5. DISCUSSION

5.1 c-DNA

We found that the affinity of some of the native or heavy atom modified oligonucleotides for their complementary sequence in the *T. thermophilus* 16S rRNA is very high (Table 1). This result agrees with previous studies which showed that some regions of the 16S rRNA from *E. coli* are accessible for the binding of short cDNA oligonucleodites (Oakes et al., 1986; Hill et al., 1988; Ricker and Kaji, 1991; Alexander et al., 1994; Wang et al., 1999).

Co-crystallisation of the 30S subunits with the oligonucleotides was not always successful. Probably because of the unstable nature of the cDNA:RNA hybrids, they may not hold during the long time needed for crystallisation. For this reason we used the labelled oligonucleotides to soak the T30S crystals. Diffusion of the oligonucleotides into the crystals is a rather short process that is terminated by the shock freezing needed for data collection. In addition, the crystal network utilises a fair part of the particle surface, thus leaving fewer regions that may compete with the region for which the cDNA oligonucleotide was designed. Clearly, the volume available within the crystal for the movement of the oligonucleotides is rather limited. In fact, such long heavy atom carriers (up to 70 Å) are not commonly used in protein crystallography. In this case the large continuous solvent region within the T30S crystals is advantageous for the diffusion of the oligonucleotides and other ligands. In this way we obtained specific labelling of the T30S within the crystals.

We observed that the oligonucleotides diffused into the T30S crystals have a marked effect on the resolution of the crystals. Some of them produced a decrease in resolution, probably because the regions where they bind are involved in the crystal network, or because their binding induced conformational changes. Others did not change or even improved the resolution of the crystals, probably by stabilising regions exposed to the solvent (see Table 1).
We could not collected data from crystals to which oligonucleotides complementing the 16S rRNA around base 1400 were diffused, since they caused a resolution drop to 12-15 Å accompanied by a dramatic increase in the mosaic spread. According to our interpretation of the map, the interparticle network forming the crystal may share some of the contacts of T30S that are normally involved in binding to the large subunit in the associated 70S ribosome. Consequently the decoding domain is masked by the crystal packing contacts, and is therefore not available for efficient cDNA binding.
In contrast, we could collect data from crystals to which oligonucleotides complementing the 3’ end region of the 16S rRNA were diffused, thus allowing us the localisation of the 3’ end of the16S rRNA (Auerbach et al., 2000). This oligonucleotide is of high functional relevance, as it contains the Shine-Dalgarno sequence, thus imitating the mRNA segment involved in the initiation of protein biosynthesis. The 3’ end region of the 16S rRNA chain is known to be rather flexible and may adopt several conformations (Müller and

Brimacombe, 1997). Hence, we suggest that the high quality diffraction obtained from the crystals derivatised by this oligonucleotide results from stabilisation of the flexible 3' arm of the 16S rRNA in a fashion that mimics its binding to mRNA.

5.2 The initiation factor 3

We found that IF3-C binds to the 30S particle at the upper end of the platform on the solvent side of 30S (Figure 18a), close to the anti-SD region of the 16S rRNA. IF3-C does not bind at the inter-subunit interface of the 30S and 50S subunits, and therefore its anti-association activity is not the product of physical blockage at the interface between the two subunits.

Electron-microscopy (EM) reconstructions of rat liver 40S in complex with the eukaryotic initiation factor 3 (eIF3) located eIF3 in a comparable region at the upper edge of the platform (Srivastava et al., 1992). The agreement with our results indicates that certain mechanisms underlying the initiation process have been evolutionarily conserved. This hypothesis is also supported by the results obtained for the 30S·edeine complex. In contrast, a more recent cryo-EM study on *T. thermophilus* small ribosomal subunit (McCutcheon et al., 1999) localised IF3-C on the side of the platform facing the subunit interface, suggesting a different mechanism for IF3-C action.

Based on the location of IF3-C that we observed, it is likely that the binding of IF3-C to the 30S subunit influences the mobility of H45. The binding at this site could affect the conformational dynamics and thus prevent the association of the two ribosomal subunits. Our suggestion is supported by the observation that the double mutant of G1530/A1531 to A1530/G1531 in H45 of the 16S rRNA reduces IF3 binding to the 30S subunit ten fold (Firpo et al., 1996). Moreover, this double mutant enhances IF3 affinity for the 70S ribosomes, and IF3 is unable to promote the dissociation of 70S ribosomes carrying this mutation (Firpo et al., 1996). Our structural results are consistent with these biochemical observations and support the hypothesis that IF3-C binding alone is not sufficient to prevent subunit association or promote dissociation, but it rather acts by changing the conformational dynamics of the subunit. These results also support our finding that IF3-C does not physically block the inter-subunit interface between the 30S and the 50S subunit.

The spatial proximity of the IF3-C binding site to the anti-SD region of the 16S rRNA suggests a connection between IF3-C binding and the mRNA molecule interacting with the anti-SD region. The interaction of mRNA with the anti-SD region and with IF3 could suppress the change in the conformational dynamics induced by IF3, thus allowing subunit association. This could also explain the observation that leaderless mRNAs are only translated at low intracellular concentrations of IF3 (Tedin et al., 1999). In the absence of the leader sequence (Shine-Dalgarno) the change in the conformational dynamics induced by IF3 would not be reversed, and thus subunits will not be able to form the 70S initiation complex.

