

# High-resolution Structures of Ribosomal Subunits: Initiation, Inhibition, and Conformational Variability

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The recent impressive progress in ribosomal crystallography has yielded insights into the mechanism of protein biosynthesis. Analysis of the high-resolution structures (Ban et al. 2000; Schluenzen et al. 2000; Wimberly et al. 2000) has led to the identification of dynamic aspects of this process and highlighted strategies adopted by the ribosomes for maintaining their structural integrity and for their survival under extreme conditions (Gluehmann et al. 2001; Harms et al. 2001). Naturally, the crystallographic studies have expanded far beyond the presentation of still pictures and are rapidly progressing toward the elucidation of snapshots describing specific functional stages during the biosynthetic process. Structures of complexes with analogs of transfer RNA and messenger RNA (Weinstein et al. 1999; Auerbach et al. 2000; Brodersen et al. 2000; Yusupov et al. 2001), compounds believed to be substrate analogs (Nissen et al. 2000), translation initiation factors (Carter et al. 2001; Pioletti et al. 2001), and antibiotics (Brodersen et al. 2000; Carter et al. 2000; Pioletti et al. 2001; Schluenzen et al. 2001) are rapidly emerging.

The ribosome is a precisely engineered molecular machine performing an intricate multistep process that requires smooth and rapid switches between different conformations. As such, it contains structural elements that allow global motions together with local rearrangements that create a defined sequence of events at the functional centers. Large-scale movements were detected by cryo-electron microscopy (Frank et al. 1995; Stark et al. 1995; Gabashvili et al. 1999), by surface RNA probing (Alexander et al. 1994), by monitoring the ribosomal activity, by numerous attempts at crystallization (see, e.g., Berkovitch-Yellin et al. 1992), and by the analysis of the high-resolution structures (Schluenzen et al. 2000; Harms et al. 2001; Ogle et al. 2001; Pioletti et al. 2001).

The small ribosomal subunit (30S in prokaryotes) is heavily involved in decoding and translocation—the dynamic aspects of protein biosynthesis—and its significant conformational variability has been correlated with its function. Analysis of the crystal structures of this subunit indicated its mobile structural elements (Gluehmann et al. 2001). Consequently, special efforts were made to identify (Wimberly et al. 2000) or to promote (Tocilj et al. 1999; Carter et al. 2000, 2001; Schluenzen et al. 2000; Pi-

oletti et al. 2001) selected conformations within its crystals. The structures of complexes of this subunit with initiation factors, antibiotics, and mRNA or tRNA analogs showed that the decoding process is accomplished mainly by the 16S ribosomal RNA, and that both the proteins and the RNA features involved in the dynamic functions can assume various conformations.

The large subunit (50S in prokaryotes) is responsible for peptide-bond formation. It is known to show less conformational variability than that found for the small one, but significant mobility can be assigned to some of its features, especially those directly involved in its functions. Both subunits may undergo reversible alterations between active and inactive conformations that may be induced by the environmental conditions. We have previously shown that only functionally active ribosomal particles yield crystals and that the dissolved crystallized material is usually highly active when tested under near-physiological conditions (Berkovitch-Yellin et al. 1992). Nevertheless, within the crystals, the ribosomes may assume a non-active conformation, if maintained under far-from-physiological conditions. Thus, there are reasons to believe that the 2.4 Å structure of the large ribosomal subunit from *Haloarcula marismortui* (H50S), which was determined under far-from-physiological conditions (Ban et al. 2000), reflects less active conformations.

In this paper, we describe our analyses on the structures of the two ribosomal subunits in several conformational states. These studies indicate the strategies that the ribosome adopts for enhancing and directing the binding of factors and substrates. They may also show how the ribosome takes advantage of the built-in flexibility of its components for preventing nonproductive interactions.

## THE SMALL RIBOSOMAL SUBUNIT

The small ribosomal subunit (30S) is responsible for the decoding of the genetic information and plays a key role in the initiation phase of protein synthesis. The refined 3.2 Å structure of the functionally activated form of this subunit from *Thermus thermophilus* contains >99% of its 16S RNA chain and most of the amino acids of the subunit's 20 proteins (Schluenzen et al. 2000; Pioletti et al. 2001). The overall fold of the RNA chain, as traced in

our map, is in almost perfect agreement with the suggested two-dimensional diagram, based on phylogenetic, protection, and cross-link studies (Gutell 1996). The global architecture of the small subunit that emerged from our crystallographic studies hints at its inherent dynamics, as it is built of loosely attached domains, radiating from one region at the active center of this particle, where the decoding is performed. We identified the elements that form the entrance to the mRNA channel and are able to close it by a latch-like mechanism (Fig. 1) (Schluenzen et al. 2000). This analysis led us to suggest an interconnected network of features that could allow a concerted movement of the subunit during translocation (for review, see Ramakrishnan and Moore 2001).

Analysis of all the available structures of the small subunit (Schluenzen et al. 2000; Wimberly et al. 2000; Pioletti et al. 2001; Yusupov et al. 2001) shows that the 16S RNA is extensively involved in the decoding process. Nevertheless, some proteins are essential for several steps

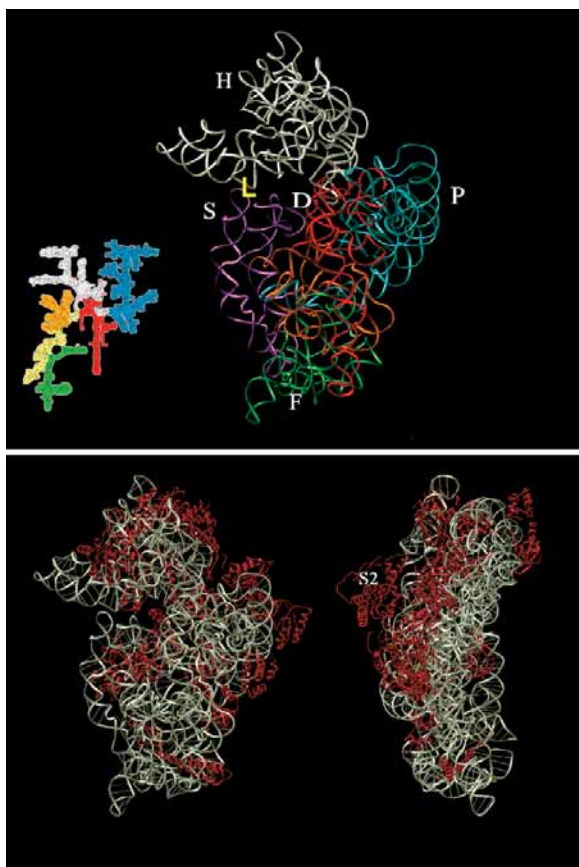
during the biosynthetic process. Almost all ribosomal proteins are built of globular domains located on the solvent side of the particle. These are connected to extended tails or loops buried within the interior of the particle and seem to stabilize the complex RNA fold. However, the tails of a few proteins are pointing into the solution and are less engaged in RNA contacts. As shown below and in Pioletti et al. (2001), some of these may make crucial contributions to the efficient binding of nonribosomal factors participating in the process of protein biosynthesis.

