

# The Structural Basis for the Action of the Antibiotics Tetracycline, Pactamycin, and Hygromycin B on the 30S Ribosomal Subunit

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## Summary

We have used the recently determined atomic structure of the 30S ribosomal subunit to determine the structures of its complexes with the antibiotics tetracycline, pactamycin, and hygromycin B. The antibiotics bind to discrete sites on the 30S subunit in a manner consistent with much but not all biochemical data. For each of these antibiotics, interactions with the 30S subunit suggest a mechanism for its effects on ribosome function.

## Introduction

The small, or 30S, ribosomal subunit has three binding sites for tRNA molecules designated the A (aminoacyl), P (peptidyl), and E (exit) sites. One of the central roles of the small subunit is to discriminate cognate from noncognate tRNAs by monitoring base pairing between the codon of mRNA and the anticodon on tRNA in its A site in a process called decoding. An overview of ribosome structure and function can be found in a recent symposium volume (Garrett et al., 2000).

The ribosome is an important target for a wide variety of antibiotics (Gale et al., 1981). Many of them, such as streptomycin and tetracycline, were of great clinical importance when they were first discovered, but unfortunately strains of bacteria with resistance to these drugs have become commonplace, limiting their effectiveness (Neu, 1992). At the same time, many other antibiotics targeting the ribosome have insufficient specificity toward bacterial (as opposed to eukaryotic) ribosomes, and hence are too toxic for routine clinical use in humans. With the emergence of new multi-drug resistant strains of bacteria, there is a real need to understand details of how these antibiotics interact with the ribosome.

Most ribosomal antibiotics work by binding to specific sites on the ribosome and interfering with its function during protein synthesis, and, in most cases, the target is ribosomal RNA rather than proteins (Gale et al., 1981). Thus, many of the binding sites for antibiotics are located at or near the mRNA or tRNA binding sites, or at locations that undergo critical structural rearrangements during decoding or translocation. The binding of antibiotics to the

ribosome has been studied extensively by biochemical methods, and the structure of two aminoglycoside antibiotics bound to a 16S RNA fragment containing their target in the 30S has been studied by NMR (Fourmy et al., 1996; Yoshizawa et al., 1998). However, until recently, the structure of antibiotics bound to an entire ribosomal subunit seemed out of reach, severely hampering an understanding of their mechanisms at the molecular level.

This situation has now changed with the determination of complete atomic structures for both the 50S and 30S subunits (Ban et al., 2000; Wimberly et al., 2000), as well as an independent high resolution structure of the 30S (Schlueder et al., 2000). These structures can be used directly to determine the location of antibiotics in difference Fourier maps by refinement against diffraction data from crystals of subunits either soaked or co-crystallized with antibiotics. Thus, although many of these antibiotics were discovered and first characterized decades ago, only now are we in a position to understand the structural basis for their action on the ribosome. This has been done recently with three antibiotics that bind to the 30S, spectinomycin, paromomycin, and streptomycin (Carter et al., 2000), in a study where the results of antibiotic action also shed light on various aspects of 30S function such as decoding and proof-reading. In this study, we investigate the structures of tetracycline, pactamycin, and hygromycin B in complex with the 30S. These antibiotics bind to quite different regions of the 30S, both with respect to each other and compared with the group of previously determined antibiotic complexes.

The tetracyclines (Tcs) form a group of antibiotics that have been used since the 1940s against a wide range of both Gram-negative and Gram-positive bacteria (Hlavka and Boothe, 1985; Chopra et al., 1992). These drugs were the first so-called “broad-spectrum” antibiotics and have been used extensively as a bactericidal agent in both human and veterinary medicine over several decades. However, in recent years, clinical use of Tcs has been limited by the emergence of widespread microbial resistance to them (Salys et al., 1990; Taylor and Chau, 1996). Tcs bind primarily to the 30S ribosomal subunit (Ross et al., 1998) where they inhibit protein synthesis by blocking the binding of aminoacylated tRNA (aa-tRNA) to the A site (Maxwell, 1967; Geigenmüller and Nierhaus, 1986). It appears likely, however, that the initial binding of a ternary complex of EF-Tu with tRNA to the A site and the process of decoding are not affected since ribosome-dependent GTP hydrolysis by EF-Tu is unaffected by tetracycline (Gordon, 1969). Tcs have no apparent effect on the binding of tRNA to the P site except during factor-dependent initiation. Consistent with the inhibition of tRNA binding to the A site during translation, Tcs also prevent binding of both release factors RF-1 and 2 during termination, regardless of the stop codon (Brown et al., 1993).

