles are investigated, with no option for averaging or enhancement.

2. *Following the advancing nascent peptide by fluorescence*

Fluorescence techniques offer unique advantages to investigate movement, either within or between components of the translation apparatus. Their sensitivity is remarkable. Generally, fluorescence from coumarin and fluorescein derivatives can be detected at minute concentrations in solutions containing relatively high concentrations of proteins and nucleic acids, if appropriate attention is given to avoid background fluorescence from contaminants. Thus, fluorescence from a probe attached to the N-terminus of nascent peptides on ribosomes at a concentration of 1-3 mg/ml can be measured as protein synthesis progresses even if synthesis occurs on only 1-10 percent of the ribosomes. Such solutions are nearly transparent to the light used to excite these fluorophores, thus radiation damage to the sample is minimal and unlabeled or inactive components do not interfere with the measurements.

Changes in the environment or position of the probes can be monitored by one or more of five different fluorescence phenomena. These are: fluorescence emission spectrum, quantum yield or fluorescence intensity, fluorescence anisotropy, non-radiative energy transfer, and fluorescence quenching or enhancement by components of the soluble phase. Each of these fluorescence parameters may provide information about the local environment, movement, relative position and accessibility of the fluorophore (Hardesty et al., 1992). Small molecules such as methyl viologen and iodide or large ones, such as antibodies interact with the probe.

The most frequently used strategy to incorporate a fluorophore into a nascent peptide is to enzymatically aminoacylate a specific tRNA species then to chemically derivatize the amino acid moiety of the aminoacyl-tRNA. The

alpha amino group of the amino acid that is destined to occupy the N-terminal position of the nascent peptide has been labeled in most cases. The epsilon amino group of lysine as Lys-tRNA (Johnson et al., 1976) and the sulfhydryl group of cysteine as cys-tRNA may be used for labeling at internal positions.

The procedure chosen for labeling alpha amino groups in the current investigations, was the formation of the mercaptoacetyl amide by reaction with the succinimide ester of dithiodiglycolic acid (the disulfide of mercaptoacetic acid), followed by reduction of the disulfide with dithioerythritol (Odom et al., 1990). The resulting sulfhydryl group is reacted with either maleimide alkyl halide derivatives of the fluorophore of choice, such as CPM [3-(4-aminoethyl) thiouredophenyl]-7-diethyl amino 4-methyl coumarin]. After purification, the resulting coumarin-N-SAc-aa-tRNA was bound to the ribosomes either non-enzymatically or with peptide initiation factors to initiate the synthesis of nascent peptides with coumarin at their N-terminus. In addition to the naturally occurring tRNAs, synthetic species were generated that could be enzymatically aminoacylated with alanine, serine, or cysteine but had AAA anticodons, that would form homopolymeric peptides such as polyalanine with poly(U) (Picking et al., 1991a; 1992b). In this way a synthetic initiator tRNA species was formed, that could be aminoacylated with alanine by the alanyl-tRNA synthetase which had an AAA anticodon (Picking et al., 1991c). It was enzymatically bound to ribosomes in the peptidyl ribosomal site with peptide initiation factors and poly(U), to form enzymatically initiated polyalanine. Essentially the same strategy was used with natural mRNA in which AUG was the initiator codon by derivatizing the amino group of Met-tRNA_f.

N-terminal coumarin nascent peptides of different lengths were formed by stopping the peptide elongation. With homopolymeric peptides this was accomplished by transferring the elongation reaction mixture to an ice-water bath after incubation for a defined time at 37°C. The average length of the nascent peptides was calculated from the ratio of N-terminal coumarin residues to the internal amino acids that were incorporated into the newly formed peptides. As there is virtually no peptide initiation during the elongation reaction and in any event only the N-labeled nascent peptides are observed, the estimated length of the nascent peptides is rather accurate, compared to the procedures used earlier, in which the lengths of growing homopolypeptides was derived by the calculation of the ratio between the total number of incorporated amino-acids and the ribosomes in the solution, not distinguishing between fully- and non-active particles.

With natural mRNAs, translational arrest was accomplished by limiting one or more amino acids that occurred at a known position in the nascent peptide or by hybridization of a specific sequence of the mRNA with a complementary oligo-deoxyribonucleotide.

3. The model building procedure

Models were built on a computer-graphics terminal of Evans and Satherland PS390, using the program FRODO (Jones, 1978), according to the algorithm described in (Berkovitch-Yellin et al., 1990). These models consist of several entities: 70S ribosomes, 50S ribosomal subunits, tRNA molecules, an alpha-helix, the MS2 coat protein (at various folding states) and an antibody molecule. 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 the electron microscopy and due to the microscopic 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 the bound state of this molecule (Moras, 1989). Each of these two conformations, namely the "open-bound" and "closed-free", was tested for possible accommodation in the inter-subunit free space. As we could not discriminate between them at the resolution limits of the current reconstructions, only the first (Sussman et al., 1978) was used for model building.

The coordinates of the MS2 coat protein were given to us by Dr. L. Liljas (Uppsala), and we thank him and his collaborators for this. Since 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 IgG1 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. In contrast, the size and the general shape of the IgG molecule and its Fab fragment did play major roles.

3. APPROXIMATING THE SHAPES OF THE RIBOSOMAL PARTICLES USING CRYSTALLINE ARRAYS

Crystalline arrays of reasonable quality were obtained within a few seconds from 70S ribosomes and 50S ribosomal subunits of *B. stearothermophilus*, using combinations of salts and alcohols as crystallizing agents. Tilt series of these arrays, negatively stained with an inert compound, gold thioglucose, were used for three-dimensional image reconstructions (Figs. 1 & 2 and in Arad et al., 1987; Yonath et al., 1987). Despite their rather low resolution (47 and 28 Å, for 70S and 50S particles respectively) and the other shortcomings of electron-microscopy studies, due to the inherently objective

nature of these reconstructions, they provided a valuable tool for further understanding of the function of the ribosome. Thus, the significant similarities in specific features of corresponding regions in the reconstructed models of the 50S and 70S particles were exploited for assessing their reliability, to suggest a possible location of the 50S subunit within the 70S ribosome, to provide a plausible description for conformational changes associated with association and dissociation of the ribosomal subunits, to approximate a model for associated 30S subunit and to tentatively assign biological functions to some structural features (details below and in Yonath & Wittmann, 1989; Yonath et al., 1990, Berkovitch-Yellin et al., 1990, 1992; Franceschi et al., 1993; Yonath and Berkovitch-Yellin, 1993).

1. *A free-space was detected in 70S ribosomes*

A free space, estimated to occupy 15-20% of the total volume, was first detected in models reconstructed from crystalline 70S ribosomes from *B. stearothermophilus* (Fig. 1 and Arad et al., 1987; Berkovitch-Yellin et al., 1990). This free space was interpreted as the separation between the small and the large subunit. Recently, a similar feature was also revealed in the model reconstructed from single 70S ribosomes from *E. coli* by the random-conical procedure (Frank et al., 1991). In fact, intersubunit-gaps of varying sizes were observed in almost all the so far reported reconstructions of whole ribosomes, regardless of the reconstruction method, the source of the ribosomes or the level of organization: single particles (Wagenknecht et al., 1989; Frank et al., 1991), *in situ* sheets (Milligan and Unwin, 1986) or *in vitro* grown two-dimensional crystalline arrays (Arad et al., 1987; Yonath and Wittmann, 1989).

Additional support for a sizable free space at the intersubunit interface was provided by phylogenetic studies, which showed no evidence for substantial direct base pairing between the two subunits (Larsen, 1992). Interestingly, even the tight intersubunit rRNA clustering, suggested from crosslinking and fingerprinting studies, could be accommodated, after slight rearrangements, in the limited area available for direct contact between the two ribosomal subunits at the interface area around the void (Mitchell et al., 1992).