### The Initiation Complex

The small ribosomal subunit is the main player in the initiation of protein biosynthesis. This step has a very important role in governing the accurate setting of the reading frame, as it facilitates the identification of the start codon of mRNA. The mechanisms whereby prokaryotic ribosomes engage mRNA and select the start site are provided by special sequence signals. The initiator mRNA in prokaryotes includes, along with the start codon, an upstream purine-rich sequence (called Shine-Dalgarno [SD]). This pairs with a complementary region in the 16S RNA (called anti-SD), at its 3'-end, thus anchoring the mRNA chains. In the high-resolution structures of the 30S subunit, the anti-SD region is located on the solvent side of the platform, the region that also contains a large part of the E (exit) site.

This intricate process requires the formation of an initiation complex, which in prokaryotes contains the small subunit, mRNA, three initiation factors (IF1, IF2-GTP, and IF3), and initiator tRNA. IF3 plays multiple roles in the formation of this complex. It influences the binding of the other ligands and acts as a fidelity factor by destabilizing noncanonical codon-anticodon interactions. It also selects the start mRNA codon (Sussman et al. 1996) and the correct initiator tRNA to be positioned at the P site (in prokaryotes, the f-met-tRNA). It stabilizes the binding of the fMet-tRNA/IF2 complex to the 30S subunit and discriminates against leaderless mRNA chains (Tedin et al. 1999). IF3 acts as an antiassociation factor because it binds with a high affinity to the 30S subunit and shifts the dissociation equilibrium of the 70S ribosome toward free subunits, thus maintaining a pool of 30S (Grunberg-Manago et al. 1975). It also seems to suppress secondary structure elements in mRNA and to be involved in mRNA shift (La Teana et al. 1995).

IF3 is a small basic protein of about 20 kD. It consists of carboxy- and amino-terminal domains (IF3C and IF3N) connected by a rather long lysine-rich linker region. The structure of the entire protein has not been determined, but nuclear magnetic resonance (NMR) (Garcia et al. 1995a, b) and X-ray structures of the amino- and carboxy-terminal domains have been reported (Biou et al. 1995; Kycia et al. 1995). The interdomain linker appears as a rigid  $\alpha$  helix in the crystal structure (of it and IF3N). The NMR studies, however, showed that even under physiological conditions, the linker is partially unfolded and displays flexibility (Kycia et al. 1995; Moreau et al.



**Figure 1.** (Top left) Secondary structure of the 16S RNA (Gutell 1996). (Top right) The small subunit seen from the interface side (the side facing the large subunit in the 70S ribosome). The colors of the domains follow the colors of the secondary structure. The major subdivisions are labeled: (H) head, (S) shoulder, (P) platform, (F) foot. L represents the latch. (Bottom) Front (left) and side (right) views of the 30S structure, showing the proteins in red and the RNA in silver (ribbon backbone and simple lines for base pairs). Protein S2 is highlighted.

1997; Hua and Raleigh 1998a,b; de Cock et al. 1999). Subsequently, the interdomain distances vary between 25 Å and 65 Å, and, as seen below and in Pioletti et al. (2001), the flexibility and ability of the linker to alter its fold are related to the functional roles of IF3.

A double mutation, G1530/A1531 to A1530/G1531, reduces IF3 binding to the 30S subunit tenfold and enhances its affinity to the 70S ribosome, so that it does not promote the dissociation of the assembled ribosome. IF3 was cross-linked to helices H25, H26, and H45 (Ehresmann et al. 1986), produced footprints in the vicinity of the P site; and enhanced the reactivity and/or altered the cross-linking pattern of specific bases on H45 (Shapkina et al. 2000). Mutations in helices H20, H23a, and H24 affect the binding of IF3, and ribosomes lacking S2 have reduced affinity to IF3 (Tappich et al. 1989; Prescott and Dahlberg 1990; Tedin et al. 1999), but no S2-IF3 cross-links have been reported. The proteins that interact directly with IF3 include S7, S11, S12, S13, S18, and S19 (MacKee et al. 1980).

It was found that IF3C binds firmly to the ribosome, whereas IF3N and the interdomain linker are loosely attached (Weiel and Hershey 1981; Sette et al. 1999). It has been suggested that the carboxy-terminal domain of IF3 (IF3C) performs many of the tasks assigned to the entire IF3 molecule, preventing the association of the 30S with the 50S subunit and contributing to the dissociation of the entire ribosome (Hershey 1987). IF3C was also shown to influence the formation of the initiation complex. The ability of IF3 to discriminate noncanonical initiation codons, or to verify codon-anticodon complementarity, has been attributed mainly to IF3N (Bruhns and Gualerzi 1980).

#### **IF3 Stretches from the P-site tRNA to the Vicinity of the Anti-Shine-Dalgarno Region**

Using crystals of T30S in complex with IF3C, we found that IF3C binds to the 30S particle at the upper end of the platform on the solvent side (Fig. 2), close to the anti-SD region of the 16S rRNA (Pioletti et al. 2001). In the absence of IF3, the region hosting IF3C includes a void of size and shape almost identical to the volume occupied by IF3C (Fig. 2), as determined for IF3C from *Bacillus stearothermophilus* in isolation (Biou et al. 1995). This location reconfirms the results of NMR and mutagenesis of the IF3 molecule (Sette et al. 1999) and is compatible with the effect of the double mutations 1503 1531 (Firpo et al. 1996). It is also consistent with almost all the cross-links, footprints, and protection patterns that were reported for the *Escherichia coli* system (MacKee et al. 1980; Moazed et al. 1995; Sacerdot et al. 1999), except for protein S12. The bound IF3C is wrapped by residues 7–21 of the central loop of protein S18, interacts with residues 21–27 of the flexible amino terminus of protein S2, with residues 153–156 of the carboxy-terminal end of protein S7, with residues 87–96 of protein S11, and with H23, H26, and H45. Support for this placement, and for the mechanism inferred from it, is also provided

by the analysis of the mode of action and the location of edeine (see Fig. 4), an antibiotic agent that interferes with the initiation process (Pioletti et al. 2001).