Bacterial resistance to Tc is usually not acquired by mutations or modifications in ribosomal RNA or proteins, or by methylation of specific RNA residues, but

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Table 1. Data Collection Statistics

Data Set	Tetracycline	Pactamycin	Hygromycin B
Resolution (Å)	99.0–3.4	99.0–3.4	99.0–3.3
Unit cell			
a,b (Å)	401.158	401.719	402.063
c (Å)	176.944	177.002	175.263
No. of observations	704,712	735,955	630,088
No. of unique reflections	193,543	184,902	201,587
R <sub>sym</sub> <sup>a</sup> (%)	17.8 (41.1)	12.5 (42.4)	15.8 (38.5)
Completeness <sup>a</sup> (%)	97.4 (95.4)	94.4 (90.7)	93.0 (83.7)
<I>/<σ <sub>I</sub> > <sup>a</sup>	4.1 (1.6)	5.6 (2.3)	4.7 (2.1)

<sup>a</sup>Numbers in parentheses indicate outermost resolution shell.

by the presence of separate enzymes that either are involved in exporting Tc across the cell membrane in an energy-dependent fashion (efflux), chemically modify the drug to render it inactive, or mimic the structure and function of the elongation factors and are thus able to release the bound antibiotic from the ribosome (Manavathu et al., 1990; Burdett, 1996; Taylor and Chau, 1996). Biochemical studies have implicated both ribosomal proteins and 16S RNA in tetracycline binding (Moazed and Noller, 1987; Buck and Cooperman, 1990; Oehler et al., 1997). Tc has one primary and multiple secondary binding sites within the small subunit (Epe et al., 1987; Kolesnikov et al., 1996), but the relevance of the secondary binding sites remains unclear.

Pactamycin (Pct) was isolated from *Streptomyces pactum* as a potential new human antitumor drug, but is in fact a potent inhibitor of translation in all three kingdoms, eukarya, bacteria, and archaea (Bhuyan et al., 1961; Mankin, 1997). For this reason, the drug is expected to interact with highly conserved regions of 16S RNA, both structurally and with respect to sequence. In bacteria, Pct inhibits the initiation step of translation. Binding of the drug prevents release of initiation factors from the 30S initiation complex, which in turn prevents the formation of functional 70S ribosomes (Cohen et al., 1969a; Kappen and Goldberg, 1976). The antibiotic interferes with factor and GTP-dependent binding of tRNA to the ribosomal P site during initiation, but factor-free initiation does not seem to be affected (Cohen et al., 1969a). It has been suggested that Pct, rather than causing a direct inhibition of the binding of tRNA, promotes structural changes in the 30S that prevent the tRNA from binding (Mankin, 1997). At higher concentrations, Pct also has an effect on elongation in prokaryotic ribosomes, but there is overlap in the range of concentrations required for affecting elongation and initiation, so Pct cannot selectively be used as an inhibitor of initiation (Tai et al., 1973).

Hygromycin B (HygB) is an aminoglycoside originally isolated as a secondary antibiotic from *Streptomyces hygroscopicus* after the discovery of another antibiotic produced by the same organism, now known as hygromycin A (Mann and Bromer, 1958). HygB is active against both prokaryotic and eukaryotic cells (Gonzales et al., 1978), and differs in structure from other aminoglycosides by having a dual ester linkage between two of its three sugar moieties resulting in a fourth, 5-membered ring (Figure 4c). The drug works primarily by inhibiting the translocation step of elongation (Cabanias et

al., 1978b; Gonzales et al., 1978; Eustice and Wilhelm, 1984b) and, to a lesser extent, causes misreading of mRNA (Eustice and Wilhelm, 1984a, 1984b). HygB has a monophasic effect on translation that is indicative of a single binding site on the ribosome (Zierhut et al., 1979) and has been shown to bind close to the A site on the small subunit (Moazed and Noller, 1987). In eukaryotes, the antibiotic affects EF-2-mediated translocation of A site bound tRNA to the P site (Gonzales et al., 1978). The inhibition of translocation is accompanied by an increase in the affinity of the A site for aminoacyl-tRNA (Eustice and Wilhelm, 1984a).

Here we report the three-dimensional structures of the 30S ribosomal subunit from *Thermus thermophilus* in separate complexes with Tc, Pct, and HygB as determined by X-ray crystallography at 3.3–3.4 Å resolution. The locations of these antibiotics in the 30S are consistent with most biochemical data while surprisingly at variance with others. The observed interactions of these antibiotics allow us to propose mechanisms for their effect on translation.

