Chapter 18
Ribosomes: Ribozymes that Survived Evolution Pressures but Is Paralyzed by Tiny Antibiotics

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Abstract An impressive number of crystal structures of ribosomes, the universal cellular machines that translate the genetic code into proteins, emerged during the last decade. The determination of ribosome high resolution structure, which was widely considered formidable, led to novel insights into the ribosomal function, namely, fidelity, catalytic mechanism, and polymerize activities. They also led to suggestions concerning its origin and shed light on the action, selectivity and synergism of ribosomal antibiotics; illuminated mechanisms acquiring bacterial resistance and provided structural information for drug improvement and design. These studies required the pioneering and implementation of advanced technologies, which directly influenced the remarkable increase of the number of structures deposited in the Protein Data Bank.

18.1 Introduction

The translation process requires a complex apparatus composed of many components, among which the ribosome is the key player, as it is actively involved in the translation process. Ribosomes are universal ribozymes performing two main tasks: decoding the genetic information and polymerizing amino acids, while providing the framework for the proper positioning of all other participants, including mRNA, its substrates (tRNAs) and initiation, elongation, release and recycling factors that ensure that protein synthesis occurs progressively and with high specificity. They operate is each living cell continuously since the constant programmed cell death, which implies constant proteins degradation, requires simultaneous production of proteins. Hundreds of thousands of ribosomes are present in typical mammalian

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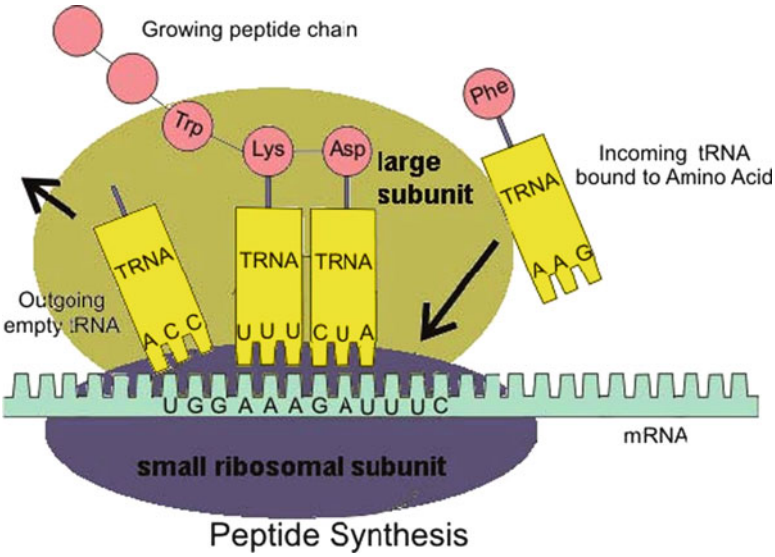

Fig. 18.1 Schematic view of the translation process

cells. Fast replicating cells, e.g. liver cells, may contain a few millions ribosomes. Even bacterial cells may contain to 100,000 ribosomes during their log period. mRNA chains, produced by the transcription of the segments of the DNA that should be translated, carry the genetic information to the ribosomes, and tRNA molecules bring the cognate amino acids to the ribosome (Fig. 18.1). The tRNA molecules from all living cells are built of double helical L-shape molecules containing an anticodon loop that matches its three-nucleotide codes on the mRNA on one of their edges, ~70 Å away, their 3′ends are single strands with the universal sequence CCA to which the cognate amino acid is bound by an ester bond. For increasing efficiency, a large number of ribosomes act simultaneously as polymerases synthesizing proteins by one-at-a-time addition of amino acids to a growing peptide chain, while translocating along the mRNA template, producing proteins on a continuous basis in an incredible speed (5–15 new peptide bonds per second, in eukaryotes and prokaryotes, respectively).