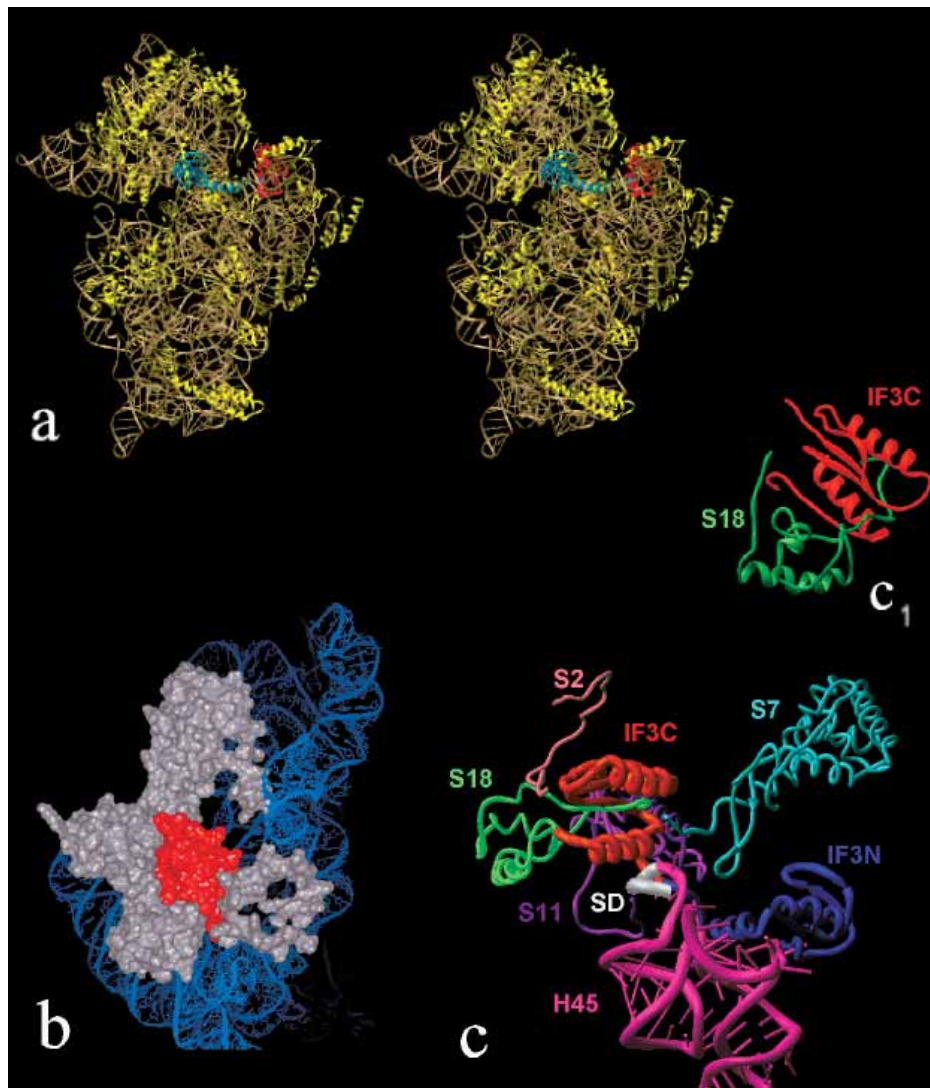
We docked the IF3N and the interdomain linker using the program MOLFIT (Eisenstein et al. 1997) by scanning the surface of the whole 30S subunit for possible binding sites. The only location found by this procedure is in close proximity to the P site. In parallel, we fitted IF3N manually, satisfying its proposed function, the constraints posed by the position of IF3C, and the existing biochemical data. The position found manually is almost identical to that identified by the computed search. In this position IF3N interacts with H28, H29, H31, H34, and H44 (via nucleotides 924–927 and 1381–1387 of H28, 1341 of H29, 966–968 of H31, 1062–1064 of H34, and 1398–1400 of H44). Thus, IF3N contacts all helices known to be involved in the peptidyl-tRNA binding and could affect the RNA cross-links C967 × C1400 and C1402 × C1501, as reported by Shapkina et al. (2000).

#### **IF3 Discriminates Initiator tRNA by Space Exclusion and Prevents Subunit Association by Affecting the Conformational Mobility of the Small Subunit**

The location of IF3C that we observed suggests that the binding of IF3C to the 30S subunit influences the mobility of H45, close to the anchoring site for the SD sequence. IF3 at this site could affect the conformational mobility of the platform that leads to the association of the two ribosomal subunits, consistent with biochemical observations. The spatial proximity of the IF3C-binding site to the anti-SD region suggests a connection between IF3 and the interactions of the mRNAs with the anti-SD region. These interactions could suppress the change in the conformational dynamics induced by IF3, thus allowing subunit association. The connection between the double mutation of G1530/A1531 to A1530/G1531 and the reduced IF3 binding to the 30S subunit, together with the enhanced affinity of IF3 to the 70S ribosomes, supports this hypothesis.

The binding of IF3C on the solvent side of the upper platform sheds light on the initial step of protein biosynthesis, which involves the detachment of the Shine-Dalgarno sequence. It has been suggested that this region is involved in the displacement of the platform that accompanies the translocation (Gabashvili et al. 1999), as part of the combined head-platform-shoulder conformational changes. The binding of IF3C and the hybridization of the anti-SD sequence are likely to limit the mobility of this region. The detachment of the SD anchor, required at the beginning of the translocation process, allows the platform to regain its conformational mobility.

The bound IF3N leaves a limited, albeit sufficient, space for P-site tRNA. Only small conformational changes are required for simultaneous binding of IF3N, mRNA, and the P-site tRNA. Docking of the P-site tRNA to the 30S-IF3N-bound structure led to close contacts between residues 31–35 of the tRNA and the amino-terminal



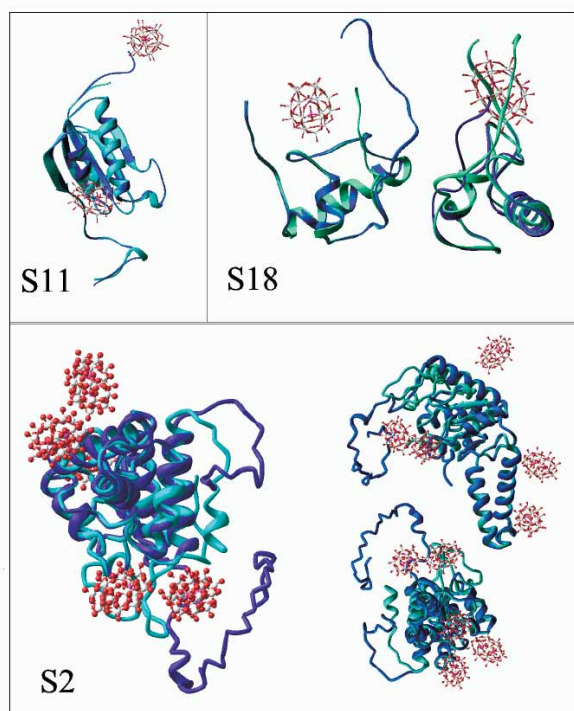
**Figure 2.** The binding of IF3 to T30S. IF3C is shown in red and IF3N and the interdomain linker in blue. (a) Stereo view of the small subunit seen from the interface side (as in Fig. 1, top) with IF3 binding site. (b) Space-filling representation of IF3C within its vicinity when bound to T30S. Ribosomal proteins are shown in gray, the RNA chain in blue. (c) Close-up of the vicinity of IF3. The RNA features and the proteins are marked by their numbers. (SD) Anti Shine-Dalgarno region. (c1) Close-up showing the contacts of S18 and IF3C.

end of IF3N, shedding light on the mechanism of IF3N-mediated discrimination of noncanonical initiation codons. Thus, it seems that the influence of IF3N on initiator tRNA binding is based on space exclusion principles, rather than on specific codon-anticodon complementarity rules, as suggested earlier (Meinzel et al. 1999).

Only indirect contacts exist between IF3N and IF3C, via the curved connection formed by the interdomain linker that wraps around the platform toward the neck. Various mutations, insertions, and deletions that cause significant modifications in the length of the linker do not have major effects on the efficiency of IF3, indicating that the linker maintains its flexibility while IF3 is bound to the 30S subunit. Consequently, it can act as a transmitting

strap between the two domains and can indirectly affect the conformation of the P site and induce its specificity for tRNA-fMet (de Cock et al. 1999). Similarly, the structural changes in IF3 could trigger conformational changes within the 30S subunit that are required for initiating the biosynthetic process and may also lead to a suppression of secondary structure elements in the mRNA. Thus, our structure is consistent with the proposal that the linker maintains its flexibility when IF3 is bound to the 30S subunit and that the flexibility and the ability of the linker region to alter its fold are related to the function of IF3.