## Results

### Structure Determination

X-ray diffraction data were collected from multiple crystals of *Thermus thermophilus* 30S subunits soaked in either Tc, Pct, or HygB post crystallization at a concentration at which they were known to block translation in vivo (Table 1) (Cohen et al., 1969a, 1969b; Cabanias et al., 1978a; Semenkova et al., 1982). The antibiotics were located in difference Fourier density maps after refinement of the native 30S structure (PDB entry 1FJF) (Wimberly et al., 2000) against measured structure factor amplitudes for complexes with each of the three antibiotics. The results of a final refinement with the antibiotics included in the model are shown in Table 2. Throughout this paper, the numbering scheme for RNA residues is that for the corresponding nucleotides in the *E. coli* 16S sequence as done previously (Wimberly et al., 2000), and we also use the standard helix numbering H1–H45 for 16S RNA (Mueller and Brimacombe, 1997). An overview of the binding of all three antibiotics to the 30S subunit is shown in Figure 1.

### Tetracycline Binds to the Head of the 30S

Tetracycline (Tc) is a flat fused-ring system with hydrophilic functional groups along one side (Figure 2). The molecule is thus able to make charged interactions with

Table 2. Refinement Statistics

Data Set	Tetracycline	Pactamycin	Hygromycin B
Resolution (Å)	99.0–3.4	99.0–3.4	99.0–3.3
No. of reflections (working set)	172,608	166,337	178,509
No. of reflections (free set)	9,128	8,776	9,454
No. of atoms	51,941	51,917	51,913
protein	19,271	19,271	19,271
RNA	32,508	32,508	32,508
ligand	64	40	36
metal ions	98	98	98
R factor <sup>a</sup> (%)	22.2 (26.4)	23.2 (28.0)	21.8 (26.1)
Coordinate error <sup>b</sup> (Å)	0.39 (0.46)	0.43 (0.51)	0.39 (0.46)
Rms deviations			
bond lengths (Å)	0.0070	0.0070	0.0068
bond angles (°)	1.26	1.25	1.25
dihedrals (°)	28.4	28.4	28.5
plane (°)	1.60	1.56	1.59

<sup>a</sup> Numbers in parentheses represent free R factor.

<sup>b</sup> Estimated Luzzati coordinate error for reflections in the resolution range 5.0 Å–3.4 Å/3.4 Å/3.3 Å. Numbers in parentheses represent the cross-validated coordinate error.

one edge and either hydrophobic or stacking interactions with the other side. We have found two binding sites for Tc within the small ribosomal subunit. The better occupied site is located near the acceptor site for aminoacylated tRNA between the head and the body of the 30S (the A site), and the less occupied site is at the interface between three RNA domains in the body of

the subunit. Throughout this paper, the binding site of tetracycline near the A site is termed the *primary* site, whereas the location in the body is called the *secondary* binding site. The discovery of a second binding site is not surprising since the drug is known to have secondary, possibly nonspecific sites on both subunits (Pestka, 1974; Vazquez, 1974).