The ribosomes are giant assemblies composed of many different proteins (r-proteins) and long ribosomal RNA (rRNA) chains. The ratio of rRNA to r-proteins (~2:1) is maintained throughout evolution, except in mitochondrial ribosome (mitoribosome) in which almost half of the bacterial rRNA is replaced by r-proteins. In all organisms ribosomes are built of two subunits, which associate to form functionally active ribosomes. In prokaryotes, the small subunit, denoted as 30S, contains an RNA chain (16S) of ~1,500 nucleotides and ~20 different proteins. The large subunit (50S in prokaryotes) has two RNA chains (23S and 5S RNA) of about 3,000 nucleotides in total, and different <31 proteins. The available three dimensional structures of the bacterial ribosome and their subunits show that in each of the two subunits the ribosomal proteins are entangled within the complex

rRNA conformation, thus maintaining a striking dynamic architecture that is ingeniously designed for their functions: precise decoding; substrate mediated peptide-bond formation and efficient polymerase activity. The structural bases for ribosomal functions, as obtained by high resolution crystallographic studies are summarized in several recommended recent reviews [34, 46, 58, 73]. Further insights obtained from the combination of the crystallographic results with those emerging from single-molecule techniques (cryogenic electron microscopic and fluorescence resonance energy transfer) are outlined in [23]. Selected topics of ribosome function are discussed below. As so far high resolution structures are available only for prokaryotic ribosomes, the discussion is confined to these ribosomes and to insights evolved from their structures.

18.2 Snapshots Along the Birth of the Nascent Chains

While the elongation of the nascent chain proceeds, the two subunits perform cooperatively. The tRNA molecules are the non-ribosomal entities combine the two subunits, as each of their three binding sites, A-(aminoacyl), P-(peptidyl), and (exit), (Fig. 18.2) resides on both subunits. Their anticodon loops interact with the mRNA on the small subunit, and their acceptor stems with the aminoacylated or peptidylated 3'ends are located on the large subunit.

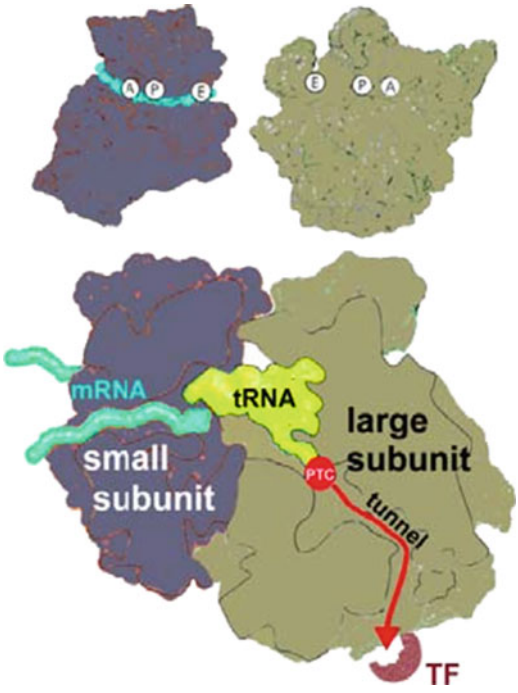

Fig. 18.2 (*Left*) The two subunits. The approximate positions of the mRNA and three tRNA sites are marked. (*Right*) A slice through the center of the translating ribosome showing the P-site tRNA (*blue*), the nascent chain path and direction are shown as a *red arrow*, and the first chaperone encountering the emerging nascent chain (*TF* trigger factor) is represented by a *half circle*

The small subunit provides the path along which the mRNA progresses, the decoding center and the mechanism controlling translation fidelity [46, 51]. Translation initiation is the rate-limiting step of the entire process. It starts by the correct selection and placement of the mRNA reading frame, with the help of the initiation factors, and then proceeds through a tightly regulated steps. Within the initiation complex the initiation codon is decoded in the P-site of the ribosomal subunit, and involves GTP-binding [57].

The large subunit contains the site for the main ribosomal catalytic function, namely polymerization of the amino acids and provides the dynamic protein exit tunnel [13]. The structure of its larger subunit revealed that the ribosome is a ribozyme with RNA at the core of its enzymatic activity. Simultaneously with the advancement of the mRNA along the path in the small subunit, peptide bonds are being formed in the large subunit. This inherently dynamic process requires small and large-scale motions of the ribosomal substrates (e.g., the intersubunit rotational movements during tRNA-mRNA translocation), coupled to conformational rearrangements of its components, facilitating the translocation of the tRNA 3′end from A- to P-site, the detachment of the P-site tRNA from the growing polypeptide chain, the passage of the deacylated tRNA molecule to the E-site and its subsequent release. The nascent proteins progress along a dynamic tunnel and emerge from the large subunit (Fig. 18.2) into a shelter formed by ribosome-bound trigger-factor, acting as a chaperone preventing aggregation and misfolding [8, 53].