The position of IF3 suggests two additional pathways for the transmission of information between the P site and the solvent side of the platform. The flexible protein S2



**Figure 3.** Conformations of proteins S2, S11, and S18 in the presence (*blue*) and the absence (*cyan*) of W18 clusters (the W atoms are shown as red balls). Note the similarity between protein S18-IF3C and S18-W18 contacts (see also Fig. 2).

(Figs. 1, 2, and 3) that interacts with the head and the body of T30S may be an appropriate candidate, consistent with the reciprocal relationship between its existence and the efficiency of IF3 (Tedin et al. 1999). The mRNA itself may also provide a long-range information channel, as successful binding of the cognate tRNA at the P site should be followed by the disruption of the hybridization at the anti-SD region.

In summary, the conformation structure of the small ribosomal subunit that was determined by us is similar to the conformation of this subunit within the initiation complex. The localization of IF3C on the 30S subunit and the docking of IF3N and of the linker region provide a connection between the functions of IF3 and the existing cross-linking, protection, and mutagenesis data. It also explains the correlation between the binding of IF3 to the small ribosomal subunit and the mRNA requirement to interact with the anti-SD region of 16S rRNA for initiating correct translation. The discriminatory function exerted by IF3 against noncanonical codons of the initiator tRNA appears to result from space-exclusion principles, rather than by specific codon-anticodon complementarity rules, since the binding of IF3N leaves limited, albeit sufficient, space for the P-site tRNA. The location of IF3C in our map indicates that the anti-association activity of IF3 is not due to physical blockage of the intersubunit interface, but rather to a change in the conformational dynamics of the subunit.

### Agents Interfering with the Initiation Process

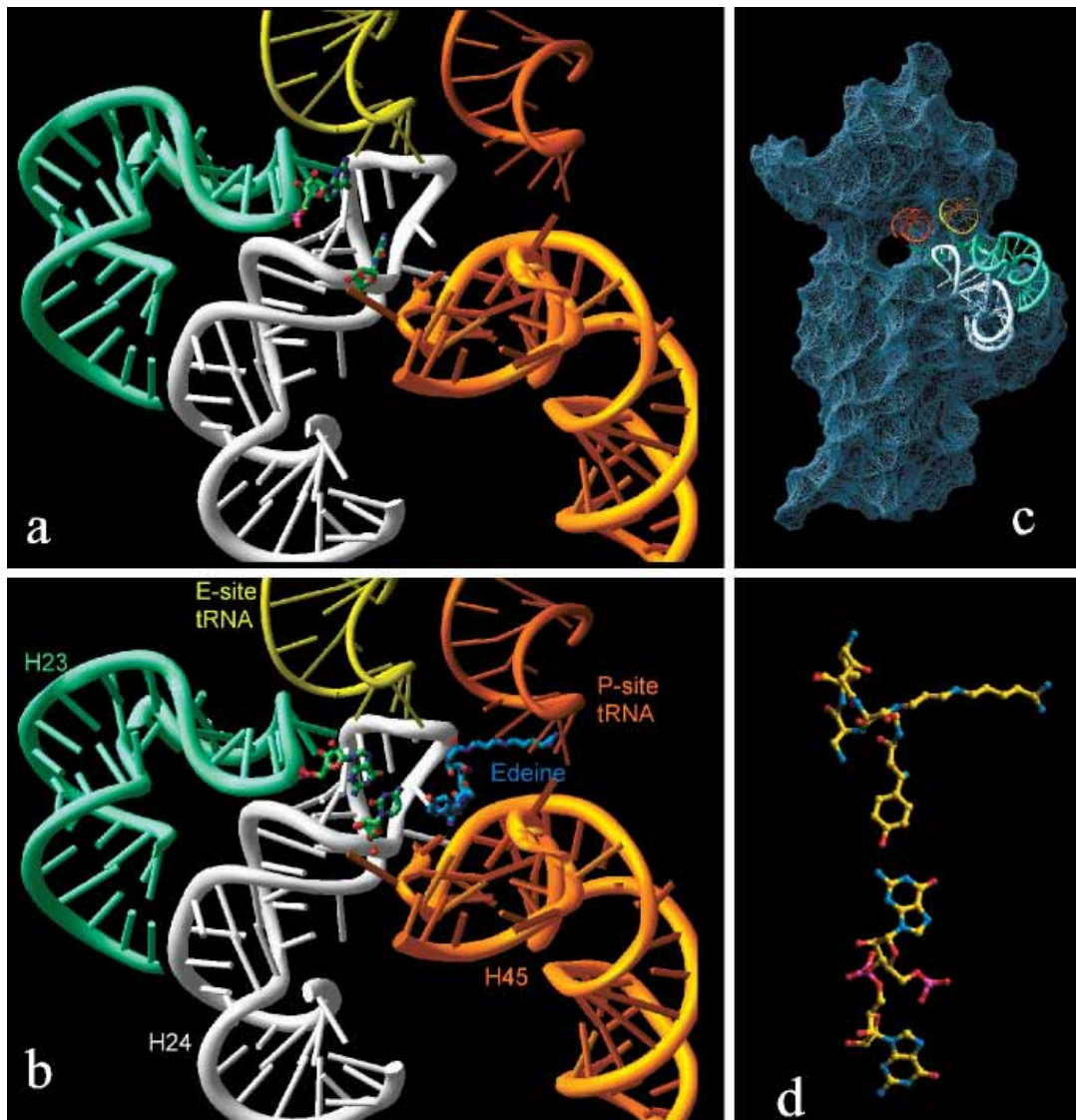
Analysis of the structure of the complex of T30S with edeine, a universal initiation inhibitor (Odon et al. 1978; Altamura et al. 1988), supports the mechanism suggested above for the initiation process. We found that edeine binds in the platform between the loop of helix 24 and helix 45 (Fig. 4) (Pioletti et al. 2001). In this position, it would not alter the binding of IF3C, but might well affect the binding of the IF3 intersubunit linker and of IF3N. At the same time, it could influence the mobility of the particle, the interaction of the 3' end with IF3C and the interactions between the 30S and 50S subunits.

Edeine is a peptide-like antibiotic agent, produced by a strain of *Bacillus brevis*. It contains a spermidine-type moiety at its carboxy-terminal end and a  $\beta$ -tyrosine residue at its amino-terminal end (Kurylo-Borowska 1975). It protects a subset of 16S rRNA nucleotides that are also protected by P-site tRNA (Moazed and Noller 1987; Woodcock et al. 1991). It also shares protections with the antibiotics kasugamycin (bases A794, G926) and pactamycin (bases G693, C795) (Woodcock et al. 1991; Mankin 1997). Mutations in G791 and A792 (H24) reduce association of the 30S and 50S subunits, and an A792 mutant is associated with loss of IF3 binding (Tapprich et al. 1989; Santer et al. 1990). G926 (H28) interacts with the tRNA bound at the P site and is protected by edeine (Woodcock et al. 1991). In addition, mutations in U1498 impair A-site function and enhance tRNA-fMet selectivity (Ringquist et al. 1993), whereas mutations in G1505 increase the levels of stop codon readthrough and frameshifting (O'Connor et al. 1995, 1997).

We found that edeine binds to nucleotides of H24, H28, and H44 at the core of the decoding region, and it is a close neighbor of H45. By physically linking these four helices, critical for tRNA, IF3, and mRNA binding, edeine could lock the small subunit into a fixed configuration (Fig. 4) and hinder the conformational changes that accompany the translation process (Gabashvili et al. 1999; VanLoock et al. 2000).