2. *Internal tunnels in large ribosomal subunits from various sources*

As early as in 1983, a hole was detected within a model 50S subunit obtained by the optimized series expansion reconstruction method (Vogel, 1983). Later on, a channel was seen in images of eukaryotic 80S ribosomes (of chick-embryos) at 50-60 Å resolution (Milligan and Unwin, 1986). A few elongated features of low density were clearly observed in the models reconstructed for the large subunit and the whole ribosome. These were named "tunnels", and assigned numbers according to the scheme shown in Fig. 3. The

mere appearance and level of clarity of these tunnels in different reconstructions were considered in subsequent assignments of functional elements in the ribosome.

One of these tunnels (t1 in Fig. 3) appears in all the reported reconstructions from crystalline 50S and 70S particles regardless of the staining procedure. This tunnel is about 100-120 Å in length and up to 25 Å in diameter (Yonath et al., 1987, 1990; Yonath and Wittmann, 1989; Berkovitch-Yellin et al., 1990), dimensions similar to those observed in the reconstruction from crystalline arrays of 50S subunits from *T. thermophilus* (Y. Fujiyoshi, personal communication). Furthermore, 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). It is noteworthy that two internal holes with no obvious functional roles were detected in the random-conical reconstructed models of single 50S subunits and 70S ribosomes from *E. coli* (Radermacher, 1992; Frank et al., 1991). Visual examination indicated that at a proper contour level, these holes may be connected to form a comparable tunnel. Interestingly, at this contour level the length of the extended arm (assigned as L7/12) is somewhat shorter than in the conventional consensus models, closer to that observed for particles embedded in amorphous ice or by reconstruction from crystalline arrays (reviewed in Yonath and Berkovitch-Yellin, 1993).

4. INTERPRETATIONS OF THE RECONSTRUCTED MODELS

1. *The original interpretation*

Two fundamentally different approaches were reported for interpreting the reconstructed models of the 70S ribosome. The first was applied to the reconstructed models from crystalline arrays. It is based solely on objective structural arguments, namely, on the similarities in specific features observed in different experiments. It was found that all models reconstructed from crystalline 50S subunits were almost indistinguishable (Berkovitch-Yellin et al., 1990, 1992; Yonath and Berkovitch-Yellin, 1993), whereas those derived from crystalline 70S ribosome were of comparable dimensions and shared common features but differed in bulkiness, and therefore called "thin" and "thick" (Fig. 1, and in Berkovitch-Yellin et al., 1990). Initially we attempted the interpretation of the more detailed and therefore more restrictive "thin" model. This interpretation served later as the basis for further functional assignments, which were found to fit even the bulkiest "thick" model. For this interpretation the 50S subunit was placed in a position which allows the matching of the direction of its main tunnel with that of tunnel t1 of the 70S ribosome (Fig. 3), 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 positioning allowed several functional assignments, described below.

a. A plausible protected path for nascent proteins

A large body of evidence derived from biochemical, fluorescence and functional experiments, obtained first in the sixties and reconfirmed recently, indicate that ribosomes mask the latest synthesized 25-70 amino acids of newly formed proteins (Malkin and Rich, 1967; Blobel and Sabatini, 1970; Smith et al., 1978; Ryobova et al., 1988; Yen et al., 1988; Kurzchalia et al., 1988; Evers and Gewitz, 1989; Kolb et al., 1990; Hardesty et al. 1990, 1993; Crowley et al., 1993, Hartl, 1993). Complementing information was obtained by immuno-electron-microscopy, showing that nascent proteins emerges out of the 50S subunit, opposite to the site of the formation of the peptide bond, the peptidyl transferase center (Bernabeu and Lake, 1982).

The feature accounting for these observations may be one of the internal tunnels, as all of them originate at the same location, span the 50S subunit and end at positions opposite to the main entrance. Our original interpretation was based on the choice tunnel t1 (Fig. 3), since its relative location, length and diameter suit all the so far available biochemical, functional, immunological, energetical and computational observations. As mentioned above, indications for the existence of such feature were derived from reconstructed images of eukaryotes (Milligan and Unwin, 1986), three different eubacteria, namely: *B. stearothermophilus* (Yonath et al., 1987) *E. coli* (Radermacher et al., 1987), *T. thermophilus* (Y. Fujiyoshi, personal communication) and an archeabacteria, *H. marismortui* (Eisenstein et al., 1991), providing convincing arguments for the universality of a protected path for the nascent polypeptide chain in the large ribosomal subunit.

b. A groove in the 30S subunit: a possible binding site for mRNA

A distinct region of crowded rRNA was revealed within the part of the model of the crystalline 70S ribosome, which was assigned as the associated 30S subunit (Yonath and Wittmann, 1989, Berkovitch-Yellin et al., 1990; Yonath et al., 1990). A similar region of high stain-density was detected by electron microscopical investigations of isolated 30S subunits (Oakes et al., 1990). In accord with cross-linking and model-building experiments which showed that the mRNA binds to the 30S subunits in an environment rich in rRNA (Brimacombe et al., 1988, 1990; Rinke-Appel et al., 1991), and with biochemical evidence showing that during translation a segment of about 30-40 nucleotides of mRNA is masked by the ribosome (Kang & Cantor, 1985), this groove was tentatively identified as the mRNA progression path. The resolution of the current reconstructed images is too low for an accurate determination of the dimensions of the groove, but a rough estimate indicated that it may

accommodate a stretch of the length comparable to that of the masked segment (i.e. 30-40 nucleotides) in random, U-shaped or helical conformations.

c. The intersubunit space as the site of protein biosynthesis

The assignments described above led to the suggestion that the intersubunit free space is the location for the various enzymatic activities of protein biosynthesis. The shape and the dimensions of tRNA allow its placement in the intersubunit space, so that its anticodon loop may associate with the mRNA, and its CCA-terminus is positioned so that the newly formed peptidyl group may extend into the common entrance to the tunnels. In this orientation the tRNA molecule may also form several non-cognate interactions (Fig. 4). At the current resolution of the reconstructions the two crystallographically determined orientations of tRNA: the native-closed and the bound-open one (for review see Moras, 1989), are indistinguishable. The positioning of the tRNA molecules within the intersubunit space, between the two subunits, accords well 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 ribosomes, the entire P-site tRNA molecule is inaccessible even to hydroxyl radicals (Huettenhofer and Noller, 1992).

Although under natural conditions no more than two tRNA molecules are bound to the ribosome simultaneously, three different binding sites were proposed for them, the A, P and E sites (for review see Nierhaus et al., 1993). Indeed, steric considerations showed that the intersubunit void is spacious enough to provide up to three tRNA binding sites, alongside with other non-ribosomal components participating in protein biosynthesis. It is noteworthy that these tRNA molecules may assume various relative orientations, ranging from parallel, the lowest space-requiring arrangement, to perpendicular (Fig. 4), the highest space-consuming one (Spirin, 1987).

2. Assessing the reliability of assignments at low resolution

a. The interpretation of the random-conical reconstructed model

The functional interpretation of the semi-objectively reconstructed image of the 70S ribosome by the random-conical procedure was based on objectively determined structural elements, mixed with comparisons with prominent contours seen in the "consensus" model of the 30S subunit (Wittmann, 1983). The latter is of significantly lower level of objectivity, as it was obtained by straightforward visualization of images in the electron microscope. Hints were also taken from the projected exterior shape of the particle and from its internal mass distribution, so that high density regions were interpreted as rich in rRNA

(Frank et al., 1991). First, some prominent contours within the random-conical reconstructed 70S ribosome were matched with the "consensus" shape of the 30S subunit. The rest of the 70S ribosome was compared with features such as the "L1 arm" and the "stalk base", characteristic to the shape of the 50S subunit viewed in the electron microscope (Wittmann, 1983) or reconstructed by the random-conical procedure at a comparable resolution, i.e. 40 Å (Radermacher et al., 1987).