The current consensus view is consistent with ribosomal positional catalysis assisted by its P-site tRNA substrate (e.g. [9, 11]) and not by acid/base mechanism [43]. All known structures indicate that the ribosomes provide the suitable stereochemistry for peptide bond formation, the guided path for the A- to P- site translocation and the appropriate geometrical means for substrate mediated catalysis. In all of the so far determined structures the ribosomal catalytic site, called the peptidyl transferase center (PTC), is situated within a highly conserved symmetrical region (Fig. 18.3) that connects all ribosomal functional centers involved in amino-acid polymerization, namely the tRNA entrance/exit dynamic stalks, the PTC, the nascent protein exit tunnel, and the bridge connecting the PTC cavity with the vicinity of the decoding center in the small subunit. Hence, it can serve as the central feature for signaling between all the functional regions involved in protein biosynthesis, that are located remotely from each other (up to 200 Å away), but must “talk” to each other during elongation [1, 2, 9]. As the symmetry relates the backbone fold and nucleotides orientations, but not nucleotide sequence, it emphasizes the superiority of functional requirement over sequence conservation.

The linkage between the elaborate architecture of the symmetrical region and the position of the A-site tRNA, as observed crystallographically [9] indicates that the translocation of the tRNA 3′end is performed by a combination of two independent, albeit synchronized motions: a sideways shift, performed as a part of the overall mRNA/tRNA translocation, and a rotatory motion of the A-tRNA 3’end along a path confined and navigated by the PTC walls, of which all nucleotides have been classified as essential by a comprehensive genetic selection analysis

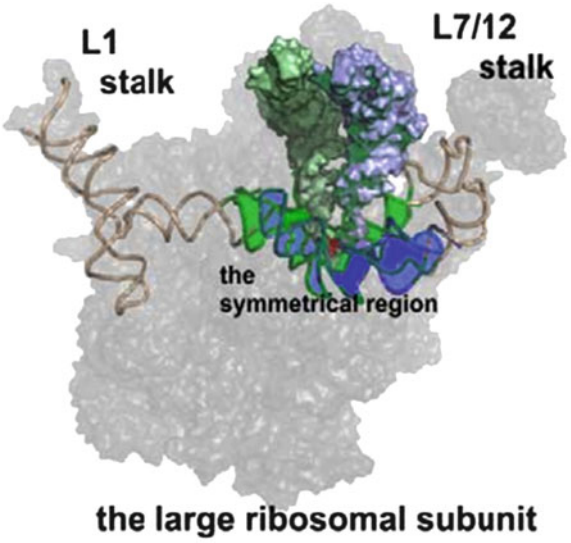

Fig. 18.3 The symmetrical region within the large ribosomal subunit (colored in *blue* and *green*). Its extensions are shown in *gold*. The A-site tRNA is shown in *metal blue* and the P-site tRNA in *light green*

[49]. This motion enables the formation of all interactions that are prerequisite for substrate positioning [33, 56, 63], for mediating acceleration [64], and for the formation of the transition state (TS) of this reaction [24]. This stunning architecture allows for the PTC remarkable ability to rearrange itself upon substrate binding, explaining the pace difference between the formation of single peptide bond by minimal substrates and possessive amino acid polymerization and verifying the finding that the peptidyl transfer reaction is modulated by conformational changes at the active site [9, 11, 12, 54, 67].

18.3 The Proto Ribosome Concept

The high level of conservation suggests that the modern ribosome evolved from a simpler entity that can be described as a pro-ribosome, by gene fusion or gene duplication. In particular, the preservation of the three-dimensional structure of the two halves of the ribosomal frame regardless of the sequence demonstrates the rigorous requirements of accurate substrate positioning in stereochemistry supporting peptide bond formation. This, as well as the universality of the symmetrical region led to the assumption that the ancient ribosome contained a pocket confined by two self folded RNA chains, which associated to form a pocket like dimer (Fig. 18.4).