We also found that the binding of edeine to the 30S subunit induces the formation of a base pair between C795 at the loop of H24 and G693 at the loop of H23. G693 has been shown to be protected when edeine is bound (Woodcock et al. 1991). H23 plays an important role in the binding of the carboxy-terminal domain of IF3, and nucleotides 787–795 of H24 are directly involved in subunit association (Tapprich and Hill 1986). This newly induced G693–C795 base pair alters the mRNA path and would impose constraints on the mobility of the platform, hence interfering with the initiation. Thus, our data suggest that the initiation process is the main target of this universal antibiotic and that edeine induces an allosteric change by the formation of a new base pair—an important new principle of antibiotic action that fits nicely with our suggested mechanism of the initiation step.

Independent studies show that pactamycin, an antibiotic agent that shares a protection pattern with edeine, bridges H23b and H24a, the same helices that are linked by the new base pair that is induced by edeine (Brodersen et al.



**Figure 4.** Binding of edeine to the small subunit. Detailed views of the edeine-binding site are shown in *a* and *b*. For both, the assignments of the different ribosomal components are shown in *b*. (*a*) The native conformation. (*b*) The same site once edeine is bound. Note the newly formed base pair. (*c*) The small subunit shown at about 75° rotation (around the vertical axis of the particle) compared to the view of Fig. 1, top. The mRNA channel is clearly seen. (*d*) The pseudo base pair formed by edeine (*top*) upon interaction with the 30S subunit.

2000). This agent is known to interfere with the initiation process, and it is likely that besides reducing the mobility of the platform by locking these two helices, it also alters the mRNA path at the E site. Like edeine, pactamycin interacts with the extended loop of *S7*—the upper border of the path of the exiting mRNA/tRNA complex—and its mode of interaction suggests that it may interfere with the pairing of the SD sequence or prevent it.

Tetracycline, a multisite antibiotic agent, also binds to protein *S7*. This is one of the less-occupied sites among the six binding sites of tetracycline that were characterized by us (Pioletti et al. 2001), but it was detected in several biochemical studies. Its influence, if any, at this site is minor, compared to its primary activity—preventing

A-site tRNA binding. Nevertheless, it may induce conformational changes in *S7*, a protein that plays an important role in initiation and in translocation. *S7* is one of the primary rRNA-binding proteins (Held et al. 1974) and is known as one of the proteins that initiate the assembly of the 30S subunit (Nowotny and Nierhaus 1988) *in vitro*. Therefore, the binding of tetracycline to it could disturb the early assembly steps of new 30S particles, contributing to the overall inhibitory effect of tetracycline.

#### On the Universality of Initiation

The universal effect of edeine on initiation implies that the main structural elements important for the initiation

process are conserved in all kingdoms (Odon et al. 1978). Analysis of our results shows that the rRNA bases defining the edeine-binding site are conserved in chloroplasts, mitochondria, and the three phylogenetic domains. In particular, the effect of edeine on the mRNA path—preventing hybridization of the incoming mRNA—is achieved by edeine's interactions with G926 and G693, two conserved nucleotides. Thus, G926 (H28) has been photo-crosslinked in *E. coli* to position +2 of the mRNA (Sergiev et al. 1997) and to position +1 in human ribosomes (Demeshkina et al. 2000), and G693 has been photo-crosslinked in *E. coli* to positions -1/-3 of the mRNA and in human ribosomes to position -3 of the mRNA (Demeshkina et al. 2000).

Cryo-EM reconstruction of a complex of the small ribosomal subunit from *T. thermophilus* with IF3 from *Thermotoga* localized IF3C at the subunit interface, suggesting that the anti-association activity of IF3 is the product of physical blockage at the interface between the two subunits (McCutcheon et al. 1999). On the other hand, EM studies on rat liver 40S in complex with the eukaryotic initiation factor 3 (eIF3) located eIF3 on the solvent side of the upper edge of the platform (Srivastava et al. 1992), in a region comparable to our findings. In this location, IF3 seems to perform its anti-association activity by effecting the conformational mobility of the small ribosomal subunit—in particular, suppressing the conformational mobility of the platform, essential for association of the two ribosomal subunits. Some aspects of the initiation process of protein biosyntheses were found to be different in eukaryotic and prokaryotic systems (Hershey et al. 1996). Nevertheless, neither of them indicates different locations of IF3. The consistency between our results and the location of the eukaryotic initiation factor may indicate that the main concepts underlying the initiation process and governing the anti-association properties of the initiation complex have been evolutionarily conserved.

### Structural Basis for the Tight Binding of IF3C

Most of the extended regions of the ribosomal proteins are buried within RNA features and are believed to stabilize or even assist in shaping the intricate ribosomal structure. In addition to these, there are many flexible tails that are pointing toward the solution, or lie loosely on the ribosomal surface. The amino-terminal ends of S18 and of S2, and the carboxy-terminal ends of S7 and S11, show strikingly different conformations when comparing our structures of T30S (Schlunzen et al. 2000; Pioletti et al. 2001) with that independently determined (Wimberly et al. 2000), presumably related to our crystal stabilization with a heteropolytungstate cluster containing 18 atoms of W (Dawson 1953). We used this cluster to minimize the conformational heterogeneity and limit the mobility of the crystallized T30S subunits (Tocij et al. 1999; Schlunzen et al. 2000). This procedure was employed for native crystals as well as for complexes of T30S with compounds that facilitate or inhibit protein biosynthesis,

mRNA analogs, initiation factors, and antibiotics. The preparation of the complexes was found to be more efficient if the crystals were soaked in solutions containing the nonribosomal compounds at elevated temperatures, following the routine heat-activation procedure (Zamir et al. 1971), and once the functional complexes were formed, the crystals were cooled down to room temperature and then were treated with the clusters.

Analysis of the structure of the tungstenated crystals (Schlunzen et al. 2000, Pioletti et al. 2001) showed that the extensions that point out into the solution are more ordered than their counterparts in the non-W18-treated crystals (Wimberly et al. 2000). Most of these protein tails bind W18 clusters, creating an extensive network of contacts between their lysines and arginines and the acidic clusters (Fig. 3), suggesting that these tails are able to act as tentacles that enhance the binding of non-ribosomal compounds participating in the process. Once the binding is no longer required, owing to their inherent flexibility, the protein tails can stretch out and release the compounds. Comparing the structure of the tungstenated 30S subunits with that of the complex with carboxy-terminal domain of IF3 shows that the W18 cluster imitates IF3C (Figs. 2 and 3). Indeed, in competition experiments it was found that crystals that were treated with W18 prior to soaking in solutions containing IF3C failed to bind IF3C. This explains why no major conformational changes were observed between the tungstenated and IF3C-bound 30S subunits, contrary to the conformational changes observed while binding IF3 to 30S at its free conformation (Gabashvili et al. 1999; McCutcheon et al. 1999). We therefore conclude that the reference structure of the 30S ribosomal subunit, as determined by using W18-treated crystals, mimics that of the small subunit at the initiation stage.

In summary, the analysis of the structure of the complex of T30S with IF3C not only sheds light on the nature of the binding and action of this factor, but also indicates that the exterior protein tails have important functional tasks that benefit from their significant flexibility. These tasks are quite different from those assigned to the protein extensions in the interior of the particle, which are assumed to be involved mainly in the stabilization of the ribosome structure.