As the original interpretation, the interpretation of the random-conical model utilizes the intersubunit void for the peptidyl transferase activity. It is noteworthy that the low-resolution assignments in the random-conical reconstructed models appear, at first glance, to be rather informative. Therefore they stimulated further efforts, aimed at accurate identifications of the exposed rRNA regions located at the subunits interface (Mitchell et al., 1992) and to somewhat speculative, albeit extremely detailed, propositions for the stereochemistry of the tRNA-mRNA interaction (Lim et al., 1992; Spirin et al., 1993).

b. An alternative interpretation

Except for the inherent low resolution there is no reason to suspect the feasibility of the original interpretation. Yet, the random-conical model gave rise to other tentative assignments. So far all the reconstructions of the 70S ribosome were performed at similar resolution limits (i.e. 47-50 Å). Furthermore, as the shape of the random-conical reconstructed 70S ribosome (Frank et al., 1991) is almost identical to the "thick" model, and its dimensions are very similar to those observed for the other reconstructed models, objective comparisons are, in principle, possible. A computer experiment was designed to facilitate this comparison. Thus, an alternative interpretation of the image reconstructed model from the crystalline arrays was suggested, accommodating the major concepts used for the interpretation of the random-conical reconstructed model (Frank et al., 1991).

The various models reconstructed from the crystalline arrays (Fig. 1 and in Arad et al., 1987) were systematically screened for alternative assignments, all based solely on the external shape and the internal features revealed in the reconstructed model of the crystalline 50S subunit (Yonath et al., 1987). It was found that positioning the reconstructed 50S subunit in the "thin" 70S model at any location other than the original one (Fig. 3), leaves hardly any density for the 30S subunit. In contrast, the "thick" model possesses sufficient density for the 30S subunit in several orientations, none of which show a convincing match between the shape of the 50S and the assigned part for it within the 70S ribosome. An acceptable fit, though far from being satisfactory, was obtained by aligning the direction of the main 50S tunnel with that called t2 in the 70S reconstructed model (Fig. 3). This tunnel is shorter

than t1 (40-60 Å in length), therefore even polypeptides of about 12-16 amino acids at an extended conformation may span it. However, as t1, t2 is of sufficient width to accommodate partially folded chains. Hence, it is suitable to provide the protected path for much longer nascent chains (>70 amino acids). It is noteworthy that the remaining part of the 70S ribosome, assigned as the 30S subunit, contains a groove suitable to host the mRNA, in a position permitting a tRNA molecule to bridge between it and the entrance to the tunnel (Fig. 4).

This exercise demonstrated clearly the uncertainties involved in attempts at the interpretation of globular and poorly detailed models, even when only objectively determined features are being considered (Franceschi et al., 1993). Yet it is most indicative. Its main lessons are that the size and shape of the intersubunit void permits the accommodation of the components associated with protein synthesis in several sterically reasonable arrangements; that the current models do not possess the level of details required for unequivocal positioning of the two subunits, and that the assignment of the intersubunit void as the site of protein biosynthesis does not critically depend on the accurate locations of the small and the large subunits. Nevertheless, the fact that all three interpretations utilize the intersubunit free space as the center of activity of the ribosome is most gratifying. This void, first reported in 1987 (Yonath et al., 1987), was until recently ignored or opposed. Even in 1992, experimental evidence indicating that the entire P-site-tRNA is placed between the two ribosomal subunits, were reported as unexpected (Huettenhofer and Noller, 1992).

5. INTEGRATING BIOCHEMICAL, FUNCTIONAL AND STRUCTURAL STUDIES

1. Homopolypeptides may choose exit paths different from that of native proteins

For over three decades the universal procedure for determining the extent of functional activity of ribosomes was based on programming them with poly(U) or poly(A) and monitoring the production of polyphenylalanine or polylysine, respectively. As such, this procedure was found to be reliable, informative, efficient and reproducible. Nevertheless, new compelling evidence indicated that these two homopolypeptides provide rather poor tools for the accurate and quantitative analysis of the structural properties of nascent proteins, due to the very nature of the measurements and to the inherent differences between the chemical properties of homo- and hetero-polypeptides.

In principle, following the growth of homopolypeptides is bound to yield average values. In quantitative investigations of the number of the amino acids incorporated into nascent chains at any given time they may introduce



Figure 1: A computer graphics display of the outer contours of the most deviating models of 70S ribosomes (the "thick" in blue and the "thin" in yellow). The models were reconstructed from negatively stained crystalline arrays of 70S ribosomes from *B. stearothermophilus* at 47 Å resolution (Arad et al., 1987).

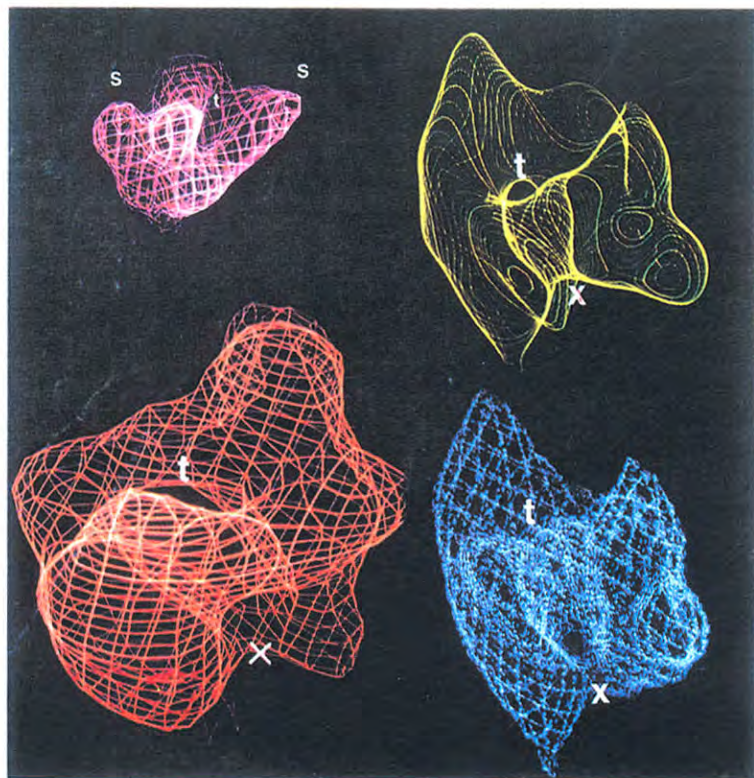


Figure 2: Several views of the model of the 50S subunit, as reconstructed from negatively stained crystalline arrays of these subunits from *B. stearothermophilus* at 28 Å resolution (Yonath et al., 1987). t points at the entrance to the internal tunnels and x shows the funnel-shape exit from the longest tunnel. This region may provide the site for the initial folding of nascent chains. s shows the approximate regions which are in direct contact with the 30S subunit. (not shown here) within the assembled 70S ribosome. The left views were taken from "top" (compared to Fig. 3), namely from the peptidyl-transferase center, and those on the right are seen from the side. In the bottom left and top right, the cleft leading into the tunnel is clearly seen.