As RNA chains can act as gene-like molecules coding for their own reproduction, it is conceivable that the surviving pockets became the templates for the ancient ribosomes. In later stage these primitive RNA genes underwent initial optimization

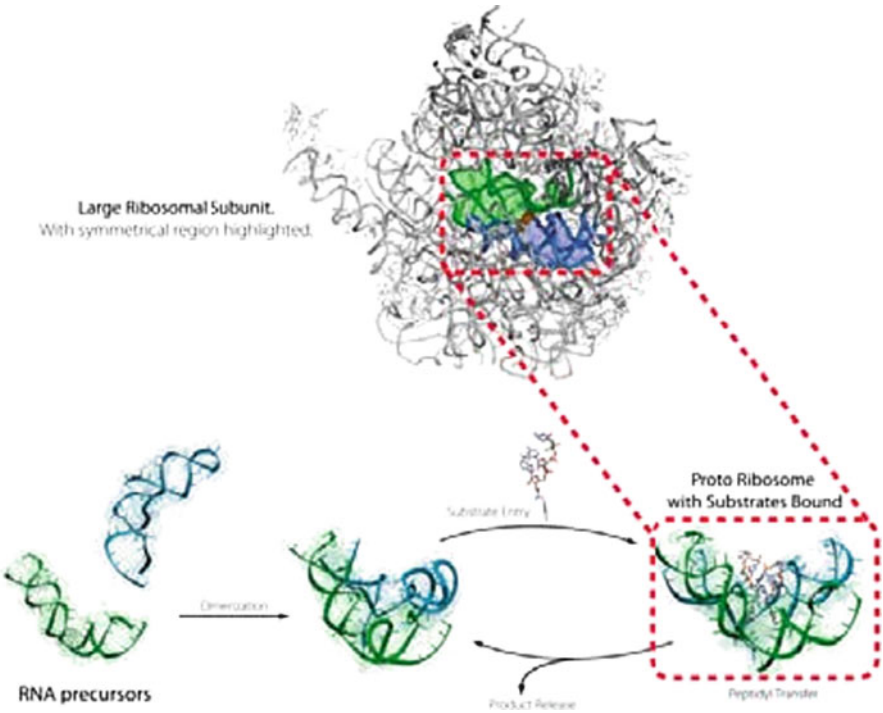

Fig. 18.4 Shows how the proto ribosome could have evolved. The symmetrical region is highlighted within the contemporary ribosome. A view showing its pocket-like nature is shown on *bottom right*

to produce a more defined, relatively stable pocket, and when a clear distinction was made between the amino acid and the growing peptidyl sites, each of the two halves was further optimized for its task so that their sequences evolved differently. In parallel, the substrates of the ancient ribosomes, which were initially activated amino acids (presumably by binding to single or oligo nucleotides), evolved to allow accurate binding. Later, for increasing specificity, these short RNA segments were extended to larger structures by their fusion with RNA stable features, to form the ancient tRNA. Later, RNA chains capable of storing, selecting and transferring instructions for producing useful proteins became available. Subsequently, the decoding process was combined with peptide bond formation. Then single molecules evolved, capable of not only carrying the amino acids while bound to them, but also translating the genomic instructions, by adding a feature similar to the modern anticodon arm to the ancient tRNA structure [10, 21]. Importantly, the notion that the ribosome evolved around an ancient core is also supported by computational and biochemical studies [15, 31].

In short: analysis of substrate binding modes to unbound ribosomal subunits and to functionally active ribosomes illuminated the significance of the PTC mobility and supported the hypothesis that the ancient ribosome could have

evolved from an RNA molecular machine that was functionally active in the RNA world era, which produced single peptides bonds and non-coded chains. Genetic control of the reaction seems to evolve after polypeptides capable of enzymatic function were created, and a stable RNA primitive carrier fold was converted into tRNA molecules.

18.4 Structures for Improving Antibiotics

The intensive research on ribosomes has practical aspects; one of them has clinical relevance since many antibiotics target the ribosome. The increasing incidence of antibiotic resistance and toxicity creates serious problems in modern medicine; combating resistance to antibiotics has been a major concern in recent years. Useful antibiotics that target ribosomes inhibit cell growth by selectively paralyzing the ribosome's activity in pathogens (always eubacteria) and not the eukaryotes. They act by diverse mechanism, all based on a common strategy: coinciding with functionally critical centers of the ribosome Examples are causing miscoding, minimizing essential mobility, interfering with substrate binding at the decoding center and at the PTC, or blocking the protein exit tunnel (Suggested reviews and recent work are: [3–5, 16–20, 32, 37–39, 45, 53, 55, 59, 61, 68, 69]).