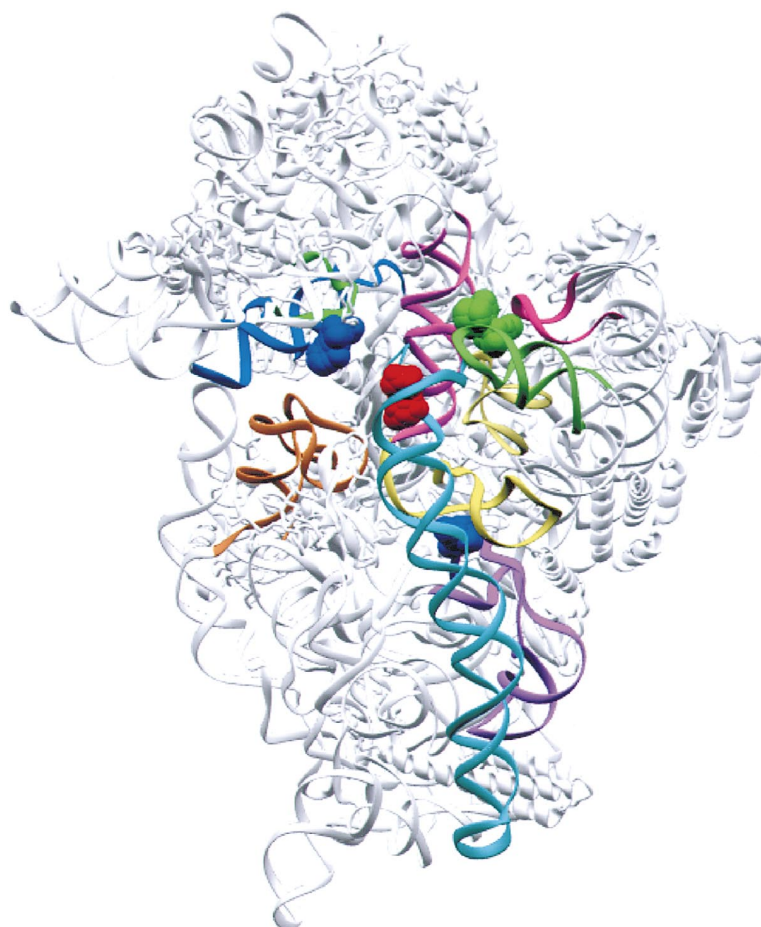
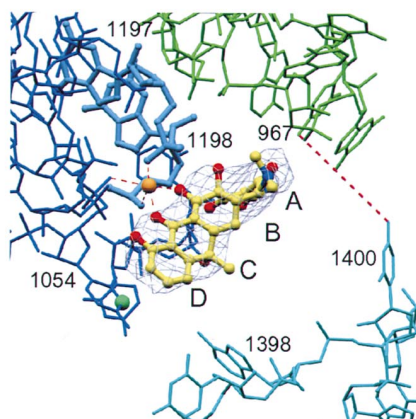
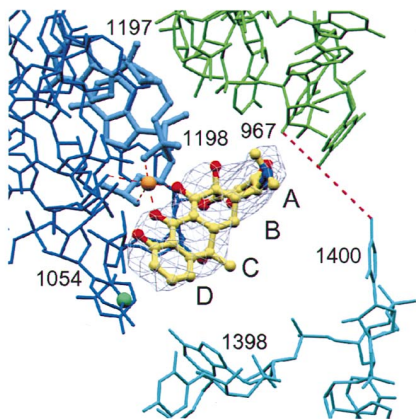
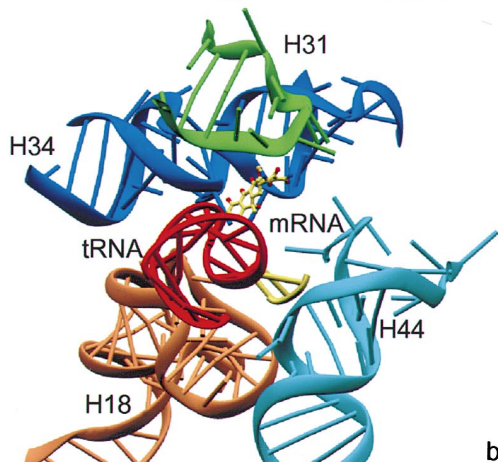


Figure 1. Overview of Tetracycline, Pactamycin, and Hygromycin B in the 30S Subunit. Antibiotics are shown as space-filling models, with tetracycline (blue), pactamycin (green), and hygromycin B (red). The parts of 16S RNA that make contacts with any of the three antibiotics are colored as in the following figures.

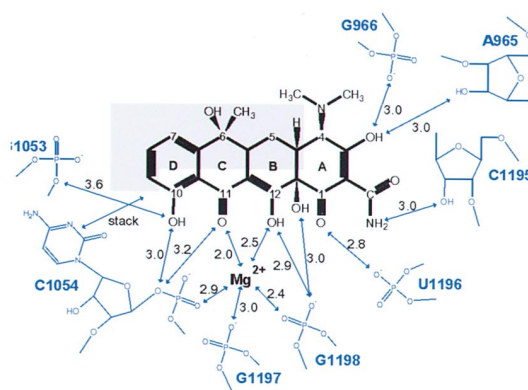




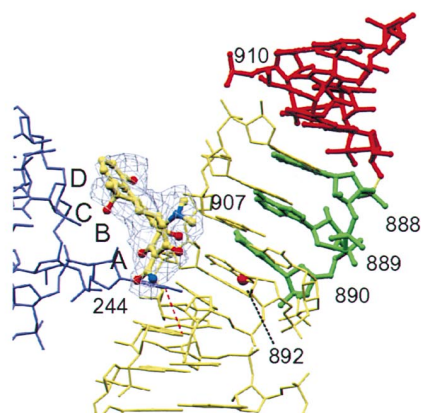
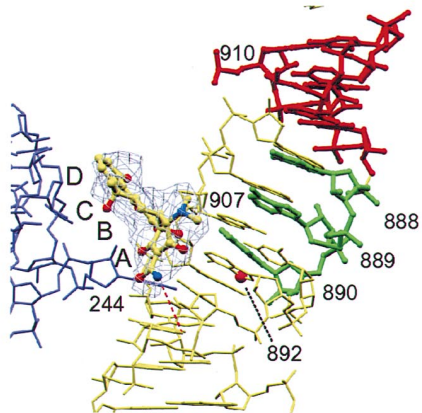
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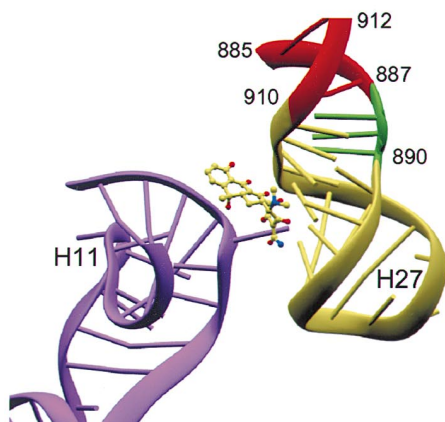
b



