Ribosome crystallography: From early evolution to contemporary medical insights

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1. Introduction

Ribosome research took off as soon as ribosomes were identified. At the end of the seventies the extensive biochemical studies yielded illuminating findings about the overall nature of ribosome function. These studies showed that all ribosomes are composed of two unequal subunits. The small subunit in bacteria, denoted as 30S, contains an RNA chain (16S) of about 1500 nucleotides and 20–21 different proteins, whereas the large subunit, denoted as 50S, contains two RNA chains (23S and 5S RNA) of about 3000 nucleotides in total, and 31–35 different proteins. In all organisms the two subunits exist independently and associate to form functionally active ribosomes.

The substrates engaged in protein formation are aminoacylated, peptidylated and deacylated (exiting) tRNA molecules. The three-dimensional structures of all tRNA molecules from all living cells across evolution are alike, although each of them has features specific to its cognate amino acid. The tRNAs are double helical L-shaped RNA molecules in a stem-elbow-stem organization, and contain a loop that comprises the anticodon complementing the three-nucleotide codon on the mRNA. About 70 Å away, at their 3' end, tRNAs contain a single strand with the universal sequence CCA, to which the cognate amino acid is attached by an ester bond. The tRNA molecules are non-ribosomal entities that bring together the two subunits, as all three of their binding sites, A (aminoacyl), P (peptidyl), and E (exit), reside on both subunits. The small subunit provides the path along which the mRNA progresses, the decoding center and the mechanism controlling translation fidelity, and the large subunit contains the site for the main ribosomal catalytic function, namely the polymerization of amino acids.

Although an overall description of protein biosynthesis was available by the end of the seventies, detailed functional information was not available because of the lack of three-dimensional molecular structures. Indeed, the common hypotheses about the mode of ribosome function underwent significant alterations once three-dimensional structures became available. Striking examples for conceptual revolutions in the understanding of ribosomal function (Wekselman et al., 2008) relate to the functional contribution of the different ribosomal components and the path taken by nascent chains.

In the middle of the last century, RNA-rich particles, called "Palade particles", were identified in the vicinity of the endoplasmic reticulum (Palade, 1955; Watson, 1963). These were proposed to be involved in gene expression, suggesting that proteins are made by an RNA machine (Crick, 1968). Nevertheless, it was commonly assumed for over four decades that decoding of the genetic code and peptide bond formation are performed by r-proteins, while r-RNA provides the ribosomal scaffold (Garrett and Wittmann, 1973). The proposition that RNA provides the ribosome catalytic activities (Noller et al., 1992) was met first with skepticism. Modest acceptance of this idea was achieved as several functional roles played by RNA molecules in various life processes were identified around the last decade of the 20th century, including peptide bond formation *in vitro* by selected ribozymes (Zhang and Cech, 1997) and spontaneous conjugation of amino acids with oligonucleotides (Illangasekare et al., 1995). Finally, at the turn of the third millennium, the emerging high-resolution structures verified the notion that both the decoding center and the site of peptide bond formation (called peptidyl transferase center or PTC) reside in regions where rRNA predominates.

M. V. Rodnina et al. (eds.), *Ribosomes*

The three-dimensional crystal structures of ribosomes and their various complexes illuminated the molecular basis for most of the mechanisms involved in ribosome function. These showed how the assembly of the initiation complex occurs (Simonetti et al., 2008; Simonetti et al., 2009), revealed the decoding mechanism (reviewed in (Ramakrishnan, 2008; Demeshkina et al., 2010)), the mRNA progression mode, including the narrowing of the downstream mRNA tunnel that occurs upon the transition from initiation to elongation (Yusupova et al., 2006; Jenner et al., 2010), identified the relative positions of A-, P- and E-site tRNAs (Yusupov et al., 2001) and shed light on the way the initiation, elongation, termination, and recycling factors modulate ribosome function (Carter et al., 2001; Pioletti et al., 2001; Wilson et al., 2005; Borovinskaya et al., 2007; Laurberg et al., 2008; Weixlbaumer et al., 2008; Schmeing et al., 2009). In addition, the positions of the tRNA molecules within the PTC (Woolhead et al., 2004; Blaha et al., 2009; Voorhees et al., 2009), the conformational rearrangements that E-site tRNA undergoes while exiting the ribosome (Jenner et al., 2007), and the architectural and dynamic elements required for amino acid polymerization were determined (Bashan et al., 2003a; Bashan and Yonath, 2008b). Thus, it appears that the main catalytic activities of the ribosome provide the framework for proper positioning of all participants in the protein biosynthetic process, thus enabling decoding, successive peptide bond formation and the protection of the nascent protein chains.

This article focuses on ribosome crystallography and on the functional implications evolving from these studies. It describes snapshots from the chronological progress of ribosomal crystallography as a semi historical report. It highlights selected events occurring during the long way from the initial ribosome crystallization including the introduction of innovations in the procedures required for the determination of the ribosomal structures, such as cryo bio-crystallography and the use of heavy atom clusters [reviewed in (Gluehmann et al., 2001)]. The article also focuses on selected structural and dynamic properties of the ribosome that enable its function as an efficient machine and illuminates several key ribosomal strategies for efficient usage of resources and for minimizing protein production under non-optimal conditions. Additionally, this article discusses modes of action of antibiotics that hamper ribosome function and suggests mechanisms for the acquisition of antibiotic resistance. It also addresses issues concerning the origin of translation, as can be deduced from the universal structural element that embraces the ribosomal active site and possesses internal symmetry within the otherwise asymmetric contemporary ribosome.

2. Hibernating bears stimulated ribosome crystallization

Once it was found that ribosomes are the molecular machines translating the genetic code and initial knowledge of the chemical composition of the *E. coli* ribosome became partially available, attempts at ribosome crystallization were made worldwide. For over two decades these attempts were unproductive. Owing to repeated failures the crystallization of ribosomes was considered a formidable task. The extreme difficulties in ribosome crystallization stemmed from their marked tendency to deteriorate, their high degree of internal mobility, their flexibility, their functional heterogeneity, their chemical complexity, their large size and their asymmetric nature.

Nevertheless, the finding that large amounts of ribosomes in hibernating bears are packed in an orderly fashion on the inner side of their cell membranes indicated that ribosomes can assemble in periodical arrangements *in vivo*. Similar observations were made in shock-cooled fertilized eggs (Unwin and Taddei, 1977; Milligan and Unwin, 1986). These phenomena were associated with cold or similar shocks, and were rationalized as a strategy taken by organisms under stress for storing pools of functionally active ribosomes that can be utilized when the stressful conditions are removed. Indeed, structural studies, performed on samples obtained from shock-cooled fertilized eggs led later to the visualization of ribosomal internal features (Milligan and Unwin, 1986) (see below).