By its nature, X-ray crystallography should be the choice method for investigating ribosome-antibiotics interactions. However, since X-ray crystallography requires diffracting crystals, and since so far no ribosomes from pathogenic bacteria could be crystallized, currently the crystallographic studies are confined to the currently available crystals of suitable pathogen models. Currently available are high-resolution structures of complexes of antibiotics with ribosomal particles from the eubacteria *E. coli*, *Thermus thermophilus* and *Deinococcus radiodurans*, all suitable to serve as a pathogen model. Also available are complexes obtained from antibiotics bound to ribosomes from the Dead Sea archaeon *Haloarcula marismortui* that resembles eukaryotes in respect to antibiotics binding site, hence requiring enormously high antibiotics concentrations for obtaining these complexes. Comparisons between the two types of complexes proved indispensable for increasing our understanding on antibiotics action (Fig. 18.5).

A major issue concerning the clinical usefulness of ribosomal antibiotics is their selectivity, namely their capabilities in the discrimination between the ribosomes of the eubacterial pathogens and those of eukaryotes. Although prokaryotic and eukaryotic ribosomes differ in size (~2.4 and 4 Mega Dalton, respectively), their functional regions, which are the targets for the antibiotics, are highly conserved. Therefore the imperative distinction between eubacterial pathogens and mammals, the key for antibiotics usefulness, is achieved generally, albeit not exclusively, by subtle structural difference within the antibiotics binding pockets of the prokaryotic and eukaryotic ribosomes. In fact, even among the pathogens, there are examples for species selectivity that determines the susceptibility and the fitness cost of the ketolides (e.g. [44]). Selectivity (and resistance) can also be obtained by exploiting induced fit mechanisms based on network of remote interactions by utilizing

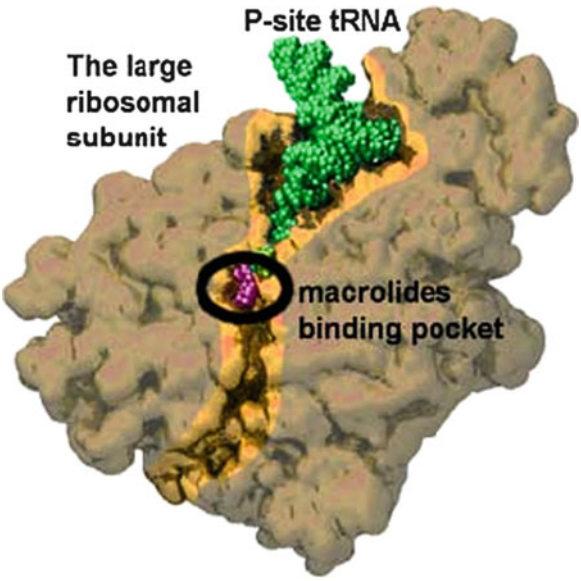

Fig. 18.5 Shows a section through the large ribosomal subunit at the level of the protein exit tunnel, together with P-site tRNA. The location of the macrolide binding pocket is circled

nucleotides that are less conserved, as they do not directly involved in the ribosome functions [19, 20]. Another intriguing issue relates to the contributions of two ribosomal proteins, namely L4 and L22. These proteins line a small part of the exit tunnel at its constriction, and do not interact directly with most of the members of the macrolides family, yet their mutations acquire resistance to them [14, 22, 42, 72], presumably by perturbing the rRNA structure at the tunnel walls [26, 35].

Current attempts to overcome antibiotics resistance and increase their selectivity are being made (e.g. [18, 66]).These include developments of synergetic antibiotics, such as the recent potent antibiotic drug, synercid [29, 69] and reviving “forgotten” antibiotics families, such as the lankacidins [5]. Other strategies are based on insertions of additional moieties that should bind to the ribosome and compensate for the lost interactions in the resistant strains. In parallel, comprehending the factors allowing for selectivity should provide powerful tools to understand many of the mechanisms exploited for acquiring resistance. Thus, the lessons learned from ribosome crystallography for combating resistance of antibiotics targeting the ribosome paved new paths for antibiotics improvement.