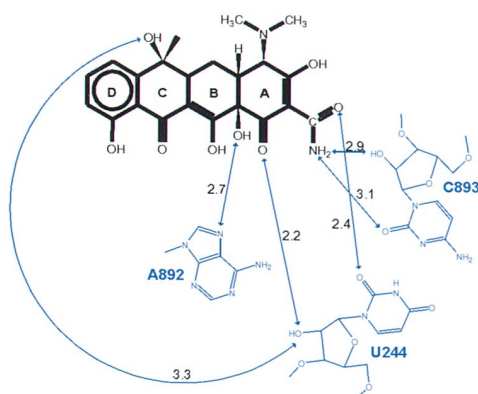
c



d



e



f

In its primary binding site within the 30S, Tc binds exclusively to the 3' major domain of 16S RNA in the upper part of the crevice between the head of the 30S and the shoulder, right above the binding site for A site tRNA located between the 530 stem-loop of H18 in the 5' domain and the long H44 of the 3' minor domain (Figures 2a and 2b). The binding pocket for Tc is about 20 Å wide and 7 Å deep and is formed by an irregular minor groove of H34 (RNA residues 1196–1200:1053–1056) in combination with residues 964–967 from the H31 stem-loop. Tc interacts primarily with the exposed sugar phosphate backbone of H34. The bases of 1054 and 1196, which bulge out from the regular double-helical structure of H34 to form one end of the binding pocket, apparently make hydrophobic interactions with the fused-ring system of Tc. However, the majority of the interactions of the drug are through hydrogen bonding interaction between oxygen atoms on one side of Tc and backbone phosphate oxygen atoms of H34. In addition, there is a clear hydrogen bond to the O2P of G966 from H31. A putative magnesium ion on the hydrophilic side of Tc makes important salt bridges to phosphate oxygen atoms of G1197 and G1198 (Figure 2c). Divalent magnesium is known to be crucial for binding of Tc to the ribosome (White and Cantor, 1971), and is found here in an identical position relative to Tc as in the structure of its complex with a class D tet-repressor, where it is also required for function (Hinrichs et al., 1994). Interestingly, this  $Mg^{2+}$  ion was also present in the 30S structure in the absence of tetracycline (Carter et al., 2000), suggesting that it is required to maintain the local structure of the 30S in that region.

The structure rationalizes data on tetracycline modifications: positions on tetracycline that abolish its antibiotic function when modified would all interfere with interactions with the 30S, while those that have no effect (shown as gray area in Figure 2c) all lie on the side of tetracycline that does not bind to the ribosome in this site (Hlavka and Boothe, 1985). Finally, of the three antibiotics studied here, the binding site for tetracycline is the least conserved between bacteria and eukaryotes, thus explaining its greater specificity for bacteria.

There is little or no overall change in the conformation of the 16S RNA upon binding of Tc to the ribosome. However, residues C1054 and U1196 appear to shift slightly to accommodate the molecule, and, in the case of C1054, the shift explains the increased reactivity of

this base toward DMS (Moazed and Noller, 1987). Increased reactivity was also reported for the nearby U1052, but this residue does not seem to move upon Tc binding, nor does it interact with the drug. Tc is known to inhibit the UV-induced cross-link C967-C1400 completely (Figure 2a), an interaction which links the H31 stem loop to the top of the functionally important H44 (Noah et al., 1999). In addition, the UV-induced cross-link between C1402 and C1501, both of which are located in H44, increases in intensity upon Tc binding (Noah et al., 1999). This suggests that the binding of the drug has subtle long-range effects on the functional center of the 30S. However, we have not been able to confirm such structural changes by comparison with the native 30S structure. Mutation of G1058 to cytosine causes resistance to Tc (Ross et al., 1998), probably due to a disruption of the base pair G1058:U1199 in H34, which leads to a local conformational change at the Tc binding site since the two residues upstream from there, G1198 and G1197, are intimately involved in hydrogen bonding with the drug as mentioned above.