Extending the level of order from membrane-supported two-dimensional monolayers produced *in vivo* to three-dimensional crystals grown *in vitro* was not trivial, but became successful with the introduction of uncommon crystallization strategies. These were based on the interpretation of the life cycle of the winter sleeping bears, which regularly pack and unpack their ribosomes each year, as part of their normal life cycle. The fact that these processes are associated with living organisms that require functionally active ribosomes immediately when awaking from a state of winter sleep suggested (i) that the integrity of highly active ribosomes can be maintained for relatively long periods without undergoing significant deterioration and (ii)

that the ribosomes can be periodically ordered in three-dimensions. In other words, ribosomes seemed likely to form crystals.

The breakthrough in ribosome crystallization was based on the assumption that the higher the sample homogeneity, the better the crystals, and secondly, that the preferred conformation is that of the functionally active ribosome. Consequently, highly active ribosomes of bacterial species that grow under robust conditions were selected and conditions for optimization and maintenance of their activity (Vogel et al., 1970; Zamir et al., 1971) were maintained throughout purification and crystallization. The first three-dimensional micro-crystals of ribosomal particles, treated as "powder samples" diffracted to relatively high resolution (3.5 Å) and had impressive internal order (Figure 1), were obtained from the large ribosomal subunit of a thermophilic bacterium, *Bacillus stearothermophilus* (B50S), at the beginning of the eighties (Yonath et al., 1980). A thorough screening of about 25,000 different crystallization conditions, performed by careful monitoring of the nucleation of crystalline region (Yonath et al., 1982a), was accompanied by a systematic search for parameters favoring crystallization (Yonath et al., 1982b). At the beginning of the eighties, *B. stearothermophilus* as an extremophile was considered to be exotic. Therefore frequent doubts about its suitability as a representative of "normal" bacterial ribosomes, such as those of *E. coli*, were expressed, despite the very high sequence homology between them. Nevertheless, the preliminary successes of the crystallization stimulated the use of ribosomes from other robust bacteria. Consequently, a few years later, three-dimensional crystals were obtained from the large ribosomal subunits of the halophilic archaeon, *Haloarcula marismortui*, that lives in the Dead Sea (Shevack et al., 1985). In 1987, seven years after the first crystallization of the large ribosomal subunits, parallel efforts led to the growth of crystals of the small ribosomal subunit (Yonath et al., 1988; Yusupov et al., 1988) and of the 70S ribosome (Trakhanov et al., 1987) from the extreme thermophilic bacterium, *Thermus thermophilus*.

At that time it was widely assumed that the three-dimensional structure of the ribosome may never be determined, as it was clear that alongside the improvement of the crystals, the determination of the structure would require the introduction and development of innovative methodologies. For instance, because of the weak diffraction power of the ribosome crystals even the most advanced rotating anode generators were not powerful enough to yield suitable diffraction patterns. Similarly, only a few diffraction spots could be recorded (Yonath et al., 1984) (Figure 1), even when irradiating extremely large crystals (~2 mm in length) by synchrotron radiation, which at the time was in its early stage. In parallel to the advances in growing ribosomal crystals of several forms (Yonath and Wittmann, 1988), the synchrotron facilities and the detection methods underwent constant (albeit rather slow) improvement. However, even when more suitable beamlines became available, the radiation sensitivity of the ribosomal crystals caused extremely fast crystal decay. Hence, pioneering data collection at cryogenic temperature (Hope et al., 1989) became crucial. Once established, this method became routine worldwide, and, although when using very bright X-ray beam, decay was observed even at cryo-temperature, interpretable diffraction patterns were ultimately obtained from the extremely thin crystals. Additionally, multi-heavy atom clusters suitable for phasing were identified (Thygesen et al., 1996). One of these clusters, originally used for providing anomalous phasing power, was found to play a dual role in the determination of the structure of the small ribosomal subunit from *T. thermophilus* (T30S). Post-crystallization treatment with minute amounts of these clusters dramatically increased the resolution from the initial 7–9 Å to 3 Å (Schluenzen et al., 2000), presumably by minimizing the internal flexibility of the particle (Bashan and Yonath, 2008a).

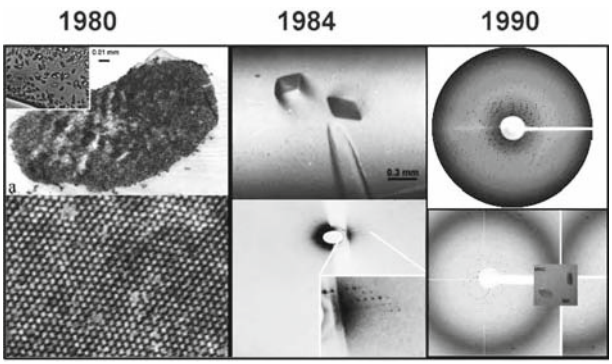

Fig. 1 From micro-crystals to three-dimensional crystals yielding useful diffraction. Left, top: The first microcrystals of B50S (Yonath at al., 1980). Left, bottom: a negatively stained section of the microcrystals, viewed by electron microscopy. Middle, top: the tip of a ~2 mm-long crystal of B50S. Middle, bottom: its diffraction pattern, obtained at 4°C in 1984 using the EMBL/DESY/Hamburg beam line. Right, top: The diffraction pattern from crystals of H50S, obtained at ID13 beamline at ESRF, Grenoble, at 95K; the diffraction extends to 2.8Å. Right, bottom: The crystals decayed completely after collecting about 0.3% of the data.