### THE LARGE RIBOSOMAL SUBUNIT Flexibility, Functional Activity, and Apparent Disorder

In ribosomal crystallography, the key to high-resolution data was to crystallize the relatively robust ribosomal particles, assuming that they deteriorate less while being prepared and therefore are expected to yield more homogeneous starting materials for crystallization. The ribosomes from the extreme halophile, *Haloarcula marismortui*, the bacteria that live in the Dead Sea, were found suitable. These bacteria have developed a sophisticated system to accumulate enormous amounts of KCl (3–5 M), although the medium contains only millimolar amounts of it (Table 1) (Ginzburg et al. 1970). Indeed, the func-

**Table 1.** Concentration of Ions within the Cells of *H. marismortui*

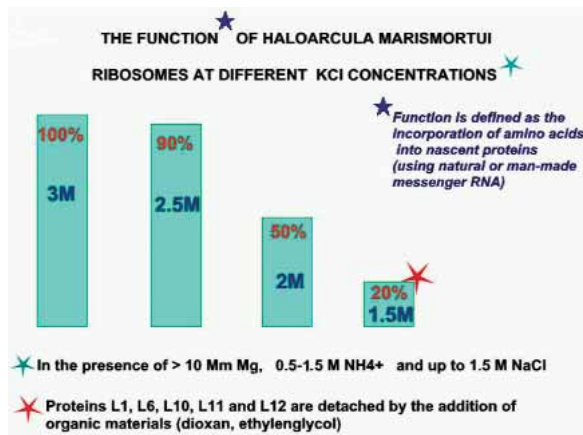
	Early log	Late log	Stationary
K in cells:	3.7–5.0 M	3.7–4.0 M	3.7–4.0 M
Na in cells:	1.2–3.0 M	1.6–2.1 M	0.5–0.7 M

(Modified from Ginzburg et al. 1970)

tional activity of these ribosomes was found to be directly linked to the concentrations of the potassium ion in the reaction mixture (Fig. 5).

Initially, we grew the crystals of the 50S subunits from this bacterium (H50S) under conditions mimicking the in situ conditions at the bacterial log period. In these experiments, crystals were grown in solutions containing 0.5 M ammonium chloride and 3 M potassium chloride. Under these conditions, nucleation occurred rapidly and yielded small disordered crystals. Consequently, we developed a procedure for crystallization at the lowest potassium concentration required for maintaining the integrity of the subunits (1.2 M KCl), although under these conditions the halophilic ribosomes have only marginal activity (Shevack et al. 1985). Once the crystals grew, we transferred them to solutions containing around 3 M KCl, so that the crystallized particles could rearrange into their active conformation. Post-crystallization rearrangement of ribosomes has also been induced in crystals of the small ribosomal subunits from *T. thermophilus*, either by W18 treatment or by binding initiation factors (Carter et al. 2001; Pioletti et al. 2001).

This procedure yielded crystals showing functional activity and diffracting beyond 2.5 Å resolution (von Bohlen et al. 1991; Yonath et al. 1998). However, the high potassium concentration within these crystals (2.8–3.0 M) caused severe problems in the course of structure determination. The combination of severe non-isomorphism, apparent twinning, high radiation sensitivity,



**Figure 5.** Functional activity of the ribosomes from *H. marismortui* at different potassium concentrations. Activity was checked by the synthesis of polypeptides and by the incorporation of 50S into 70S. In both cases, the ribosomal particles underwent heat activation at 55° for 40 minutes, and homo- or heteronucleotides served as mRNA chains.

unstable cell constants, nonuniform mosaic spread, and uneven reflection shape hampered the collection of data usable for structure determination. As these problems became less tolerable at higher resolution, the structure determination under near-physiological conditions stalled at resolutions lower than 5 Å (Yonath et al. 1998; Ban et al. 1999; Harms et al. 1999; Weinstein et al. 1999).

Improved crystals were obtained by drastic reduction of the salt concentration in their stabilization solution, and the exchange of a high concentration of KCl for a relatively low concentration of NaCl (called here L-Na). These far-from-physiological conditions yielded a structure at 2.4 Å resolution (Ban et al. 2000) and even allowed the binding of compounds believed to be substrate analogs, such as CCdA-phosphate-puromycin (Welch et al. 1995; Nissen et al. 2000). Thus, in contrast to the wealth of crystallographic information already obtained for factors and antibiotic-bound complexes of the small subunit, no reports have appeared of high-resolution crystallographic studies of H50S in such complexes. This is consistent with previous observations, showing that extreme halophiles are resistant to most of the antibiotic agents that inhibit bacterial and eukaryotic ribosomes, even when tested under their physiological conditions (Mankin and Garrett 1991). Clearly, the less active conformations are likely to show even lower binding affinities for natural substrates like acylated-tRNA molecules and antibiotics.

The nucleotides that could not be traced in the electron density map obtained from the L-Na crystals, since there was no density that could account for them, were considered to be disordered. These account for less than 10% of the total structure but comprise a significant portion of regions involved in functional activity. They include helix H1, the distal end of helix H38, the tip of H69, the entire regions of H43-H44 and H76-H78. Among these, the L1 stalk (H76-H78 with their bound protein L1) and the L12 stalk (H43-H44 and their bound proteins L10 and L12) create the prominent features of the typical shape of the large ribosomal subunit. These are two lateral protuberances that are readily observed in all EM models and reconstructions, using negative staining, dark-field, or cryo-EM reconstruction. They were also detected in electron density maps obtained from crystals of H50S that were grown and maintained under near-physiological conditions (Ban et al. 1998; Yonath et al. 1998), albeit at lower resolution.

All the untraceable regions of the RNA, together with the proteins that bind to them, contribute to the process of protein biosynthesis. H38 and H69 form intersubunit bridges within the assembled ribosome and interact with the tRNA molecules. L12 and L10 are involved in the contacts with the translocational factors and in factor-dependent GTPase activity (Chandra Sanyal and Liljas 2000). L11 is known to be involved in elongation factor activities (Cundliffe et al. 1979). L1 is a translational repressor binding mRNA (Nikonov et al. 1996), and its absence has a negative effect on the rate of protein synthesis (Subraminian and Dabbs 1980). Since these regions were detected in the maps of the assembled 70S ribo-



some, their absence in the L-Na electron density of H50S stimulated the notion that the functionally important regions are disordered in the free particles, and become fixed in place upon subunit association (Yusupov et al. 2001).

All four proteins (L1, L10, L11, L12) that were not observed in the L-Na map match the list of proteins that we detached selectively from halophilic ribosomes (Franceschi et al. 1994). Evidently, these proteins are less well bound to the core of the large subunit, but it is not clear whether they are indeed disordered, as suggested by Ban et al. (2000), or partially or fully removed from the large subunit. Interestingly, the conditions used for the stabilization of the L-Na crystals are similar to those developed by us for the detachment of these proteins from the ribosome, in solution (Franceschi et al. 1994). It is conceivable that these loosely held proteins were partially or fully detached once the crystals were exposed to the far-from-physiological conditions. The free proteins could then float within the unusually large and continuous solvent regions of these crystal forms (Yonath et al. 1998).