It has been suggested that the flexibility and the ability of the linker region to alter its fold are partially related to the function of IF3 in the formation of the initiation complex, and thus has a functional importance. Assuming that our IF3-N placement is correct, the

linker region between IF3-C and IF3-N needs to be flexible. Indeed, the extreme flexibility of the linker region of IF3 was shown biochemically (de Cock et al., 1999), as well as by NMR studies (Moreau et al., 1997). Our results also agree with the proposal that the linker maintains its flexibility when IF3 is bound to the 30S subunit. Thus, it is possible that once the IF3-N and the IF3-C domains are bound, the linker acts as a strap between the two domains. In this manner the IF3 linker could indirectly affect the conformation of the P-site, and induce its specificity for $tRNA^{fMet}$ (de Cock et al., 1999). The location of the docked IF3-N (Figure 18a) suggests that codon-anticodon recognition may be based on space-exclusion principles, rather than specific interactions of IF3 with the codon-anticodon-complementarity mechanism, as suggested (Meinnel et al., 1999).
In summary, our localisation of IF3-C on the 30S subunit and the modelling of the linker region and of the IF3-N binding site, provides a connection between IF3 function and the existing biochemical data. Our IF3-C location shows clearly that the anti-association activity of IF3 is not due to physical blockage of the inter-subunit interface, but rather to a change in the conformational dynamics of the subunit. 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 efficient translation.

5.3 Antibiotics

Edeine. We found one 30S binding site for edeine (Figure 19, Figure 20a), in contrast to the six sites of tetracycline. All the rRNA bases defining the edeine binding site are conserved in chloroplasts, mitochondria, and the three phylogenetic domains, explaining why edeine is said to be a universal protein synthesis inhibitor (Altamura et al., 1988). Interestingly, the 16S rRNA bases involved in edeine binding are also conserved in *Brevibacillus brevis*, the organism that synthesises it. Thus, edeine acts equally well as a protein synthesis inhibitor on *B. brevis* ribosomes (Kurylo-Borowska, 1975), which resolves this apparent paradox by rapidly releasing the active edeine into the growth medium, and by only maintaining low concentrations of inactive edeine attached to the internal part of the cell membrane (Kurylo-Borowska, 1975).
The binding of edeine involves nucleotides situated in H24, in H28, the neck helix, in H44, the core of the decoding region, and in its close neighbour H45, which is also involved in the decoding process (Figure 20a). 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 (Santer et al., 1990; Tapprich et al., 1989). G926 (H28) interacts with the tRNA bound at the P-site and is protected by edeine (Woodcock et al., 1991). Furthermore, mutations in U1498 impair A-site function and enhance $tRNA^{fMet}$ selectivity (Ringquist et al., 1993), while mutations in G1505, increase the levels of stop codon read-through and frameshifting in (O'Connor et al., 1995; O'Connor et al., 1997). By physically linking these four helices, critical points for tRNA, IF3, and mRNA binding, edeine could lock the small subunit in a fixed configuration and hinder the conformational changes that are thought to accompany the translation process (Gabashvili et al., 1999b; VanLoock et al., 2000).

In addition to the direct interactions of edeine with the 16S bases (Table 4), our structure shows that edeine induces the formation of a base pair, between C795 at the loop of H24 and G693 at the loop of H23 (Figure 20a). Although, neither G693 nor C795 are directly involved in edeine binding, G693 has been shown to be protected when edeine is bound (Woodcock et al., 1991). Thus, the formation of this base pair explains the protection against chemical attack of G693 upon edeine binding.

H23 plays an important role in the binding of the C-terminal domain of IF3 (Table 4), and nucleotides 787-795 of H24 are directly involved in 30S-50S subunit association (Tapprich and Hill, 1986). Analysis of our structure shows that the G693-C795 base pair would impose constraints on the mobility of the platform, which is believed to move during translation (Gabashvili et al., 1999a). Based on these findings, it is conceivable that the formation of the G693-C795 base pair interferes substantially with both elongation and initiation. Existing biochemical data on edeine binding to the 30S subunit supports this hypothesis. These data suggest that edeine blocks not only initiation (Odon et al., 1978), but also elongation, by interfering with the P-site tRNA (Moazed and Noller, 1987). It is possible that in prokaryotes and archaea the G693-C795 base pair blocks the path of the mRNA between the decoding region and the anti-SD region of the 16S rRNA.

This blockage would make the anti-SD region on the 16S rRNA less accessible and thus it would not base pair with the incoming mRNA during the initial stages of initiation. This effect on the mRNA path seems to be universal since edeine interacts with G926 and G693, two universally conserved nucleotides. 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). G693, on the other hand, has been photo-crosslinked in *E. coli* to positions –1/-3 of the mRNA (Bhangu and Wollenzien, 1992) and in human ribosomes to position –3 of the mRNA (Demeshkina et al., 2000).