18.5 Historical Comments

Owing to the huge size and the complexity of the ribosome, it was widely assumed that ribosomes cannot be crystallized. Twenty years passed from the first indications for potential high resolution by examining the initial microcrystals that diffracted to

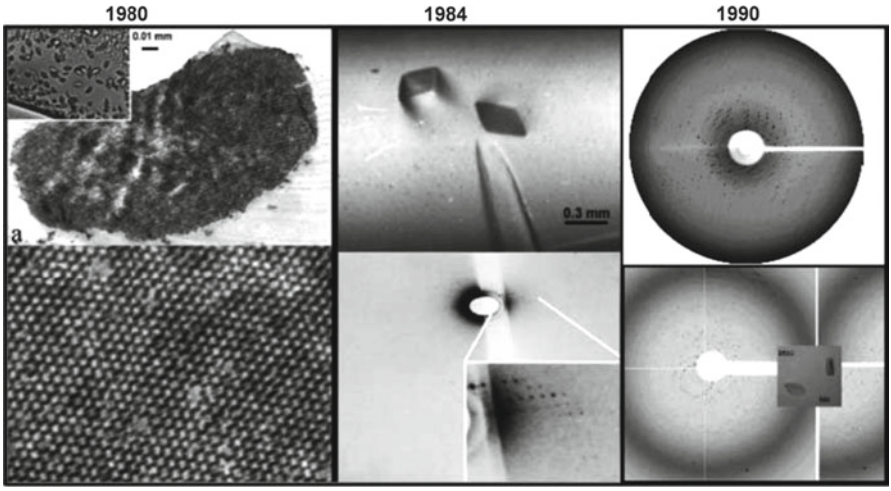

Fig. 18.6 Shows the progress in crystallization. On the *left*, the microcrystals are shown in an insert (*top left*), and a positively stains section of these micro crystals as seen by EM

relatively high resolution, namely 3.5 Å [71] to the first 3D structures. The shift from poorly diffracting microcrystals to high-resolution structures was achieved gradually, based on the assumptions that the higher the conformational homogeneity the better the crystals, and that the preferred conformation is that of functionally active ribosomes. Assuming that the ribosomes of bacteria that grow under robust conditions are less sensitive to external conditions, we focused on such sources, and, indeed, the first three dimensional microcrystals were obtained (Fig. 18.6) from the large ribosomal subunits from *Bacillus stearothermophilus* [71], a source considered to be an extremophile at the beginning of the eighties. Extensive systematic explorations for suitable bacterial sources indicated that the key for obtaining crystals suitable for crystallographic studies is to use ribosomes from relatively robust bacteria, such as *H. marismortui*, *T. thermophilus* and *D. radiodurans* [25]. A parallel strategy is to crystallize complexes of ribosomes with substrates, inhibitors and/or factors that can trap them at preferred orientations. Among such complexes are the initial crystals of the whole ribosome from *T. thermophilus* with mRNA and tRNA molecules [27]. Efforts aimed at crystals improvement included a thorough examination of the influence of the relative concentrations of mono- and di-valent ions [62] and constant refinements of bacterial growth pathways [6]. Remarkably, flexible functional regions could be traced in maps obtained from crystals grown under conditions mimicking their physiological environment [28], whereas in crystals obtained under far from physiological environment these regions are highly disordered [7].

While developing crystallographic procedures, we obtained a starting model by electron microscopy, using three-dimensional image reconstruction from two dimensional sheets (Fig. 18.7). These studies revealed that nascent proteins progress zcated protection of nascent chains by the ribosome [36, 48]. However, the common notion