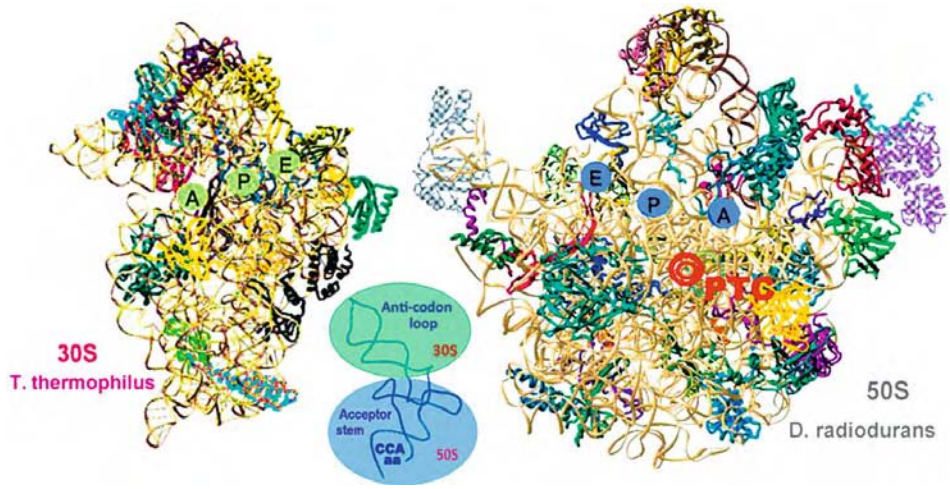

Fig. 2 The three-dimensional structures of the two ribosomal subunits from the bacteria *Deinococcus radiodurans* and *T. thermophilus*. The interface faces are facing the reader. The rRNA is shown in brownish colors, and each of the r-proteins is painted in a different color. The approximate site of the PTC is marked in red. Insert: the backbone of a tRNA molecule. The circles designate the regions interacting with each of the ribosomal subunits.

Continuous efforts were aimed at improving crystals, including the assessment of the influence of the relative concentrations of mono- and divalent ions (von Bohlen et al., 1991) on crystal properties. These efforts led to dramatic improvements in the quality of the crystals of the large ribosomal subunits from *H. marismortui* (H50S). In addition, constant refinements of bacterial growth protocols (Auerbach-Nevo et al., 2005) alongside a thorough investigation of crystallization conditions (Zimmerman and Yonath, 2009), indicated a noteworthy correlation between the conditions at which the ribosome functions and the crystal quality. Along these lines it is worth mentioning that flexible ribosomal regions were detected in electron-density maps obtained from crystals of ribosomal particles that were obtained under conditions that supported optimal functional activity (Harms et al., 2001), whereas the same regions were significantly more disordered in crystals obtained under non-physiological conditions (Ban et al., 2000).

An alternative strategy for improvement of crystal quality was the crystallization of complexes of ribosomes with substrates, inhibitors and/or factors, presumably because these factors trap the ribosomal particles in preferred conformations. Indeed, the initial diffracting crystals of the 70S ribosome from *T. thermophilus* (T70S) with mRNA and tRNA molecules (Hansen et al., 1990) a decade later led to impressive advances in resolution from crystals of functional ribosome complexes (Yusupov et al., 2001; Korostelev et al., 2006b). Importantly, these techniques also enabled the structural analysis of snapshots of ribosomes trapped in specific conformations, albeit not necessarily functional ones (Schuwirth et al., 2005).

3. The ribosome is a polymerase

The crystal structures of bacterial ribosomes showed that the interface surfaces of both ribosomal subunits and their active sites (the decoding center and the peptidyl transferase center) are rich in RNA (Figure 2). These observations verified previous biochemical suggestions that the ribosome is a ribozyme. Particularly, as seen below, the striking architecture of the ribosome governs all tasks related to nascent protein elongation: namely the formation of peptide bonds, the processivity of this reaction, and the detachment of the growing polypeptide chain from the P-site tRNA. Thus, in addition to factor-assisted movements, i. e. the entrance and exit of the tRNA molecules and the progression of the mRNA, amino acid polymerization requires several major motions including peptidyl-tRNA translocation from the A to the P site, entry of the growing chain into the ribosome exit tunnel (Figure 3), passage of the deacylated tRNA molecule from the P to the E site and its subsequent release. However, it should be kept in mind that, although single peptide bonds can be produced by mixtures containing mainly rRNA and traces of r-proteins (Noller et al., 1992), the production

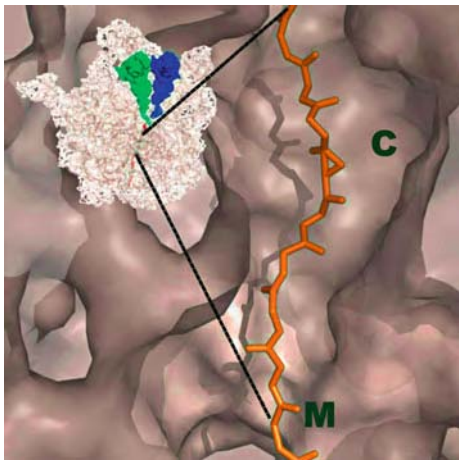

Fig. 3 The ribosomal exit tunnel. The entire large subunit, viewed from its interface surface with A- and P-site tRNAs (blue and green, respectively) and with polyalanine (orange) modeled in the tunnel, is shown in the top left panel. The main view is a zoom into the upper end of the tunnel. C denotes a crevice where co-translational initial folding may occur (Amit et al., 2005), and M shows the tunnel constriction, add.

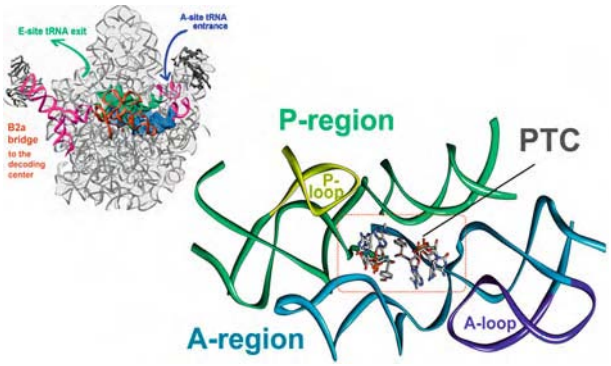

Fig. 4 The symmetrical region within the ribosome. Left: The symmetrical region within the ribosome and its details. The A-region is shown in blue, the P-region in green, and the non-symmetrical extensions are depicted in magenta. The bridge to the small subunit is shown in light brown. Right: Zoom into the symmetrical region, highlighting the basic structure that can form the active-site pocket and the loops that accommodate the C74 of the 3' ends of the A- and P-site tRNAs.

of peptide bonds by pure ribosomal RNA has not yet been demonstrated (Anderson et al., 2007).