#### Conformational Variability as a Functional Tool

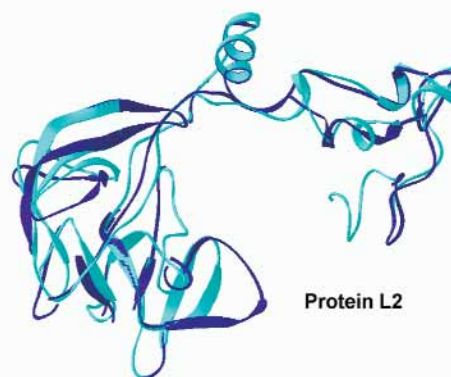
Recently, we optimized the experimental conditions and minimized the harm caused by the high potassium concentration. Using data collected from crystals of H50S that were grown and kept under conditions mimicking the physiological environment throughout their life span (called here H-K crystals), we constructed a 3.6 Å electron density map. Phases were obtained from anomalous dispersion of several heavy atoms (Harms et al. 1999; Bashan et al. 2000) in combination with crystal averaging and molecular replacement studies based on the 2.4 Å resolution structure (I. Agmon et al., in prep.). Because of the difficulties caused by the high potassium content, the resolution of these studies is somewhat lower than that obtained for the L-Na crystals. Nevertheless, the electron density map is interpretable and enabled a rather detailed comparison between the two structures, aimed at the identification of conformational elements that are required for functional activity in order to reveal how the inherent flexibility of specific features is being exploited by the ribosome to enhance productive, and prevent non-productive, interactions.

In general, the skeleton of the H-K structure is similar to that determined at low NaCl. However, slight but distinct changes in inter-helix packing, and consequently in the compactness of the structure, were observed. In addition, significant parts of the RNA regions that were not detected in the L-Na map could be traced in the H-K map. These include the tip of H38, which forms the A-site finger and the intersubunit B1a bridge, and H42–H44, the helices forming the GTPase center. We also observed significant discrepancies in the locations of the globular parts of the proteins L10e (L16 in *E. coli*), L37e, L24e, L23, L44e, and L6 in the two maps. Among these, proteins L16 and L6 are of particular interest. L16 is known to be involved in protein biosynthesis in a yet-unknown

way. Protein L6 belongs to the group of five proteins that can be stoichiometrically detached from the particle. It is the only one among this group (L1, L6, L7/12, L10, and L11) that is seen in the L-Na map.

The conformations of almost all the proteins in the H-K structure are different from those observed in L-Na. Some of the differences are subtle, localized within inter-domain loops. In order to fit these proteins, minor adjustments were required, and it is likely that such rearrangements may occur after changes in the environment (e.g., salt concentration, type of ion). More interesting are the differences in the locations and the conformations of the termini extensions. Notable are L24, L16 (*E. coli* numbering), and L2. The latter seems to play a key role in facilitating the formation of the peptide bond and in binding both A- and P-tRNA. It was found to increase the hydrolysis rate of peptidyl-tRNA. Mutations in its highly conserved His-229 are fully or conditionally lethal (for review, see Uhlein et al. 1998; Khaitovich and Mankin 1999; Diedrich et al. 2000), and its affinity labeling with an analog of chloramphenicol—an antibiotic known to inhibit protein biosynthesis—caused an irreversible loss of peptidyl-transferase activity (Sonenberg et al. 1973). It was also found to resist extensive digestion with potent proteases in combination with phenol treatment, a procedure that disrupted all other ribosomal proteins (Noller et al. 1992).

Figure 6 shows that the carboxy-terminal extension of L2 loops toward the interior of the particle in the L-Na structure. In the H-K structure, however, there is no density for it, indicating its flexibility. It could therefore extend toward the tRNA molecules located in its vicinity (Yusupov et al. 2001). In this way, it may enhance the binding of the tRNA molecules and effect their accurate positioning as long as they are involved in the peptide-



**Figure 6.** Protein L2 as in the L-Na (in cyan) and H-K (in blue) structures. Note the differences in the general fold. The carboxyl terminus of the L-Na structure loops toward the main part of the protein, whereas in the H-K structure, there is no density for it for the last 15 residues, indicating its potential flexibility.

bond formation, then, by a small conformational change, relieve the tight binding and allow translocation, acting in a fashion similar to that observed for S18 upon binding of IF3C (Figs. 2 and 4) (Pioletti et al. 2001). This may be the reason for the tight binding of L2 to the large subunit, and for the difficulties encountered in attempts to separate it from the RNA core (Noller et al. 1992).

One conclusion that can be drawn from both structural studies of the large ribosomal subunit *H. marismortui* is that the 2.4 Å L-Na structure represents a conformation that differs from that of the native particle. The absence of almost all the structural features of the 50S subunit that are involved in noncatalytic functional aspects of protein biosynthesis in the 2.4 Å structure of H50S pose an additional shortcoming. These features were not seen in the electron density map and, therefore, were assumed to be disordered. Biochemical, functional, and EM studies indicated that these features are inherently flexible. However, flexibility is not necessarily synonymous with disorder. Thus, flexible parts of molecules or assemblies may assume several well-defined conformations, and the switch from one conformation to another is related to their functional state. In such cases, well-diffracting crystals may be obtained from one of the specific conformations. Therefore, large features that are disordered in the 2.4 Å L-Na H50S map may indicate a special ribosomal strategy to avoid inefficient binding of nonribosomal components. As mentioned above, a similar discriminative binding fashion was detected for the binding of IF3 by the small subunit: The amino-terminal tail of protein S18 is disordered unless it binds the carboxy-terminal domain of IF3 or its mimics.

*H. marismortui* is an archaea bearing low compatibility with *E. coli*, the species yielding most of our knowledge on ribosomes. Despite the suitability of the ribosomes from *H. marismortui* for high-resolution crystallography, they have not become a subject of many biochemical studies. Consequently, only a small part of the vast amount of data accumulated over almost half a century of ribosomal research can be related directly to its structure. In addition, the antibiotics from the macrolide family hardly bind to the halophilic ribosomes, since a key nucleotide, A2508, is a guanine in their 23S RNA. They are also rather resistant to other antibiotic agents (Mankin and Garret 1991), even under suitable conditions. Thus, it is not surprising that, contrary to the wealth of crystallographic information already obtained about binding of factors and antibiotics to the small subunit (Brodersen et al. 2000; Carter et al. 2000, 2001; Ogle et al. 2001; Pioletti et al. 2001), so far only complexes of H50S with materials believed to represent substrate analogs were found to be suitable for high-resolution crystallographic studies (Nissen et al. 2000). Furthermore, despite extensive studies exploiting these complexes (Nissen et al. 2000), the structural basis for peptidyl-transferase activity is still not well understood (Barta et al. 2001). Thus, in contrast to the strict requirements for antibiotic binding, all nucleotides believed to be crucial for the catalytic activity according to the suggested mechanism (Nissen et al. 2000) could be

mutated with little or no effect on peptide-bond formation in vitro (Polacek et al. 2001) and in vivo (Thompson et al. 2001).

For these reasons, we initiated crystallographic studies on the large ribosomal subunit from a eubacterial source that shows a high homology with *T. thermophilus* and *E. coli*. Crystals of the 50S particles from *Deinococcus radiodurans* (D50S) were grown and maintained under conditions that are almost identical to the in situ environment. These crystals, as well as those grown from complexes of these subunits with antibiotics, diffract to higher than 2.9 Å resolution, are relatively stable in the X-ray beam, and yield crystallographic data of very high quality. Thus, they provide an excellent system to investigate antibiotic binding and functional flexibility (Harms et al. 2001; Schlunzen et al. 2001).