#### Tetracycline Also Binds in the H27 Switch Region

The second binding site of Tc is located in the body of the subunit, in close proximity to H44 and sandwiched between the functionally important H27 in the central domain and the very top of H11 in the 5' domain of 16S RNA (Figures 2d and 2e). The binding site is confined on one side by a major groove of H27 (residues 891–894:908–911) and the edge of H11 (residues 242–245). The bulged-out base U244, which reaches across and makes an important interdomain interaction with C893 in H27, forms the bottom of the binding site. Again, all interactions between the antibiotic and the ribosome are mediated by the RNA component. The binding pocket is approximately 14 Å wide and 7 Å deep, and as in the primary binding site, it is the hydrophilic side of Tc that is involved in contacts with the RNA. We see no evidence for a magnesium ion in the secondary binding site. Contacts are mainly to the backbone of RNA, especially at G906, but, in this case, the interaction is with the 2' OH and 3' OH groups of the sugar moiety rather than to phosphate oxygen atoms as in the primary binding site (Figure 2d). Also, the binding of Tc at the second site to a greater extent involves the nitrogen and oxygen atoms attached to ring A, and there are a number of se-

Figure 2. Tetracycline

- (a) Stereo figure of the primary Tc binding site (A site region) with rings A, B, C, and D of the fused-ring system. H34 (top left, blue) and H31 (top right, green) are shown together with H44 (cyan). The enhanced reactivity of C1054 (green sphere) and the reduced UV cross-link C967x C1400 (dashed red line) are indicated. The bound magnesium ion (gold sphere) is shown with residues involved in its coordination (thick sticks, light blue). The initial difference electron density map ( $mF_o - DF_c$ ), calculated before inclusion of Tc in the model, is shown at  $6\sigma$ .
- (b) Overview of primary binding site of Tc indicating the RNA components close to the site and the interaction with A site tRNA, H34 (blue), H31 (green), H18 (orange), and H44 (cyan). The model of A site tRNA (red) and mRNA (yellow) is shown.
- (c) Chemical structure diagram of Tc and possible interactions with 16S RNA at the primary site (blue). The shaded area represents positions on the molecule that can be modified without affecting its inhibitory action (Hlavka and Boothe, 1985).
- (d) Stereo figure of the secondary tetracycline site (H27 switch region) with rings A, B, C, and D. H27 is yellow/green/red and H11 violet. The 885–887:910–912 base pairs are shown in red, whereas the bases 888–890, involved in a proposed alternative base-pairing scheme are green. The reduced reactivity towards DMS at A892 (red sphere) and the reduced cross-link G894-U244 (red dashed line) are also shown. The initial difference electron density map ( $mF_o - DF_c$ ) is shown at  $4\sigma$ .
- (e) Overview of secondary binding site of Tc along with the RNA elements it interacts with, H11 (violet), H27 (yellow, red, and green as above).
- (f) Possible hydrogen bond interactions with 16S RNA at the secondary tetracycline binding site (blue).

quence specific interactions with e.g., A892:N1, C893:O2, and possibly A907.

The observed weakening of the UV-induced cross-link between U244 and G894 upon Tc binding (Noah et al., 1999) can easily be understood in that the antibiotic effectively sits on top of U244. The direct interaction of Tc with A892 rationalizes the observed strong protection against attack by DMS at this position (Moazed and Noller, 1987). A direct cross-link from Tc to G890 has been observed at high Tc concentrations (Oehler et al., 1997), but this base is approximately 9 Å from the Tc in our structure.

In contrast to the conclusions of earlier biochemical studies (Buck and Cooperman, 1990; Oehler et al., 1997), there are no proteins involved in binding of Tc in either site. In the primary binding site, the nearest protein components are the C-terminal extension of S13, which is about 9 Å away (Lys-122), the 50–60 loop of S10, which is about 8.5 Å away (Lys-55), and the 155–165 loop region of S3 (8.8 Å, Gln-162). Similarly, in the second site, the nearest protein, S12, is 8 Å away. Tc has also been reported to cross-link to residues G1300 and G1338, which are located in the head of the 30S (Oehler et al., 1997), close to the binding site of S7, a protein that has also been implicated in binding of this antibiotic (Buck and Cooperman, 1990). However, S7, G1300, and G1338 are all located far from either Tc binding site in our structure. It is possible that these discrepancies arise from weak or transient binding sites that are not seen in our difference Fourier maps and are unrelated to the physiological effects of tetracycline.