The PTC is situated within a highly conserved universal symmetrical region that is embedded in the otherwise asymmetric ribosome structure (Figure 4). This region provides the machinery required for peptide bond formation, for the translocation of the A-site tRNA 3' end, for the detachment of the free substrate after peptide bond formation, and for the entry of the growing chain into the ribosome tunnel. This region is composed of 180 nucleotides, the fold, but not the sequence, of which is related by an internal pseudo two-fold symmetry. This region contains the two conserved nucleotides, G2552 and G2553, which form symmetrical Watson-Crick G-C base-pairs with the universally conserved CCA termini of the P and A-site tRNAs, respectively (Samaha et al., 1995; Kim and Green, 1999), and has been identified in all known ribosome structures, regardless of the source or functional state of the ribosomes (Bashan et al., 2003a; Zarivach et al., 2004; Agmon et al., 2005; Baram and Yonath, 2005). More specifically, the same sub-structure was identified in the cores of ribosomes from mesophilic, thermophilic, radiophilic and halophilic bacteria and archaea, regardless of their functional state or the ligands bound to them (Agmon et al., 2005). It is conceivable that the central location of the symmetrical region allows it to serve as the central signaling feature between all the functional regions involved in protein biosynthesis that are located remote from one another (up to 200 Å away), but must communicate during elongation (Uemura et al., 2007).

The PTC is built as an arched void located at the bottom of a V-shaped cavity that hosts the helical portion of the acceptor stems and the 3' ends (Figure 2) of both A- and P-site tRNAs (Figure 5). In the crystal structure of the large ribosomal subunit from *Deinococcus radiodurans* (D50S) in complex with a substrate analog that mimics the acceptor stem and the 3' end of the A-site tRNA (called acceptor stem mimic, ASM), the acceptor stem interacts extensively with the walls of the cavity (Bashan et al., 2003b), forming an elaborate network of interactions (Figure 5). These interactions dictate a specific orientation that facilitates the processivity of peptide bond formation. Thus, although the PTC has some tolerance in the positioning of "fragment reaction substrates" (Hansen et al., 2002), the interactions of the tRNA acceptor stem seem to be crucial for accurate substrate positioning in the PTC in a configuration allowing for peptide bond formation (Yonath, 2003). This structural observation supports the finding that the tRNA core region contributes to interactions with the ribosome (Pan et al., 2006). The linkage between the elaborate architecture of the symmetrical region and the position of the A-site tRNA suggests that the translocation of the tRNA 3' end within the PTC is related to the overall tRNA/mRNA translocation, assisted by EF-G, which is performed by a combination of two synchronized motions: a side-

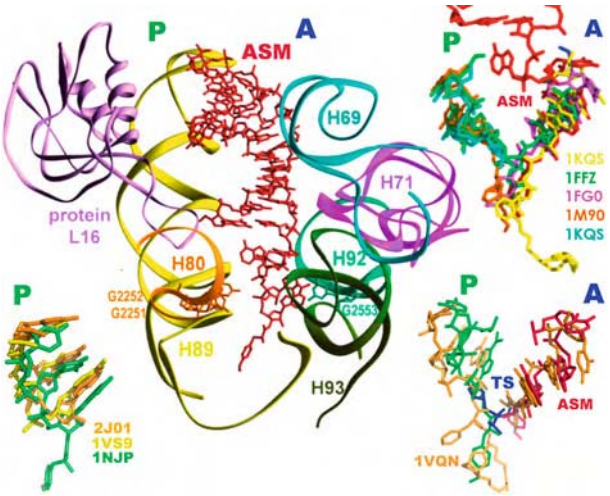

Fig. 5 Various substrates bound at the PTC. The central image shows the PTC and the cavity leading to it. The position of a mimic of A-site tRNA acceptor stem and 3' end (ASM, red) is shown. The components of the base pairs between the PTC upper rim (one at the A site and two in the P site) are highlighted. The RNA helices and the nucleotides located at the walls of the cavity are identified using *E. coli* numbering. Bottom left: Superposition of the 3' ends of P-site tRNAs: two experimentally determined (yellow and orange) and one derived (by rotation) moiety, in green. PDB entries are indicated by numbers. Top right: Positions and orientations of ASM and of various "minimal fragments" (puromycin derivatives) in the PTC; a dipeptide (yellow), produced by a minimal fragment that could not rotate into the P site because of its minimal size and its specific orientation, is shown in the A site. Bottom right: Superposition of ASM, the computed transition state (Gindulyte et al., 2006) (TS) and a chemically designed TS analog. Note that the extension of the chemical TS (Schmeing et al., 2005b), which is supposed to represent the nascent chain, is originating in the P site.

ways shift (the main component) and a rotatory motion of the A-site tRNA 3' end along a path confined by the PTC walls.

This rotatory motion appears to be navigated and guided by the ribosomal architecture, mainly the PTC rear wall that confines the rotatory path. Two flexible nucleotides, A2602 and U2585, seem to anchor and propel this motion. This means that the ribosomal architecture and its mobility provides all structural elements enabling ribosome function as an amino acid polymerase, including the formation of a symmetrical universal base pair between each of the tRNAs and the PTC (Bashan et al., 2003a; Agmon et al., 2005; Pan et al., 2006), alongside an additional base pair between the P-site tRNA and the PTC, a prerequisite for substrate-mediated acceleration (Weinger et al., 2004).

Importantly, all nucleotides involved in this rotatory motion have been classified as essential by a comprehensive genetic selection analysis (Sato et al., 2006). Furthermore, the rotatory motion positions the proximal 2'-hydroxyl of A76 of the P-site tRNA in the same position and orientation as found in crystals of the entire ribosome with mRNA and tRNAs (Korostelev et al., 2006a; Selmer et al., 2006) (Figure 5) and allows for chemical catalysis of peptide bond formation by A76 of the P-site tRNA (Weinger et al., 2004).

Simulation studies of the rotatory motion indicated that during this motion the rotating moiety interacts with ribosomal components confining the rotatory path, along the "PTC rear wall" (Agmon et al., 2005; Agmon et al., 2006). Consistently, quantum-mechanical calculations, based on D50S structural data, indicated that the transition state (TS) of peptide bond formation is formed during the rotatory motion and is stabilized by hydrogen bonds with rRNA nucleotides (Gindulyte et al., 2006). Importantly, the TS location suggested by quantum-mechanics is close to the location of a designed TS analog in the crystal structure of its complex with H50S (Schmeing et al., 2005) (Figure 5).