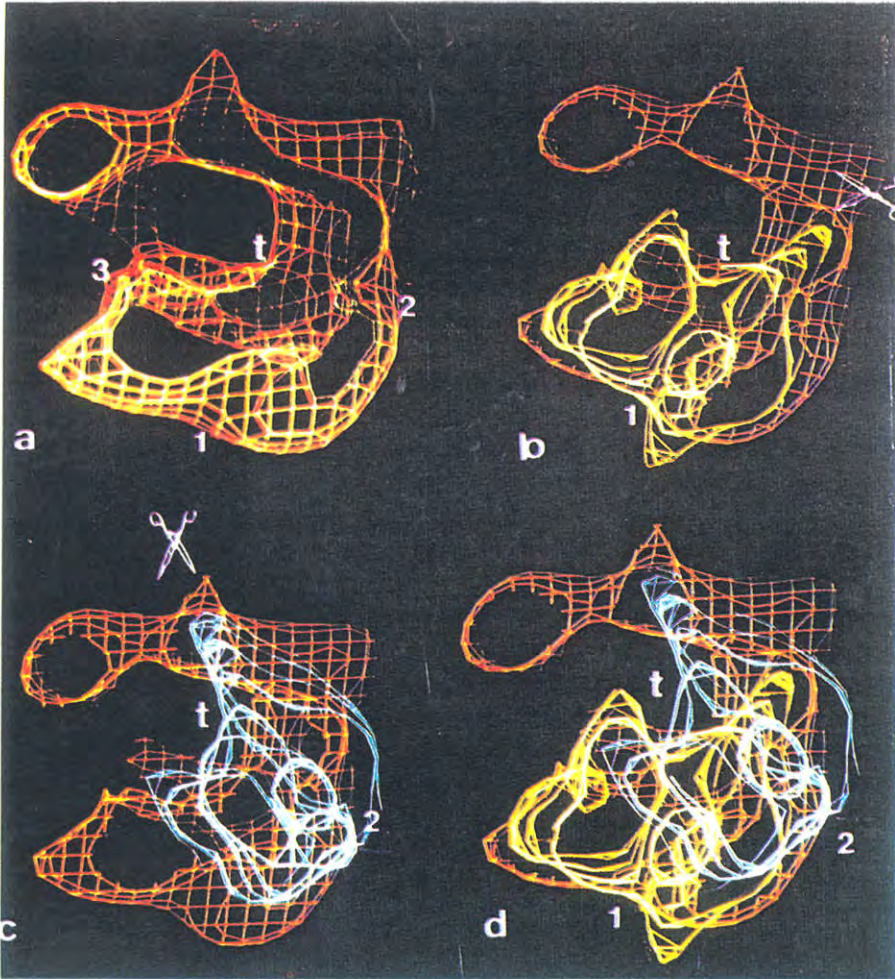


Figure 3: A slice of about 50Å in thickness, through the "thin" model of the 70S ribosomes (the yellow 70S in Fig. 1). **t** points at the common entrance to the three internal tunnels, and the numbers **1**, **2** and **3** show the exit sites from tunnels t1, t2 and t3 respectively. (b) (c) and (d) are superpositions of comparable slices of the reconstructed model of the 50S subunit, on the slice 70S ribosome (in orange) aligned so that its main tunnel coincides with (b): tunnel 1, as in the original interpretation, (c): tunnel 2, as in the alternative interpretation, and (d): both tunnels, for comparative inspection. The scissors point at the assumed contact region between the 30S and the 50S subunits.

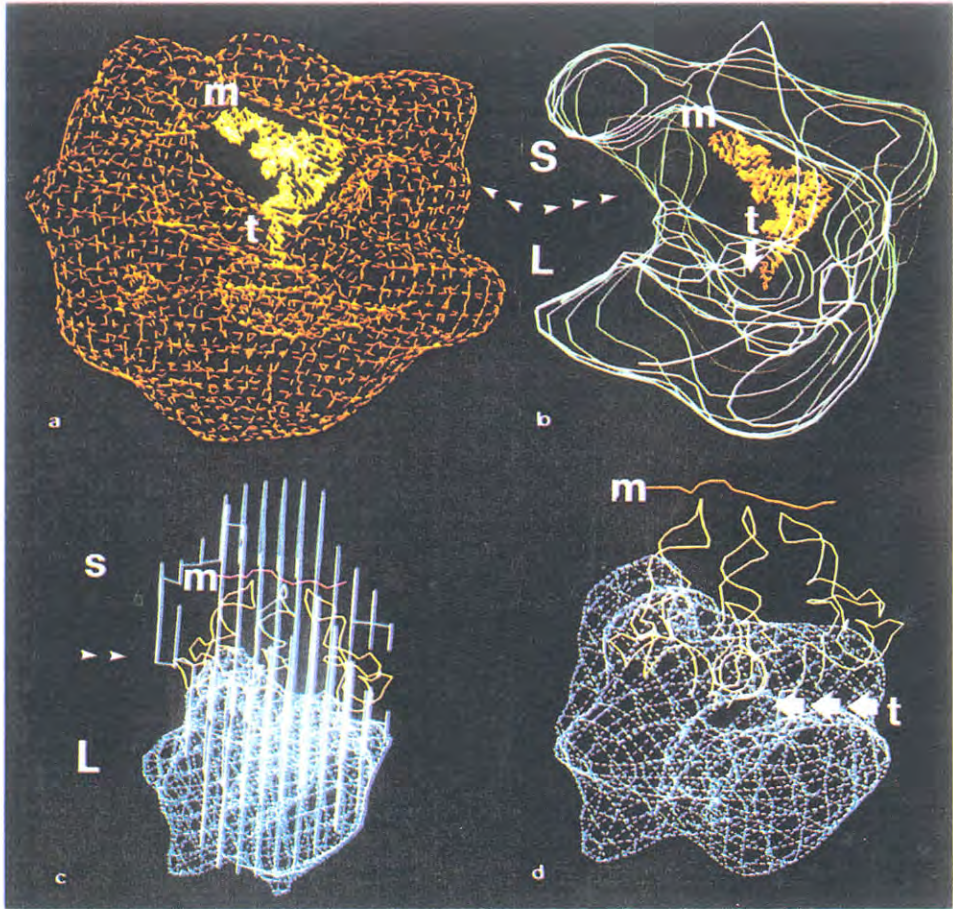


Figure 4: The original interpretation, based on the position and direction of tunnel t1 (Fig. 3). The arrow heads show the approximate directions of the interface between the S (small=30S) and L (large=50S) subunits, t points at the entrance to the tunnel. The presumed positions of the bound part of the mRNA chain are marked by m. A model built tRNA is shown in the "thin" (a), and "thick" (b) models (the yellow and blue in Fig. 1). (c) A perpendicular view of the "thin" ribosome (contours shown in light blue lines), showing that the free space at the intersubunit interface is large enough to accommodate three binding sites for tRNA molecules, two with their 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 left tRNA molecular in this figure). The aim of this exercise was solely to show the availability of space for three sites, not to suggest that three tRNA molecules are bound to the ribosomes simultaneously. The 50S subunit is highlighted as a blue net. (d) Same as (c), but only the 50S subunit is shown.



Figure 5: (a) A slice of 50Å thickness of the 50S subunit (in blue), into which a theoretical, undeformed stretch of an alpha-helix (in white) of 67 residues was model built. The helix was positioned along tunnel t1 (Fig. 2). A molecule of tRNA (in violet) 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 (in pink) 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.