#### **Pactamycin Mimics the Structure of Two Consecutive RNA Bases**

Pactamycin (Pct) binds in a single site on the 30S in the upper part of the platform, very close to the cleft in the subunit that is responsible for binding of the three tRNA molecules (Figure 1). The antibiotic interacts primarily with residues at the tips of the stem loops H23b and H24a in the central domain of 16S RNA. In this region, H24a forms a regular helical stem loop that the H23b stem loop is packed against with interactions mainly between bulging bases in H23b and the backbone of H24a. The bases near the apex of H23b curve around and pack into the major groove of H24b.

Within the ribosome, the antibiotic folds up to mimic an RNA dinucleotide. The two distal aromatic rings (rings I and II) stack against each other and on G693 at the tip of H23b, like consecutive, stacked RNA bases (Figure 3a). The central ring to some extent mimics the RNA sugar-phosphate backbone and it interacts with C795, C796 in H24a, and, to a lesser extent, A694 in H23b (Figure 3b). These interactions are in excellent agreement with biochemical experiments showing that binding of Pct protects the N1 and N7 atoms of G693 as well as N3 of C795 from attack by kethoxal and DMS, respectively (Egebjerg and Garrett, 1991; Woodcock et al., 1991). In addition, resistance to Pct has been shown to be caused by either of the mutations A694G, C795U, or C796U (Mankin, 1997). This indicates that the interaction between N6 of A694 and Pct is crucial for binding of the drug since this is the only interaction with that particular base. In addition, N-methylation of this resi-

due causes resistance, but this probably results from distortions of the local structure since N1 of A694 is involved in a tight hydrogen bond to the 2' OH of A787. By a similar argument, the hydrogen bond between N4 of C795 and Pct has to be crucial since its disruption (via the mutation C795U) leads to resistance. On the other hand, the resistance mutation C796U is probably due to an allosteric effect caused by disruption of the canonical G:C base pair since this residue does not interact directly with Pct. It is interesting that no resistance mutations have been found that involve G693. This could indicate that interaction with Pct at this position is via nonspecific hydrophobic stacking of the bases or that this base is crucial for translation and thus cannot be mutated.

#### **Pactamycin Interacts with the Ribosomal E Site and Displaces mRNA**

Even though Pct was described as binding primarily to the ribosomal P site (Cohen et al., 1969a; Woodcock et al., 1991), in light of an analysis of the atomic structure of the 30S, the observed protections for this antibiotic actually belong to the E site (Carter et al., 2000). In the native structure (Carter et al., 2000), the 3' end of 16S RNA binds in the message binding cleft and mimics the codons for P and E site mRNA. The two aromatic moieties of Pct displace part of this message, and lie in the position originally occupied by the last two bases of the E site codon in the native structure. In the native 30S structure, the overall path of the mRNA leads between the long and highly conserved  $\beta$  hairpin of S7 and the stem loops of H23b and H24a of the platform (Figure 3c). However, in the presence of Pct, the difference density indicates that the mRNA in the E site would be pushed upwards and toward the back of the subunit in between H28 of the head and the protruding hairpin of S7. This remarkable distortion leads to a displacement of about 12.5 Å for the last base in the E site codon. The new position would not only have consequences for initiation and mRNA movement through the 30S, but would preclude any possible interaction with an E site bound tRNA (Figure 3d).

#### **Hygromycin B Binds Near the Decoding Center**

HygB has a single binding site within the 30S (Figure 1) consistent with the finding that it has a monophasic effect on translation (Zierhut et al., 1979). It binds close to the very top of H44, in a region that contains the A, P, and E sites for tRNA and also the binding site for other aminoglycoside antibiotics (Carter et al., 2000). The molecule is located in the major groove of the helix, very close to the helical axis, and makes contact with nucleotides from both RNA strands in the region 1490–1500 and 1400–1410 (Figure 4a). These contacts are exclusively to the RNA bases and not the backbone, so that it binds in a highly sequence-specific manner. Binding of HygB does not seem to induce any significant alterations in the structure of RNA, and appears to be governed by strong base-specific hydrogen bonds spanning more than three sequential bases in one strand of H44. This is possible because the structure of the three-ring antibiotic is relatively extended in its binding site within the 30S, and is about 13 Å long.