In short, the structure of D50S in complex with a substrate analog mimicking the part of the A-site tRNA that interacts with the large subunit advanced the comprehension of peptide bond formation by showing that ribosomes position their substrates in stereochemical configurations suitable for peptide bond formation (Bashan et al., 2003a; Agmon et al., 2005). Furthermore, the ribosomal architecture that facilitates positional catalysis of peptide bond formation, promotes substrate-mediated chemical acceleration, in accord with the requirement of full-length tRNAs for rapid and smooth peptide bond formation observed by various methods, including the usage of chemical, genetic (Polacek et al., 2001; Weinger et al., 2004; Youngman et al., 2004; Beringer et al., 2005; Polacek and Mankin, 2005; Brunelle et al., 2006; Sato et al., 2006), computational (Sharma et al., 2005; Gindulyte et al., 2006; Trobro and Aqvist, 2006) and kinetic studies (Beringer et al., 2005; Wohlgemuth et al., 2006; Beringer and Rodnina, 2007; Rodnina et al., 2007).

4. Structural disorder with functional meaning

The significance of the interactions of the acceptor stem with the cavity leading to the PTC was indicated by biochemical studies and clearly demonstrated crystallographically. Thus shedding light on the differences between the binding modes of full-size tRNA to 70S

ribosomes (or acceptor stem mimics to 50S subunits) and the binding modes of the various minimal substrates used for the fragment reaction. In functional experiments, the ribosome activity is determined by the reaction between substrate analogs capable of producing single peptide bonds. These "fragment reaction substrates" (Figure 5) are basically derivatives of puromycin, an A-site analog which acts as an inhibitor of the ribosomal polymerase activity. In many biochemical and structural studies, puromycin derivatives were used because they are good substrate analogs for a single peptide bond formation and because of the relative ease to detect in vitro single peptide bonds formed by them. However, caution is required when treating them as suitable to mimic the natural ribosome polymerase function. Interestingly, despite being small and consequently presumed to diffuse swiftly into its binding site within the ribosome, the rate of puromycin reaction with fMetPhe-tRNA as P-site substrate in 50S is comparable with rates of peptide bond formation with full-size tRNA (Wohlgemuth et al., 2008). It appears, therefore, that conformational rearrangements that were found to be required for productive positioning (Selmer et al., 2006) also play a role in determining the reaction rate. This idea is consistent with the biochemical finding that the peptidyl transfer reaction may be modulated by conformational changes at the active site (Youngman et al., 2004; Beringer et al., 2005; Schmeing et al., 2005; Brunelle et al., 2006; Beringer and Rodnina, 2007).

In the crystal structure of H50S with reactive fragment reaction substrates (Schmeing et al., 2002), the dipeptide resides in the A site (Figure 5) in an orientation hardly suitable for entrance into the tunnel. This complex was named "pre-translocational intermediate", meaning that in each elongation step the nascent protein has to translocate from the A to the P site. As the A to P site translocation within the PTC is performed by a 180 deg rotation, the translocation of the suggested "pre-translocational intermediate" requires that in each elongation step (15–20 times a second) such rotation is performed by the 3' end of A-site tRNA. If the entire growing chain is attached to its 3' end, it would have to rotate with it. Such motion would require a lot of space, which is probably not available in the dense PTC environment. Similarly, the energetic requirements of such a complicated operation should be very high. It appears, therefore that the dipeptide was trapped in the A site since the 3' end of the A-site tRNA did not undergo the rotatory motion because it, like all minimal substrate analogs, is too short and hence lacks moieties that facilitate the rotatory motion. Notably, the substrate was detected near the P site in the crystal structure of H50S with a substrate designed to mimic the transition state (Schmeing et al., 2005). As mentioned above, this finding is in accord with the results of the quantum mechanical computations that placed the transition state of the peptide bond in the PTC, close to the P site (Gindulyte et al., 2006). This result and the correlation between the rotatory motion and amino acid polymerization rationalize the detection of the dipeptide at the A site (Schmeing et al., 2002).

Hence, it appears that for the ribosome's polymerase activity the A-site substrate needs to be a full-length tRNA with its 3' end accurately positioned in the active site of the ribosome. This conclusion is supported by the study of a crystal structure of H50S in complex with a tRNA "mini-helix" (similar to the ASM described above) which led to the suggestion that specific rRNA nucleotides catalyze peptide bond formation by the general acid/base reverse mechanism (Ban et al., 2000), a proposition that was challenged by a number of biochemical and mutational studies, e. g. (Polacek et al., 2001).

Notably, in this structure, only the tip of the 3'-end is resolved, whereas the entire acceptor stem is disordered, presumably because its interactions with the partially disordered cavity leading to the PTC could not be formed (Nissen et al., 2000). Importantly, the H50S crystals used for this study were obtained under far from optimal functional conditions, namely rather low KCl concentration, while it was found that a very high KCl concentration is essential for the function of the ribosomes from the halophile *H. marismortui* (Shevack et al., 1985; Gluehmann et al., 2001).

The observed disorder in otherwise very well ordered crystals of H50S suggest that ribosomes kept under far from the conditions allowing for their efficient activity, can form peptide bonds but may not be capable of elongating nascent chains. This finding seems to point to a natural strategy for avoiding or minimizing the formation of proteins under stressful or far from physiological conditions. Specifically, the disorder of almost all of the functional regions of H50S may reflect a natural response of the halophilic ribosomes to a salt deficient environment, potentially indicating a natural mechanism for conserving cellular resources under stressful circumstances. In support of this suggestion, structural comparisons showed that the H50S active site contains key PTC components in orientations that differ significantly from those observed in empty D50S

(Harms et al., 2001) as well as in functional complexes of T70S ribosomes (Selmer et al., 2006).The main conclusion from the analyses of all of these structures is that single peptide bond formation can be performed even when the initial substrate binding is not accurate. However, for elongating the nascent proteins, accurate positioning of the A-site tRNA 3' end is mandatory. It appears, therefore, that the choice of substrate analogs for the various studies as well as the discrepancy between the definitions of ribosomal activity (namely the mere ability to form single peptide bonds versus the requirement for elongating nascent proteins) are the main reason for different structures, and potentially for their interpretation. It is clear that accurate substrate positioning within the ribosome frame, accompanied by the P-site tRNA interactions with 23S rRNA that allow for substrate catalysis (Weinger et al., 2004), plays the key role in ribosome catalytic functions, a notion that is currently widely accepted [e. g. (Beringer et al., 2005; Beringer and Rodnina, 2007; Simonovic and Steitz, 2008; Bashan and Yonath, 2008b)].