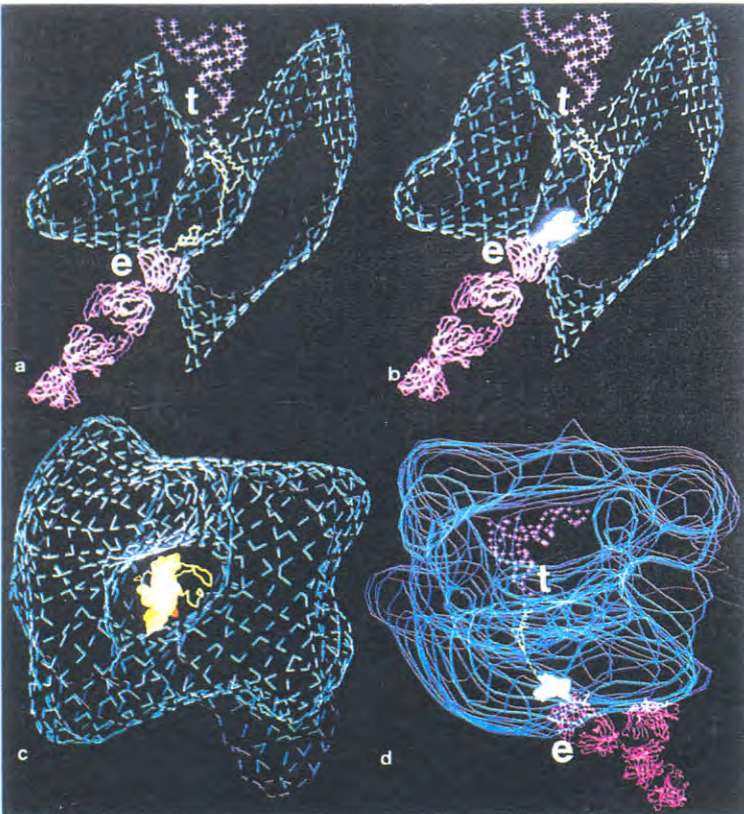


Figure 6: (a) A slice of 50Å thickness of the 50S subunit (in blue), into which the main chain of the MS2 coat protein was modeled. The protein was placed along the tunnel in a partially unfolded conformation, maintaining the native fold of its beta-stretches and the native (crystallographically determined) conformation of the segment 1-47. The C-terminus was placed in the vicinity of the proposed peptidyl transferase center and the N-terminus at the exit domain of the tunnel. The rationale of the positioning of the tRNA and the IgG molecules is similar to that described in Figure 5. t shows the entrance to the tunnel and e, its exit. (b) As (a), but the region 1-47 includes all atoms and is shown as a space-filling structure. (c) A view into the entrance of tunnel t1 from its entrance. The MS2 molecule is model built in the tunnel as in (b). For clarity, only the 50S subunit is shown. (d) As in (b) but the whole 70S ribosome is shown.

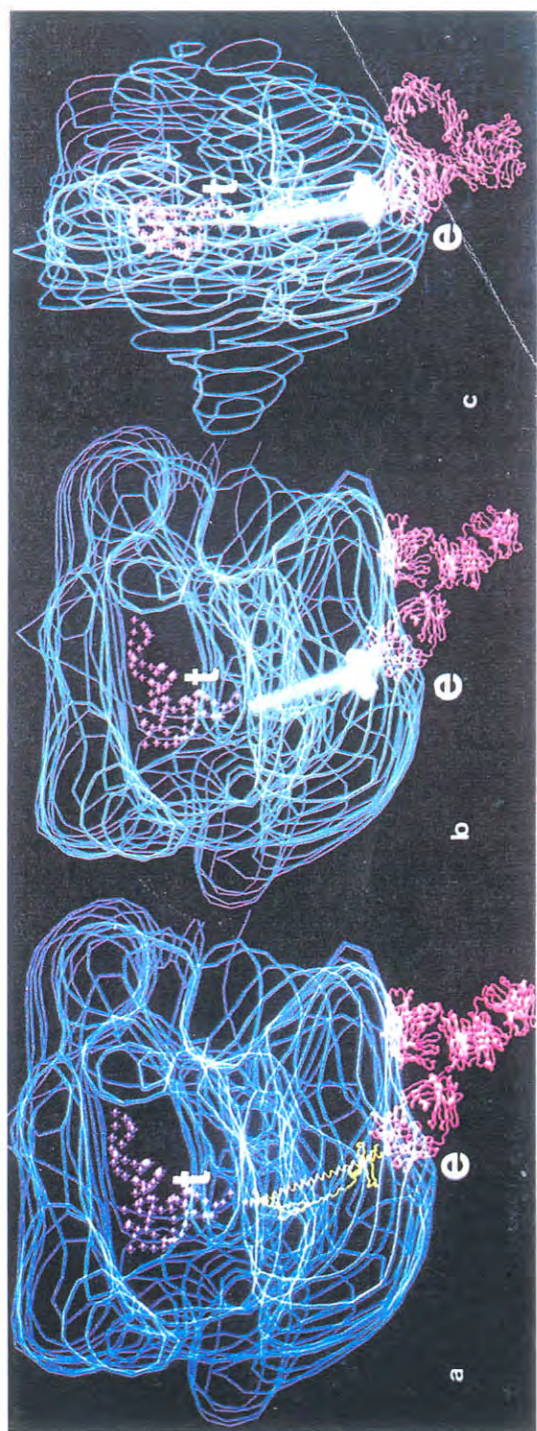


Figure 7: An imaginary composite of Figures 5 and 6. Both the alpha-helix and the MS2 protein were model built simultaneously, showing that even within tunnel t1 there is a possibility for different modes of migration. (a) shows the alpha-helix together with Figure 6(a), (b) with Fig. 6(b). (c) is a perpendicular view of (b). t shows the entrance to the tunnel and e, its exit.

conceptual and practical ambiguities, because the fraction of active particles in different preparations may vary tremendously, and in many cases it does not exceed 10-40%. In contrast to the rather accurate estimation of the average lengths of nascent homopolymeric peptides, which is made possible by the incorporation of a fluorescent probe, the values reported in some of the previous studies (reviewed in Yonath et al., 1990) were somewhat misleading. These were usually derived by a straight-forward calculation of the number of amino acids incorporated into peptides (using radioactive amino acids), and dividing them by the number of ribosomes in the solution, the latter being, by definition, rather inaccurate.

A large volume of experiments have been designed for analyzing the sequences, conformations and maximum lengths of the growing chains which can be accommodated in the tunnel. For instance, it was found that the rigidity introduced to proline-rich chains, presumably by the very nature of the closed proline ring, slows its progression (Evers and Gewitz, 1989). This finding is in accord with the fold suggested for segments of globular proteins rich in prolines. Thus, an extended rather stiff conformation was postulated for the sequence (pro-x)₄ in sorbitol dehydrogenase (Eklund et al., 1985).

Focusing on polyphenylalanine and polylysine by incorporating fluorescently derivatized amino acids into the N-terminus of nascent peptides, striking differences were observed in their progression, and the uniqueness of the path utilized by polyphenylalanine was firmly established (Picking et al., 1991b; Hardesty et al., 1990; 1993). Thus, it was concluded that in contrast to naturally occurring proteins, polyphenylalanine is formed as an insoluble mass located initially in the vicinity of the peptidyl transferase center (Odom et al., 1991). It is interesting to note that the synthesis of polyphenylalanine, unlike most other peptides, is insensitive to inhibition by erythromycin. A single molecule of this antibiotic binds tightly to a specific site on the 50S subunit so that it perturbs the environment and thereby the fluorescence from coumarin-N-Sac-aa-tRNA bound in the peptidyl transferase center (Odom et al., 1991). Synthesis of most peptides is blocked by erythromycin after one or two peptide bonds have been formed. Thus, the erythromycin inhibition does not appear to affect the peptidyl transferase center directly but rather a reaction or process that occurs after the peptidyl transferase reaction and at a close but physically distinct point.