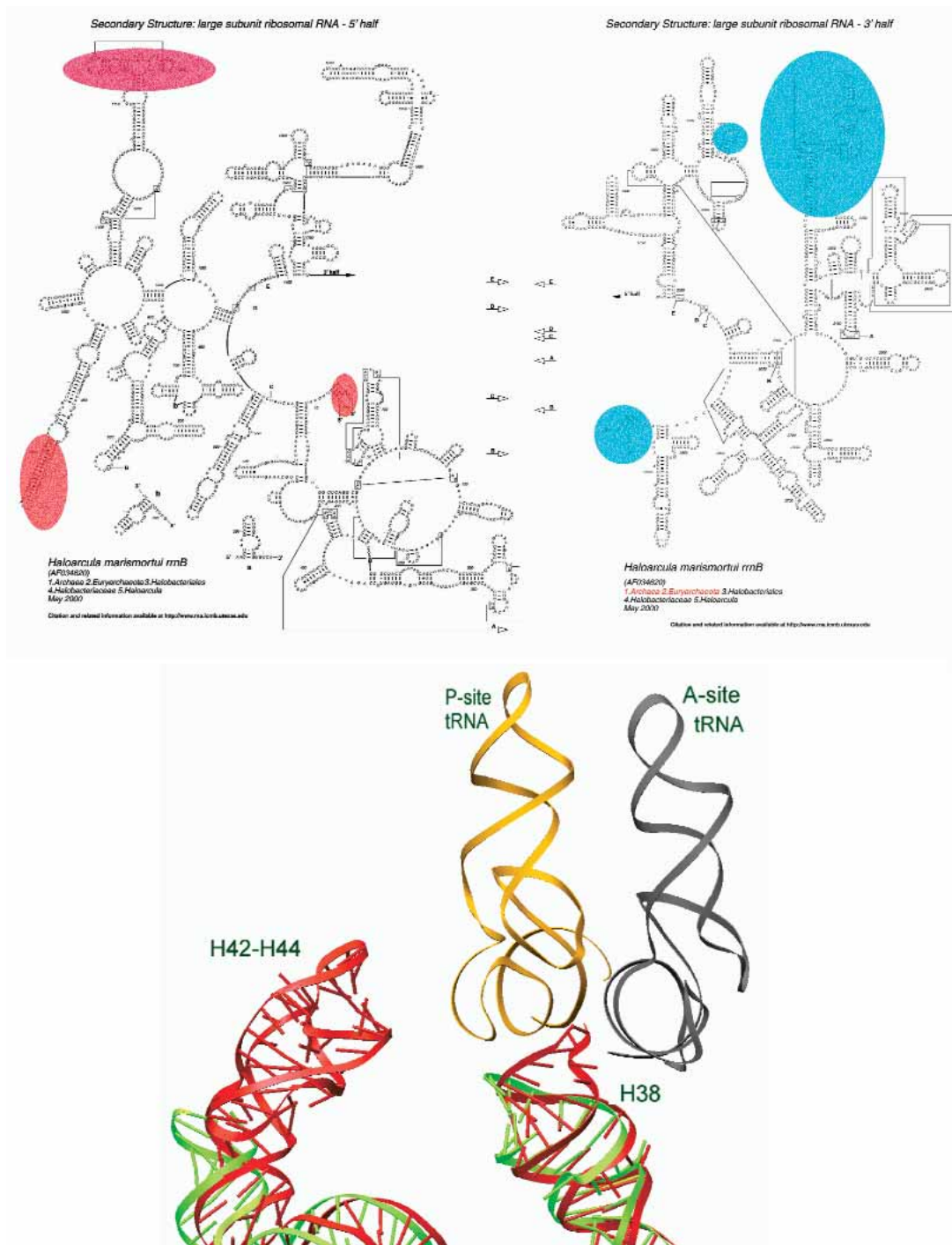
The structure of the large ribosomal subunit from this source is significantly more ordered than that of H50S, and many of the functional relevant features that are disordered in H50S are well resolved in D50S. These include the intersubunit bridges, such as those formed by L69 (the bridge to the decoding center), the upper half of H38 (called the A-site finger or the intersubunit B1a bridge) (Fig. 7), the L1 arm (helices 76–78), the GTPase center (helices H42–H44 and protein L11). All are flexible, and most assume orientations that differ, to varying extents, from those seen in the 70S ribosomes, as determined at 5.5 Å (Yusupov et al. 2001).

Of interest is protein L27, which was shown to be important for the biosynthetic process (Sonenberg et al. 1973; Wower et al. 1993). As seen in Figure 8, its location in D50S is consistent with its footprinting in *E. coli* (A.S. Mankin, pers. comm.). In this location it can reach the area between the P and A sites, whereas in *H. marismortui*, its homolog (called H21e) folds toward the interior, consistent with the hypothesis that the tails of the ribosomal proteins that bind factors and substrates fold backward when conditions are not suitable for productive protein biosynthesis.

Many unexpected developments occurred during our long-lasting involvement in ribosomal crystallography. One of them is reported here. Thus, owing to crystallographic difficulties, not only the structures of two conformations of the large subunit from *H. marismortui* are now available for comparative studies, but also a detailed structure of the large ribosomal subunit from a mesophilic source has been determined. Consequently, the gate is opened to detailed comparisons between free and 70S-bound large subunit, to thorough implementation of the biochemical knowledge obtained for *E. coli* ribosomes, and perhaps also to the design of potent antibiotics.

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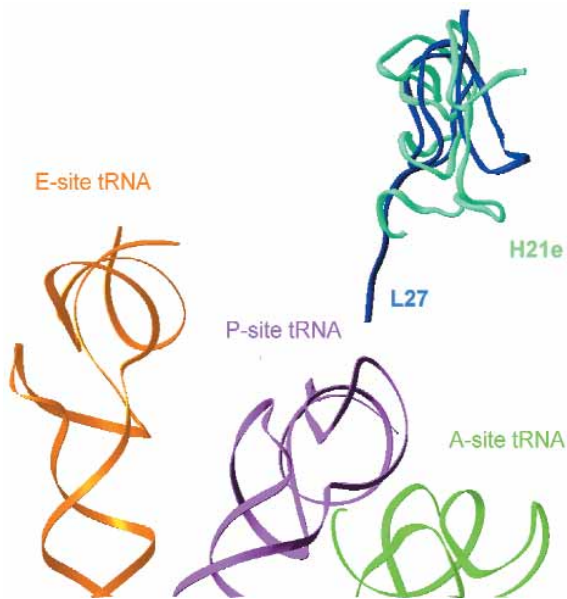
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**Figure 7.** (Top) The disordered RNA regions in structure obtained from the L-Na H50S crystals (Ban et al. 2000), marked in red on the 5' half and in cyan on the 3' half of the secondary structure diagram of the 23S RNA of *H. marismortui*. (Bottom) The conformations and locations of two of these RNA regions in the D50S structure are shown in red. For comparison, the portions of the RNA helices that could be traced in the L-Na structure are shown in green. Note the proximity of H38 to the docked A- and P-tRNA molecules.

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**Figure 8.** Comparison between D50S protein L27 (in blue) and HmL21e (in light green), the protein that was found to occupy the same location in the L-Na structure. The amino-terminal end of HmL21e folds away from the tRNA-binding sites, whereas that of EcL27 almost reaches them.

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