5. On the ribosomal tunnel and initial nascent protein folding

It was widely assumed that nascent proteins advance on the surface of the ribosome until their maturation. Even after biochemical experiments indicated that nascent chains are masked (hence protected) by the ribosome (Malkin and Rich, 1967; Sabatini and Blobel, 1970) and a tunnel was visualized in EM reconstructions from two-dimensional sheets at 60 and 25 Å resolution (Milligan and Unwin, 1986; Yonath et al., 1987), the existence of a tunnel was not generally accepted (Moore, 1988). Furthermore, it was assumed that nascent proteins are not degraded during protein synthesis because all of them adopt the conformation of an alpha helix (Ryabova et al., 1988). Doubts regarding the existence of the ribosomal tunnel were removed when it was visualized by cryo electron microscopy (Frank et al., 1995; Stark et al., 1995). Remarkably, the tunnel is of variable width and shape (Figure 3), suggesting its possible involvement in the fate of the nascent chains in accord with previous observations [e. g. (Crowley et al., 1993; Walter and Johnson, 1994)]. Furthermore, results of biochemical, microscopic, and computational experiments verified the existence of the tunnel and in several cases indicated that it may participate actively in nascent chain progression and its initial compaction, as well as in translation arrest and cellular signaling (Gabashvili et al., 2001; Gong and Yanofsky, 2002; Nakatogawa and Ito, 2002; Berisio et al., 2003; Gilbert et al., 2004; Johnson and Jensen, 2004; Woolhead et al., 2004; Amit et al., 2005; Ziv et al., 2005; Berisio et al., 2006; Cruz-Vera et al., 2006; Mankin, 2006; Mitra et al., 2006; Tenson and Mankin, 2006; Voss et al., 2006; Woolhead et al., 2006; Deane et al., 2007; Schaffitzel and Ban, 2007; Bornemann et al., 2008; Petrone et al., 2008; Starosta et al., 2010; Nakatogawa and Ito, 2004; Chiba et al., 2011).

While emerging from the ribosome, nascent chains may interact with chaperones that assist their folding and/or prevent their aggregation and misfolding. In bacteria the first chaperone that encounters the nascent proteins, called trigger factor, forms a shelter composed of hydrophobic and hydrophilic regions that provide an environment that can compete with the aggregation tendency of the still unfolded chains (Baram et al., 2005; Schluenzen et al., 2005; Kaiser et al., 2006). Interestingly, free trigger factor seems also to rescue proteins from misfolding and to accelerate protein folding (Martinez-Hackert and Hendrickson, 2009).

6. Antibiotics targeting the ribosome: strategies, expectations and problems

Because of the major significance of the ribosomes for cell viability many antibiotics target them. An immense amount of biochemical studies performed over four decades, alongside medical research and recent crystallographic analysis, showed that despite the high conservation of the ribosomal active sites, subtle differences facilitate their clinical relevance (Wilson, 2004; Yonath and Bashan, 2004; Polacek and Mankin, 2005; Yonath, 2005; Tenson and Mankin, 2006; Bottger, 2007; Sohmen et al., 2009). As so far there are no crystals of ribosomes from pathogenic organisms, structural information is currently obtained only from the crystallizable bacterial ribosomes that have been shown to be relevant as pathogen models, namely *E. coli, D. radiodurans,* and *T. thermophilus*. Antibiotic action on ribosomes from these bacteria, in conjunction with data obtained from the ribosomes of other organisms such as *Mycobacterium smegmatis* (a reasonable mimic of *Mycobacterium tuberculosis*), were found to be useful for determining antibiotic modes of action directly (described below) or indirectly (Pfister et al., 2005; Tu et al., 2005; Bommakanti et al., 2008; Hobbie et al., 2008).

The crystallographic analyses have shown that antibiotics targeting ribosomes exploit diverse strategies with common denominators. All antibioitcs target ribosomes at distinct locations within functionally relevant sites, mostly composed solely of rRNA. Each exerts its inhibitory action by competing with a crucial step in the biosynthetic cycle, including substrate binding, ribosomal dynamics, progression of the mRNA chain and decoding. Examples include hindering tRNA substrate accommodations at the PTC, stabilizing the tRNA in the A site in the pre-translocation state, preventing interactions of the ribosomal recycling factor and blocking the protein exit tunnel.

The identification of the various modes of action of antibiotics targeting ribosomes and an analysis of the ribosomal components comprising the binding pockets confirmed that the imperative distinction between ribosomes from eubacterial pathogens and mammalian cells hinges on subtle structural differences within the antibiotic binding pockets (Yonath and Bashan, 2004; Yonath, 2005; Pyetan et al., 2007; Auerbach et al., 2009; Auerbach et al., 2010). Apparently, fine tuning of the binding pocket can alter the binding parameters. These subtle sequence and/or conformational variations enable drug selectivity, thus facilitating clinical usage. Furthermore, the available structures illuminate features that are distinct between ribosomes from bacteria and non-pathogenic archaea that may be of crucial clinical importance.

Noteworthy are the results of comparisons between the crystal structures of different ribosomal particles complexed with the same antibiotics. Although leading to effective binding, disparities observed between the binding modes to pathogen models, namely *D. radiodurans* and *T. thermophilus*, may point to species specificity. Furthermore, comparison of the modes of antibiotic binding to ribosomal particles from the pathogen models (*D. radiodurans* and *T. thermophilus*) with modes observed in the archaeon *H. marismortui* (which shares properties with eukaryotes) indicated some variability in the binding modes, and in specific cases showed that binding is not synonymous with inhibitory activity. These comparisons highlighted the distinction between mere binding and binding leading to inhibitory activity. Specifically, for the macrolide family, these studies indicated that the identity of a single nucleotide can determine the strength of antibiotic binding, whereas proximal stereochemistry governs the antibiotic orientation within the binding pocket (Yonath and Bashan, 2004; Yonath, 2005) and consequently its therapeutic effectiveness. This is in accord with recent mutagenesis studies showing that mutation from guanine to adenine in 25S rRNA at the position equivalent to *E. coli* A2058 does not confer erythromycin sensitivity in *Saccharomyces cerevi*sae (Bommakanti et al., 2008). Thus, it was clearly demonstrated that the mere binding of an antibiotic is not sufficient for therapeutic effectiveness and that minute variations in the chemical moieties of the antibiotics can lead to significantly different binding modes. An appropriate example is the extreme difference between the modes of function of erythromycin, which competes with lankacidin binding, and lankamycin, which acts synergistically with lankacidin (Figure 6) (Auerbach et al., 2010; Belousoff et al., 2011).