It was found that polyphenylalanine can be formed on ribosomes to which erythromycin was previously bound. If the erythromycin is covalently labeled with coumarin, its fluorescence undergoes a pronounced shift to the blue when polyphenylalanine is formed. The emission spectrum is similar to that of coumarin in hydrophobic solvents such as benzene. Fluorescence from coumarin at the N-terminus of polyphenylalanine chains undergoes similar shift as the chain is extended. Bound erythromycin is rapidly displaced from empty

ribosomes if unlabeled erythromycin is added to the solution. However, the exchangeability decreases to nearly zero as the peptide is extended to a length of one hundred residues or more (Picking et al., 1991b). Conversely, erythromycin does not bind to ribosomes bearing nascent peptides of either phenylalanine or other amino acids. These results were interpreted to indicate that the bound erythromycin is buried under a mass of insoluble polyphenylalanine that accumulates very near to the peptidyl-transferase center as it is formed. Could the erythromycin binding site be the entrance to a tunnel in the 50S subunit through which most polypeptides are channeled?

Most natural proteins and their structural domains are globular and nearly spherical whereas, especially polyalanine but also polyserine to a somewhat lesser extent, tend to form alpha-helices which would be linear rods or nearly so. Polylysine is at the other extreme in that fluorescence, anisotropy, and quantum yield from its N-terminus decline to the level of a free peptide as the peptide is extended to a length of 5 to 10 residues. A relatively high proportion of the chains are lost from the ribosomes (Picking et al., 1992b). In contrast to polyphenylalanine and polylysine fluorescence anisotropy from the N-terminus of polyalanine chains remains relatively high to lengths greater than 60 amino acids whether they were initiated with an initiator or an elongator tRNA species (Picking et al., 1992b). Corresponding experiments in which polyserine was formed show a gradual decline in anisotropy that is intermediate between polyalanine and polylysine. The change in anisotropy appears to reflect movement of the N-terminal probe and flexibility of the nascent peptide. Polyalanine has a high propensity to form alpha-helix whereas polylysine tends to exist as a random coil (Creighton, 1983). If the nucleation step of alpha-helix formation is carried out as the peptide bond is formed, as has been convincingly hypothesized (Lim and Spirin, 1986), it appears very probable that nascent polyalanine chains exist as relatively stable alpha-helices within the ribosome. Fluorescence anisotropy for nascent polyserine is intermediate between the values for polyalanine and polylysine.

It was found that during peptide synthesis, the accessibility curves for Fab fragments derived from the same IgG are displaced towards shorter polyalanine chains by about 15 to 20 residues. Thus, probed N-termini became accessible to their IgG molecules (Mr 150 Kda) at a nascent peptide length of about 80 alanine or 60 serine residues, and to the corresponding Fab fragments (Mr 50 Kda) at lengths of 55 and 45 amino acids, respectively. Assuming that the polyalanine chains are in the form of an alpha-helix, the N-terminal coumarin becomes accessible to IgG and Fab at a distance of about 100 Å and 75 Å, respectively (1.5 Å/residue). In addition, by monitoring the sensitivity of these homopolypeptides to proteinase K (Mr 27 Kda), probed N-termini were released from the nascent peptide at an average length of about 40 alanines or 25-30 serines (Picking et al., 1992b; Hardesty et al., 1993).

Fig. 5 shows a theoretical, undeformed helix of 67 amino acids, placed within the main tunnel in the 50S subunit, so that its C-terminus is close to the tRNA binding site, and the N-terminus "interacts" with a hypothetical IgG (see above, in Experimental Procedures). Aesthetic considerations were the reason for choosing a chain-length of 67 rather than 60 or 80 residues, as the low resolution of the reconstruction permits accommodation of alpha-helical chains of either length. It is noteworthy that proteins of much longer length can be accommodated in the main tunnel in the 50S subunit as well as in its corresponding one in the 70S ribosome (t1, see Fig. 3). Thus, the entire MS2 coat protein (130 amino acids) could be fitted into this tunnel at a partially folded state (see below and in Figs. 6 and 7).

The views shown in Figs. 5 and 7 indicate clearly that by interacting with an alpha-helix of 67 amino acids, the IgG molecule makes tight contacts with the ribosome, whereas only a marginal increase (of 5-8 Å) in the diameter of the tunnel at the exit domain is sufficient for modelling the deeper penetration of an Fab molecule. Such positioning may allow the detection of probed N-terminus in shorter chains, of 45-55 residues, by the Fab fragment. As proteinase-K is significantly smaller than Fab, it may penetrate even deeper into the exit domain, releasing fragments of the nascent chains of somewhat shorter lengths.

2. Chemical and structural properties of the ribosomal tunnel

In experiments aimed at the verification of the mere existence of an internal ribosomal path for nascent polypeptide, N-termini of nascent polypeptides were detected by immuno-electron-microscopy at two distinct patches on the 50S subunit. Short polypeptides were observed close to the subunit interface, whereas longer ones at the far end of this particle (Rybova et al., 1988). It is conceivable that a specific common feature, or a combination of several features, located at the amino termini of natural proteins has a role in guiding them into the tunnel. It is still unclear whether this feature should bear a special structural motif, a chemical affinity to the entrance of the tunnel, or both. A few suggestions have been made, mostly associated with the occurrence of methionines and formyl-methionine at the N-terminus of native proteins, as well as with specific chemical affinities and/or electrostatic properties, but none led to a conclusive hypothesis.

A failure in entering the tunnel may be fed back into the biosynthetic machinery at early stages and lead to premature termination of the process. This hypothesis may explain the inhibition of peptide synthesis by erythromycin as well as some of the above findings. It also sheds light on the discrepancy between the rather small fraction (around 50%) of well prepared ribosomes found to be active in *in vitro* production of relatively long polypeptides, and the almost quantitative binding of mRNA and tRNA of similar preparations

(Rheinberger & Nierhaus, 1990; Hansen et al., 1990). In addition, it may shed light on the differences observed in the migration of short and long chains of newly synthesized polylysine or polyphenylalanine, respectively (Hardesty et al., 1990).

It was found that under appropriate experimental conditions, newly formed polyphenylalanine and polylysine may adhere to the large ribosomal subunits of *E. coli* (Gilbert, 1963), *B. stearothermophilus* (Gewitz et al., 1988; Yonath et al., 1990) and *H. marismortui*, (Yonath et al., 1987; 1990), even after the interruption of the biosynthetic process and dissociation of the ribosome. This attachment is tight enough to allow co-crystallization of short nascent homopolypeptides with 50S subunits. Thus, it seems that the exit path of these nascent homopolypeptides is composed of rRNA and hydrophobic patches, the components which are most likely to interact with polylysine and polyphenylalanine, respectively.

Fluorescence from coumarin at the N-terminus of homopolymers such as polyalanine and polyserine and of nascent natural proteins should provide information about the physical and chemical character of the tunnel. The hydrophobicity of the immediate environment of a fluorescent probe on minoacyl-tRNA in the peptidyl transferase center appears to be high as judged by the fluorescence emission spectrum and quantum yield but declines as polyalanine is formed (Picking et al., 1992b). Static quenching of fluorescence due to methyl viologen bound to ribosomal RNA also declines to nearly zero as the polyalanine chain is extended to a length of about 60 residues. The change in static quenching indicates that either there is a decrease in the proportion of RNA to protein in the region encountered by the N-terminal probe as the nascent polyalanine chain is extended, or that the N-terminal probe enters a cavity within the 50S subunit (Fig. 2). The change in apparent hydrophobicity and especially the results of studies in which the accessibility of N-terminal coumarin to anticoumarin antibodies was tested, strongly favor the latter interpretation.

Polyclonal antibodies that are produced against various coumarin derivatives of peptides and proteins appear to react primarily with coumarin ring itself and upon binding cause a large change in the fluorescence emission spectrum, quantum yield, and anisotropy any of which may be used to measure binding. As discussed above, the N-termini of nascent polyalanine chains start to become accessible to anticoumarin IgG or Fab fragments derived from it at an average length of about 40 residues and are partially shielded to an average length of about 80 residues (Hardesty et al., 1992). Considered together we interpret these results to indicate that the N-terminus of the polyalanine chain enters a cavity, possibly funnel shaped, into which Fab can penetrate further than IgG. A corollary of this hypothesis is that certain proteins, generally smaller in size, can penetrate into this cavity (Figs. 5,6 and 7).