The only mutant resistant to edeine that has been functionally characterised, is a mutant of *Saccharomyces cerevisiae*. This mutant overcomes edeine inhibition by an increased affinity for mRNA (Herrera et al., 1984). In eukaryotes, where the initial docking of the mRNA is independent of base complementarity between the mRNA and the 18S rRNA, an increased affinity of the small subunit for mRNA could indeed promote edeine resistance. The crosslink of G693 with the positions –1/-3 of the mRNA together with the formation of the cross-helix base pair (G693-C795) induced by edeine in our structure offers a good explanation as to why edeine interferes with the AUG recognition process in eukaryotes. It was shown that in the presence of edeine the 40S ribosome complex (tRNA-eIF2-GTP and other factors) scans along the mRNA without recognising the AUG start codon (Kozak and Shatkin, 1978). The structure of the 30S-edeine complex shows that the path of the mRNA is altered, mainly in the region of the small subunit that should interact with the mRNA initiation codon, thus should hamper the formation of the 80S initiation complex. Based on these findings, we conclude that the effect of edeine is to alter the path of the mRNA through the small ribosomal subunit, thereby affecting initiation as well as tRNA binding to the P-site and possibly evens to the E-site.

In summary, our structural results are not only consistent with the biochemical data on edeine binding but can also explain the involvement of edeine in the inhibition of the initiation and elongation processes. Our data suggests that the initiation process is the main target of this universal antibiotic. The fact that edeine induces an allosteric change

of this nature – forming a new base pair - could be an important principle of antibiotic action never reported previously.

Tetracycline. We have identified six binding sites for tetracycline in the 30S subunit (Table 4, Figure 19) but found no common structural trait among the binding sites. The question arises, which sites could affect translation and to what extent they are involved in the inhibitory action of tetracycline. Tet-1, the site with the highest relative occupancy (Figure 21a) interferes with the location where the A-site tRNA (Cate et al., 1999) was docked onto the 30S structure (Schlünzen et al., 2000). Thus, tetracycline can physically prevent the binding of the tRNA to the A-site. This mode of interaction is consistent with the classical model of tetracycline as an inhibitor of A-site occupation, and hence offers a clear explanation for the bacteriostatic effect of tetracycline.

There are two ribosome-related mechanisms of tetracycline resistance in bacteria. Both are linked to the Tet-1 site. In one, the resistance is mediated by ribosomal protection proteins (Roberts, 1996) and in the other by the mutation 1058G->C on 16S rRNA (Ross et al., 1998). Ribosomal protection proteins, such as TetM, TetO, and TetS, confer resistance only at low concentrations of tetracycline, and show some sequence and structural homology with the elongation factors G and EF-Tu (Dantley et al., 1998). It has been proposed that TetM binds to the A-site and upon GTP hydrolysis actively releases the tetracycline bound to it (Dantley et al., 1998). The G1058->C mutation could hamper the base pairing of G1058 with U1199 and might lead to a conformational change that results in closing the Tet-1 binding pocket. This conformational change may be due to the release of the co-ordinating magnesium ion.

These two tetracycline resistance mechanisms reflect the importance of the Tet-1 binding site in the antibiotic action of tetracycline. However, the presence of five additional binding sites, the biochemical evidence for different locations of tetracycline, and the low level of resistance conferred by the ribosomal protection proteins, demand more complex explanations about the possible functional relevance of the five additional sites. Binding of tetracycline to Tet-4, Tet-5, and Tet-6 sites is supported by biochemical evidence (Table 4), but there are no biochemical data on Tet-2 and Tet-3 sites. Tet-5 site is located between H11 and H27, the switch helix (Lodmell and Dahlberg, 1997), which is a functional hot spot (Figure 21e). Tetracycline bound to Tet-5 position could limit the mobility needed by H27 to switch between the 912-885, or error prone conformation, to the 912-888, or restrictive conformation (Lodmell and Dahlberg, 1997). Therefore, tetracycline bound at Tet-5 could lock the 30S subunit into one of these two conformations.

Only four proteins, namely S4 for Tet-2, S7 for Tet-6, S9 for Tet-4 and Tet-6, and S17 for Tet-5, come in contact with tetracycline. S4, S7, S9, and S17 are primary rRNA binding proteins (Held et al., 1974). S4 and S7 are the two proteins that initiate the assembly of the 30S subunit (Nowotny and Nierhaus, 1988). Therefore, tetracycline binding at the Tet-2, Tet-4, Tet-5, and Tet-6 sites, may not influence the decoding process, but could disturb the early assembly steps of new 30S particles, contributing to the overall inhibitory effect of tetracycline.

Overall, our data for the six positions can well explain the sometimes contradictory reported biochemical and functional data for tetracycline binding to the 30S subunits (Table 4). We show that physical blockage of the A-site tRNA binding by tetracycline

bound at Tet-1, can account for the inhibitory action of tetracycline, but we can not say with certainty that any of the five minor sites is involved in tetracycline action. Nevertheless, we hypothesise that, with the exception of the tetracycline at the Tet-3 site, these sites could act synergistically to contribute to the bacteriostatic effect of tetracycline.