In addition to rationalizing genetic, biochemical, and medical observations, the available structures have revealed unexpected inhibitory modes. Examples are the stabilization of the pre-translocation state (Stanley et al., 2010), as well as exploitation of the inherent ribosome flexibility for antibiotic synergism (Harms et al., 2004; Yonath, 2005; Auerbach et al., 2010) and for triggering an induced-fit mechanism by remote interactions that reshape the antibiotic binding pocket (Davidovich et al., 2007). Among the ribosomal antibiotics, the pleuromutilins are of special interest since they bind to the almost fully conserved PTC, yet they discriminate between bacterial and mammalian ribosomes. To circumvent the high conservation of the PTC, the pleuromutilins exploit the inherent functional mobility of the PTC and stabilize a conformational rearrangement that involves a network of remote interactions between flexible PTC nucleotides and less

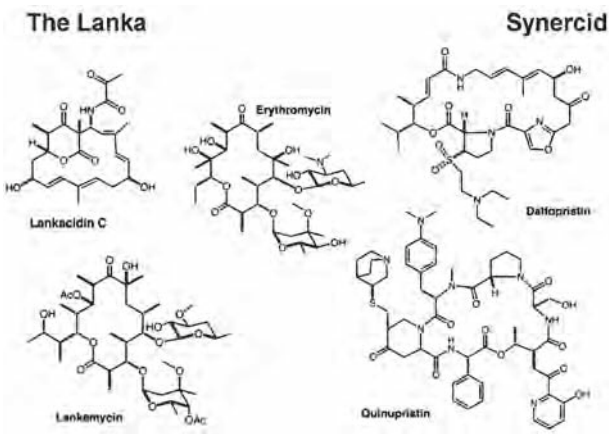

Fig. 6 The chemical compositions of antibiotic pairs acting on the ribosomal PTC and the exit tunnel. Both the lankacidin-lankamycin and the Synercid pairs display synergism. However, erythromycin (middle), which resembles lankamycin, competes with lankacidin.

conserved nucleotides residing in the PTC-vicinity (the second and third shells around the PTC). These interactions reshape the PTC contour and trigger its closure on the bound drug (Davidovich et al., 2007). The uniqueness of this pleuromutilin binding mode led to new insights as it indicated the existence of an allosteric network around the ribosomal active site. Indeed, the value of these findings is far beyond their perspective on clinical usage, as they highlight basic issues, such as the possibility of remote reshaping of binding pockets and the ability of ribosome inhibitors to benefit from the inherent flexibility of the ribosome.

Similar to the variability in binding modes, seemingly identical mechanisms of drug resistance can indeed be different, as they are dominated, directly or via cellular effects, by the antibiotics' chemical properties (Davidovich et al., 2007; Davidovich et al., 2008). The observed variability in antibiotic binding and inhibitory modes justifies expectations for structurally based improvement of the properties of existing compounds as well as for the development of novel drug classes. Detailed accounts can be found in several reviews (Auerbach et al., 2004; Yonath and Bashan, 2004; Poehlsgaard and Douthwaite, 2005; Yonath, 2005; Bottger, 2006; Tenson and Mankin, 2006; Bottger, 2007).

In short, over two dozen three-dimensional structures of ribosome complexes with antibiotics have revealed the principles allowing for clinical use, have provided unparalleled insight into the mode of antibiotic function, have illuminated mechanisms for acquiring resistance and have shown the basis for discrimination between pathogens and host cells. The elucidation of common principles of the mode of action of antibiotics targeting the ribosome combined with variability in binding modes led to the uncovering of diverse mechanisms for acquiring antibiotic resistance.

7. The ribosomal core is the optimized vestige of an ancient entity

A high level of sequence conservation in the symmetrical region has been maintained throughout ribosome evolution, even in mitochondrial ribosomes in which half the ribosomal RNA has been replaced by proteins (Mears et al., 2002; Thompson and Dahlberg, 2004; Agmon et al., 2006; Davidovich et al., 2009). This conservation and the observation that the symmetrical region provide all structural elements required for performing polypeptide elongation led us to suggest that the modern ribosome evolved by gene fusion or gene duplication (Figure 7). We refer to this ancestral entity as the proto-ribosome (Agmon et al., 2005; Davidovich et al., 2009; Belousoff et al., 2010a; Belousoff et al., 2010b).

In particular, the preservation of the three-dimensional structure of the two halves of the ribosomal frame, independent of the sequence, emphasizes the superiority of functional requirements over sequence conservation and demonstrates the rigorous requirements of accurate substrate positioning for peptide bond formation. As mentioned above, this, as well as the universality of the symmetrical region, led to the assumption that the ancient ribosome was composed of a pocket confined by two RNA chains that formed a dimer, and that this pocket is still embedded in the heart of the modern ribosome (Figures 4, 7). In fact, as mentioned above, suggestions that proteins are made by an RNA machine have been made already in the sixties (Crick, 1968), and extensive research over the past three decades (summarized in a recent book (Yarus, 2010)) has supported the idea that nucleic acids are capable of independent replication, selection, and self splicing [e. g. (Been and Cech, 1986; Abelson, 1990; Ellington and Szostak, 1990; Tuerk and Gold, 1990; Pino et al., 2008; Costanzo et al., 2009; Lincoln and Joyce, 2009)].

In accord with these findings we have proposed (Agmon et al., 2006; Davidovich et al., 2009; Belousoff et al., 2010a; Belousoff et al., 2010b; Turk et al., 2010) that the ancient machinery that could form peptide bonds was made exclusively from RNA molecules, utilizing substituents available in the primordial soup, namely RNA chains that could acquire conformations sufficiently stable to survive evolutionary stress.

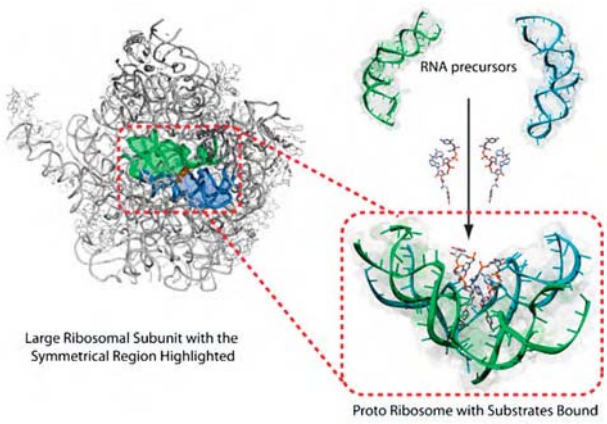

Fig. 7 The suggested proto-ribosome. Regions hosting A- and P-site tRNAs are shown in blue and green, respectively. The A-site tRNA mimic (Bashan et al., 2003) is shown in blue, and the derived P-site tRNA (by the rotatory motion) is shown in green.