As mentioned above, all three tunnels detected within the part of the 70S ribosome assigned as the 50S subunit (tunnels t1, t2 and t3) originate at the same location, at the "walls" of the intersubunit free space, in the proximity of the site assigned as the peptidyl transferase center in both the original and the alternative interpretations (Fig. 3). Each of these tunnels spans the large subunit and terminates at a location which can be described as opposite to the peptidyl transferase center. Therefore, in principle, each of them may serve as the protected path of the nascent protein, regardless of the exact orientations of the bound tRNA molecules and the location of the mRNA groove.

From considerations based on structural objectivity it was concluded that tunnel t1 (Fig. 3) is the most suitable one for shielding nascent chains, since its location within the 70S ribosome could be best matched with that found in free 50S subunit (Franceschi et al., 1993; Yonath and Berkovitch-Yellin, 1993). In addition, this tunnel provides the proper width and length to account for the observed shielding of newly formed peptides by the ribosome. It is noteworthy that large variations in the precise (or average) number of protected residues have emerged from different biochemical experiments. Thus, some indicated as little as 25 protected residues, whereas in others only chains of over 70 amino acids became exposed to a large variety of compounds, some rather large, like IgG (e.g. Hardesty et al., 1993), DnaJ or DnaK molecules (Hartl, 1993), the other very small (e.g. iodine, Crowley et al., 1993). The dimensions of the t1 tunnel may shed light on these puzzles, as the length of this tunnel is comparable to the length of 60-70 residues folded a perfect alpha-helix (see also Fig. 5) or 25-30 residues at an extended beta configuration.

Interestingly, tunnel t3 (Fig. 3) is of comparable dimensions to those of the t1 tunnel, discussed above. Hence, from size and shape considerations, the t3 tunnel may also provide the exit path of nascent proteins. So far no attempt has been made to utilize this tunnel for any functional activity, due to the fact that it was resolved only in one reconstructed image of the 70S ribosome, the so called "thin" model (Franceschi et al., 1993).

Tunnel t2, the basis for the alternative interpretation, is significantly shorter and somewhat wider than t1 or t3. From its dimensions it is clear that it can shelter chains of a minimum length of 12-15 residues (in beta conformation). As for t1, currently there is no way to determine the maximum length of chains which may be accommodated by the t2 tunnel, but it is clear that it may provide space for partially folded chains of over 70 residues, as t1 does.

3. Does early folding occur within the ribosome?

The MS2 coat protein is an optimal candidate for studying the progression of nascent chains not only because of the availability of its mRNA,

but also because in its native conformation its N-terminal methionine is pointing toward the outer solution (Valegard et al., 1990). This protein contains 130 amino acids, folded into a nearly spherical, relatively compact structure, composed of seven antiparallel beta-strands and two short alpha-helices (Valegard et al., 1990). It was found that a coumarin at the N-terminus of the nascent MS2 peptide was shielded by the ribosome from IgG to a length of at least 120 amino acid residues, the longest nascent peptide that was obtained by translational arrest (Hardesty et al., 1993). The N-terminal probe on all lengths of nascent peptides that were aborted and released from the ribosomes by reaction with puromycin were readily accessible to IgG. In contrast to the results with nascent polyalanine, static quenching by methyl viologen of fluorescence from the N-terminal probe remained relatively high with nascent peptides of nearly full length.

The results of the IgG accessibility and methyl viologen quenching experiments indicate that nascent MS2 peptides are folded into some type of relatively compact, globular-like conformation in or on the ribosome. This hypothesis was tested directly by non-radiative energy transfer between coumarin covalently attached to cysteine that was incorporated into position 47 and fluoresceine on the N-terminus of the nascent peptides. With ribosome-bound nascent peptides of about 125 residues energy transfer of more than 75% was observed. Similar levels of energy transfer were observed with nascent peptides that were released by reaction with puromycin. A high level of energy transfer, probably greater than 95%, would be expected between coumarin to fluorescence based on the distance between the N-terminus and cysteine 47 in the crystal structure. The amount of energy transfer actually observed, at least 75% was likely to be a minimum value due to technical difficulties with the energy transfer experiments. In any event, they indicate a distance that is much shorter than anticipated if the nascent peptides were in the form of an alpha-helix, beta-strands or a random coil (Creighton, 1983).

Experiments similar to those described above, investigating nascent MS2 peptides with anticoumarin IgG, were carried out with the Fab derived from it. In contrast to IgG, significant Fab binding was observed with nascent chains of about 45 residues and half maximum binding was observed with nascent chains of 60 to 75 residues (Hardesty, et al., in press, 1993). These results were in sharp contrast to those observed for the differential shielding of the N-terminal probe by the ribosome as monitored by interactions with IgG. Considered together with the results of the fluorescence experiments, they appear to indicate that the nascent MS2 peptides are folded within a cavity or folding domain in the 50S subunit that can be at least partially penetrated by Fab but not by IgG.

Fig. 6 is a visual presentation of the modeling of these findings. The crystallographically determined structure of MS2 was the basis for this

modelling experiment. The C-terminus of the MS2 protein was placed in the vicinity of the suggested peptidyl transferase center. As described above, this site was assigned at the same location at the intersubunit free space by both the original and the alternative interpretations. The main chain of the MS2 protein was placed along the tunnel in a partially unfolded conformation, while maintaining the native fold of the long stretches of the beta-structures as well as the native conformation of the segment 1-47. The latter was placed at the exit domain of the tunnel, in accord with the experiments concerned with the extension of polyserine and polyalanine (Fig. 5).

From Figures 6 and 7 it is clear that a protein of 130 residues can be accommodated within the t1 tunnel, even when its prominent features are beta rather than alpha-helices, and more than a third of the protein assumes close to native conformation. Hence, it seems that the tunnel may provide the needed space for early folding (the so called folding cavity or folding domain, above). Furthermore, the graphic representation validates the suggestion derived from the immuno-fluorescence experiments, that the folded portion of the protein is located at its N-terminus (including at least residues 1-47), hence is expected to be detected at the exit domain of the tunnel.

This modeling exercise also shows that different modes of progression are possible even within tunnel t1 (Fig. 7). Thus, although both the alpha-helix and the MS2 protein originate at the same site (the peptidyl transferase center, where the CCA end of the tRNA was placed) and emerge out of the ribosome at approximately the same location, the size and shape of the t1 tunnel allows their progression along different internal paths (Fig. 7). As suggested above, this tunnel ends in a funnel-shaped domain, allowing Fab fragments or smaller molecules to interact with either natural proteins or homopolypeptides of a length shorter than that required for attachment to IgG molecules.

An additional benefit from the modeling experiments is the highlighting of the differences in the shapes of free 50S subunits, and those assigned as it in the associated 70S ribosome. Although these differences were carefully analyzed in our previous studies (Yonath and Wittmann, 1989; Berkovitch-Yellin et al., 1990, 1993), it is still not clear whether they reflect real conformational changes occurring by the association of the two ribosomal subunits to form the 70S ribosome, or that they result from the resolution limits at which the two images were reconstructed (28 vs. 47 Å). Some of these differences are well illuminated in the Figures presented here. Noteworthy are those in the vicinity of the exit of the tunnel t1 (Figs. 3, 4 and 6). If indeed they indicate conformational changes, these may be exploited for proposing a mechanism for the natural detachment of the ribosome from membranes upon the termination of the biosynthetic event.