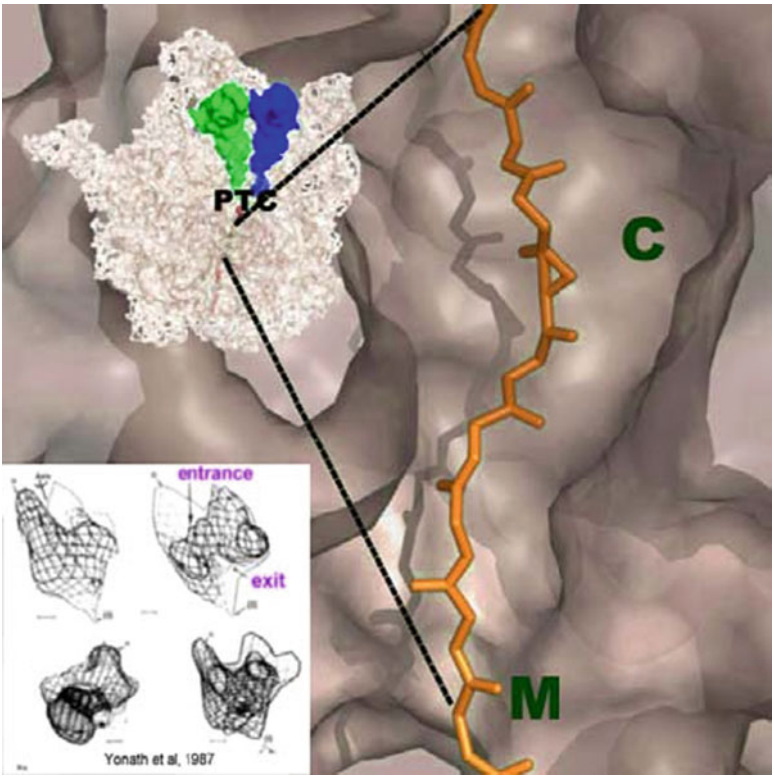

Fig. 18.7 A section through the large subunit (*top left*) and the protein exit tunnel, in which poly-alanine is docked. *C* denotes a crevices where initial folding can take place and *M* is the location of the macrolides binding pocket. The initial three dimensional reconstructions (from 2D sheets) are shown in the *bottom-left* inset

that nascent proteins progress on the ribosome surface until its maturation, raised doubts in the existence of the tunnel [41, 47], even after its visualization.

Alongside the improvement of the quality of the micro- or poorly diffracting crystals, our studies required the development of innovative methodologies. Among these is the pioneering of bio-crystallography at cryogenic temperatures, which was introduced because of the extreme radiation sensitivity of the ribosomal crystals [30] and became almost instantaneously the routine method all over the world, thus enabling structure determination from crystals considered not useful previously. Also, we introduced an unconventional use of multi-heavy atom clusters [60] (Fig. 18.8).

One of them, the heteropolytungstate (NH4)6P2W18O62 was found to play a dual role in the determination of the structure of structure of the small ribosomal subunit from *T. thermophilus*. Thus, in addition to significant phasing power and anomalous signal, post crystallization treatment with minute amount of one of this cluster increased dramatically the resolution of the X-ray diffraction from the initial low resolution (7–9 Å) to ~3Å [25, 50] presumably by minimizing the internal

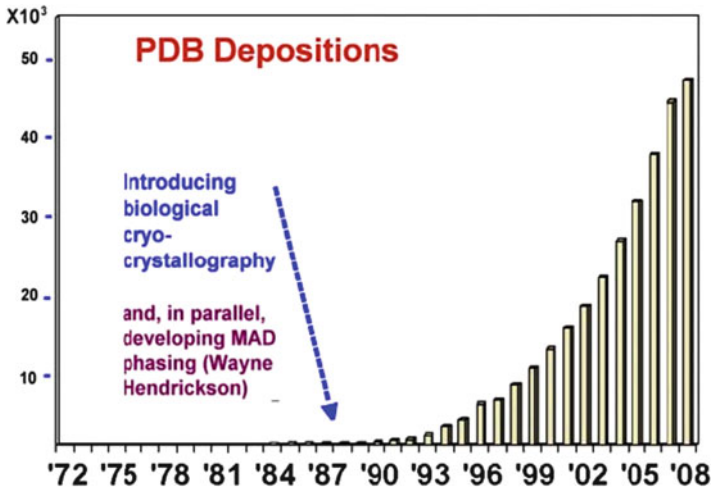

Fig. 18.8 PDB depositions by the year

flexibility involved naturally in mRNA binding to the ribosome and its progression through the ribosome.

18.6 Conclusions

By providing molecular snapshots of various intermediates in ribosome-mediated translation in atomic detail, the high resolution structures have revolutionized our understanding of the mechanism of protein synthesis. Despite this impressive progress, countless new questions arose. Many of which concern structural dynamics and intricate localized rearrangements, with answers that may emerge by combination of approaches like X-ray crystallography, cryo EM, FRET and biochemistry. An striking advance in this direction is the recent ability to follow translation by single ribosomes, one codon at a time) using mRNA hairpins tethered by the ends to optical tweezers [65].

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