These ancient RNA chains could fold spontaneously and then dimerize. The products of the dimerization yielded three-dimensional structures with a symmetrical pocket that could accommodate small substrates (e. g. amino acids conjugated with mono or oligo RNA nucleotides) in a stereochemistry suitable for spontaneous formation of various chemical bonds, including the peptide bond. These dimeric RNA complexes could have become the ancestors of the symmetrical region in the contemporary ribosome. The most appropriate pockets for promoting peptide bond formation survived.

The surviving ancient pockets became the templates for the ancient ribosomes. In a later stage, these initial RNA genes underwent optimization to produce more defined, relatively stable pockets, and when the correlation between the amino acid and the growing peptidyl sites was established, each of the two halves was further optimized for its task so that their sequences evolved differently. The entire ribosome could have evolved gradually around this symmetrical region until it acquired its final shape (Bokov and Steinberg, 2009).

The substrates of the ancient ribosomes, which were initially spontaneously produced amino acids conjugated with single or short oligo-nucleotides (Illangasekare et al., 1995; Turk et al., 2010), could have evolved in parallel to allow accurate binding, as occurs for aminoacylated CCA 3'-end. Later on, these could have been converted into longer compounds with a contour that can complement the inner surface of the reaction pocket. For increasing specificity, these short RNA segments could have been extended to larger entities by their fusion with stable RNA features, to form the ancient tRNA, presumably capable of storing, selecting and transferring instructions for producing useful proteins. The structural entity for decoding could have been combined with the structural machinery able to form a peptide bond in a single entity. Adding a feature similar to the modern anticodon loop allowed some genetic control, and could have led to the modern protein-nucleic acids world.

8. Conclusion

The currently available high-resolution structures of ribosomes and their subunits proved that the ribosome is a ribozyme. All functions of the ribosome, including decoding, peptide bond formation, protein elongation and tRNA release, are performed by ribosomal RNA while being assisted by ribosomal proteins.

A key requirement for highly efficient processivity of the ribosome's main catalytic activities hinges on accurate positioning of the ribosomal substrate, which hinges on the maintenance of the functional conformations of all ribosomal regions involved in ribosomal function, despite their high flexibility. Hence, disorder of these regions has functional meaning, and may be the result of a natural strategy to minimize cell function under hostile conditions.

The ribosomal active site, namely where the peptide bonds are being formed and where the nascent chain is elongated, is situated within a universal symmetrical region that is embedded in the otherwise asymmetric ribosome structure. This symmetrical region is highly conserved and provides the machinery required for peptide bond formation as well as for the ribosome polymerase activity. Therefore, it may be the remnant of the proto-ribosome, which seems to be a dimeric prebiotic machine that initially catalyzed prebiotic reactions, including the formation of chemical bonds, and then produced non-coded oligopeptides.

Structures of complexes of ribosomes with antibiotics revealed principles allowing for the clinical use of antibiotics, identified resistance mechanisms, and pointed at the structural basis for discriminating pathogenic bacteria from hosts. Thus, structural analyses provided valuable information for the improvement of antibiotics and for the design of novel compounds that can serve as antibiotics.

9. Future prospects

Ribosome research has undergone astonishing progress in recent years. The high-resolution structures have shed light on many of the functional properties of the translation machinery and revealed how the ribosome's striking architecture is ingeniously designed as the framework for its unique capabilities: precise decoding, substrate mediated peptide-bond formation and efficient polymerase activity. By analyzing these structures it appears that the ribosomal tasks are performed by the ribosomal RNA and may be supported by the ribosomal proteins.

Among the new findings that emerged from the structures are the intricate mode of decoding, the mobility of most of the ribosomal functional features, the symmetrical region at the core of the ribosome, the dynamic properties of the ribosomal tunnel, the interactions of the ribosome with the progressing nascent

chains, the signaling between the ribosome and cellular components, and the shelter formed by the first chaperone that encounters the nascent chains (trigger factor) for preventing nascent chain aggregation and misfolding. Novel insights from these new findings include the suggestion that the translocation of the tRNA involves at least two concerted motions: sideways shift (which may be performed in a hybrid mode) and a ribosome-navigated rotation. The linkage between these findings and crystal structures of ribosomes with over two dozen antibiotics targeting the ribosome, illuminated various modes of binding and action of these antibiotics. They also deciphered mechanisms leading to resistance and identified the principles allowing for the discrimination between pathogens and eukaryotes despite high ribosome conservation. Further studies enlightened the basis for antibiotic synergism (Figure 6), indicated correlations between antibiotic susceptibility and fitness cost, and revealed a novel induced-fit mechanism exploiting inherent ribosomal flexibility for reshaping the antibiotic binding pocket by remote interactions. Thus, the high-resolution structures of the complexes of the ribosomes with the antibiotics bound to them address key issues associated with the structural basis for antibiotic resistance, synergism, and selectivity and provide unique structural tools for improving antibiotic action.

The availability of the high-resolution structures has stimulated an unpredictable expansion in ribosome research, which in turn has resulted in new insights into the translation process. An appropriate example is the study on real-time tRNA transit on single translating ribosomes at codon resolution (Uemura et al., 2010). However, despite the extensive research and the immense progress, several key issues are still unresolved, some of which are described above. Thus, it is clear that the future of ribosome research and its applications hold more scientific excitement.

Acknowledgements

Thanks are due to all members of the ribosome groups at the Weizmann Institute and at the Unit for Ribosome Research of the Max Planck Society at DESY/Hamburg for their experimental efforts and illuminating discussion. Support was provided by the US National Inst. of Health (GM34 360), the German Ministry for Science and Technology (BMBF 05–641EA), GIF 853–2004, Human Frontier Science Program (HFSP) RGP0076/2003 and the Kimmelman Center for Macromolecular Assemblies. AY holds the Martin and Helen Kimmel Professorial Chair. X-ray diffraction data were collected the EMBL and MPG beam lines at DESY; F1/CHESS, Cornell University, SSRL/Stanford University, ESRF/EMBL, Grenoble, BL26/PF/KEK, Japan, and 19ID&23ID/APS/Argonne National Laboratory.

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