It is most tempting to take advantage of the existence of three different tunnels for speculating a specific role for each of them. One may assume that

the main difference between the three tunnels is in the environment which they provide, and the nascent proteins are directed into specific paths according to their chemical affinities. This "multiple choice" hypothesis may explain the differences observed between the progression of different proteins (and between them to artificial hetero- and homopolypeptides), and should lead to careful experiments, investigating the correlation between the progression of proteins and their chemical nature. A wild, albeit rather logical, hypothesis is that one of the tunnels is used as a "mistake collector", for disposal of wrongly synthesized proteins, once a fatal mistake is introduced into them. Many artificial sequences may fall into this category, including some (although not necessarily all) homopolypeptides. Naturally the paths for these should be shorter than that designed for maximum protection of nascent proteins, and explain why in some experiments (e.g. when the production of polyphenylalanine or polylysine were monitored), only relatively short chains appeared to be shielded by the ribosomes.

Another speculation is that each tunnel is involved in a different activity. Perhaps one of them may provide an exit path for the naked tRNA. Furthermore, assuming one of the tunnels to be the "folding domain" for globular nascent proteins, the second and/or the third may host a chaperonin or a chaperonin-like protein. Although the latter hypothesis is most appealing, it should not be forgotten that most chaperonins are rather large, therefore may not fit into the tunnel, but may well be situated near their exit funnels. Unfortunately these dilemmas can not be resolved at present. They, as well as some others, must wait until models of much higher resolution become available.

A word of caution is due. So far only tunnel t1, or indications for its existence, were resolved in all reconstructions. Furthermore, only according to the original interpretation of the crystalline 70S model (Yonath and Wittmann, 1989; Berkovitch-Yellin et al., 1990) do all three tunnels belong to the 50S subunit. According to the alternative interpretation, which is based on that suggested for the random-conical model (Frank et al., 1991), the large subunit contains only tunnel t2 (the short tunnel, as seen in Fig. 3). In fact, according to this interpretation, tunnel t1 is located at the fused borders of the 50S and 30S subunits, and t3 is buried well within the latter. At this location it may provide the path for the mRNA.

It is noteworthy that in one reconstruction of the 50S subunit (from *B. stearothermophilus*) a second, shorter tunnel was revealed. It was named the "side tunnel" (Yonath et al., 1987). As is the case for the three tunnels seen in the 70S ribosome, the main and the side tunnel originate at the same place, and terminate at the other side of the 50S subunit. So far no attempt has been made to match its direction with that of any of the tunnels detected in the "thin" 70S ribosome.

4. The influence of the tentative assignments on the design of biochemical experiments: The design of crystallizable complexes that mimic ribosomes in defined functional states.

Despite the speculative nature of the current assignments, they significantly advanced the crystallographic studies, as complexes in which the intersubunit void should be fully or partially filled (containing charged tRNA molecules and mRNA analogues), were designed.

All three-dimensional crystals of 70S particles obtained so far, namely from *E. coli*, *B. stearothermophilus* and *T. Thermophilus*, are either too small or diffract to a very low resolution, 20-45 Å (Wittmann et al., 1982; Trakhanov et al., 1989; Berkovitch-Yellin et al., 1991). This property was related to the possible inherent flexibility of the ribosome, which is made possible by its large void. This free space may provide the flexibility, dynamics, and the mobility needed for the process of protein biosynthesis, leading to significant conformational heterogeneity. Thus, it is believed that each preparation of 70S ribosomes includes a collection of conformations, including some trapped rigid conformational states alongside with others, devoid of non-ribosomal biosynthetic components, which may assume high flexibility.

Active ribosomes, constructed from purified 50S and 30S subunits which are expected to be fairly homogenous, yielded crystals containing solely 50S subunits (Yonath et al., 1990; Berkovitch-Yellin et al., 1991), indicating that the interparticle interactions in the crystals of 50S subunits are stronger than the affinity between the large and small subunits in 70S particles, kept for long periods without being active in protein biosynthesis. These findings are in accord with observations made using two-dimensional arrays of 80S ribosomes, which could be depleted of the small subunits and still maintained their packing integrity (Milligan and Unwin, 1982), and highlight the readiness of the large ribosomal subunits to crystallize, a property which is also reflected in the large number of crystal forms obtained from bacterial 50S subunits (Yonath et al., 1980, 1986, 1990; Makowski et al., 1987; Muessig et al., 1989; Volkmann et al., 1990; von Boehlen et al., 1991).

To minimize the flexibility and to increase the homogeneity of the crystallized material, complexes were prepared, containing ribosomes trapped in conformations mimicking defined stages in protein biosynthesis. A simple complex, composed of 70S ribosome from *T. thermophilus* with two p^{he}tRNA^{phe} molecules and a chain of about 35 uridyl residues, was crystallized. In this complex the mRNA is of a length which may fit in the groove found in the 30S subunit so that no long stretches of it project into the solvent, the intersubunit space is rather occupied, and, above all, most of the ribosomes are "trapped" in a similar conformation.

Despite the non optimal composition of this complex (e.g. a homopolynucleotide rather than an RNA chain of a designed sequence was used

as mRNA), dramatic improvements in the reproducibility of crystal growth and in the internal order of the crystals were observed. Whereas the best three-dimensional crystals of 70S ribosomes (of *T. thermophilus*) diffract to 20-24 Å resolution (Trakhanov et al., 1989; Berkovitch-Yellin et al., 1991), those grown from this complex exhibit sharp diffraction patterns to 12-14 Å (Hansen et al., 1990; von Boehlen et al., 1993). To assess the individual contributions of the different components to the stability of this complex, 70S ribosomes were cocrystallized with a chain of 35 uridines. Only poorly shaped three-dimensional crystals were occasionally grown, indicating the larger contribution of the tRNA to the stability of the complex.

The studies reported above aimed at investigating the chemical properties of the exit path of the nascent chains by identifying compounds that adhere to it, led to the crystallization of a second type of complexes, composed of large ribosomal subunits, short nascent polypeptide and tRNA molecules. As expected, the time needed for the translation of a given length of mRNA depends on its sequence (Evers and Gewitz, 1989), and various polypeptides differ in their ability to adhere to the ribosome. Using poly(U) or poly(A) as messenger RNAs, small crystals have been grown from 50S subunits of either *H. marismortui* and *B. stearothermophilus*, together with a short nascent polyphenylalanine or polylysine (of 8-18 amino acids in length) and one molecule of their cognate tRNA (Gewitz et al., 1988; Muessig et al., 1989). In addition, conditions were determined for stoichiometric binding of tRNA_{phe} to several ribosomal particles (Weinstein et al., 1992).

Since tRNA is a part of all these complexes, it is an obvious target for indirect attachment of heavy-atom clusters to ribosomal crystals. As most of the interactions of tRNA with the ribosome are well characterized biochemically, crystallographic determination of the position of the heavy-atom clusters attached to it should provide information, useful not only for phase determination but also for the localization of the tRNA binding site on the ribosome.

6. CONCLUSIONS AND PROSPECTS

Despite the exciting observations reported in this chapter, it is evident that models of a higher resolution, coupled with careful and sophisticated biochemical studies, are essential for a better understanding of the fate of the nascent proteins as well as for more accurate assignments of functional centers within the ribosome. Except for the crystallographic studies, which are expected, in the long run, to yield such detail, cryo-temperature image reconstructions from unstained crystalline arrays is the most promising procedure. Preliminary efforts in this direction have already led to the growth of arrays of 50S subunits, diffracting to about 15 Å resolution (Avila-Sakar et al., 1993). The models which are expected to emerge from these monolayers,

accompanied by highly sophisticated biochemical and functional studies, are bound to add substantially to our understanding of this system.

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