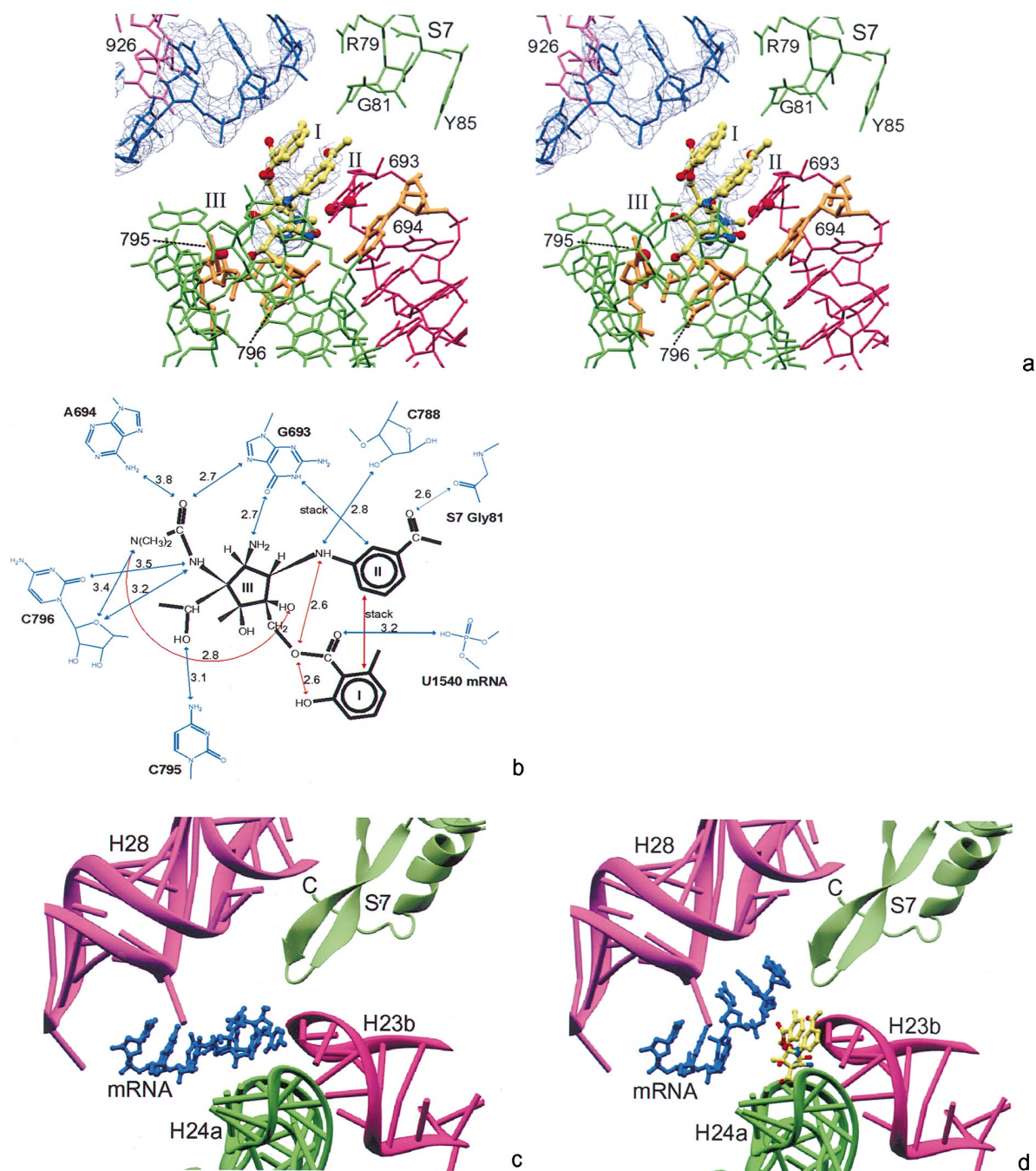


Figure 3. Pactamycin

(a) Stereo figure of Pct bound at the 30S E site with rings I, II, and III. H23b is red (lower right), H24a green, and H28 magenta (upper left). The altered position of the 3' end of 16S RNA that mimics mRNA (blue) and a part of the conserved  $\beta$  hairpin of protein S7 (yellow-green) are also shown. Resistance mutations at C795, C796, and A694 (yellow) and decreased reactivity towards DMS and kethoxal at C795 and G693, respectively (red spheres), are shown. The initial difference electron density map ( $mF_o - DF_o$ ), calculated before inclusion of Pct and mRNA in the model, is shown at  $8\sigma$  (Pct) and  $6\sigma$  (mRNA mimic).

(b) The chemical structure diagram of pactamycin and its proposed interaction with 16S RNA (blue). Internal interactions are shown in red.

(c) Overview of the E site of the native structure of the 30S along with the original path of mRNA mimic (blue) showing the RNA elements involved in binding, H28 (magenta), H23b (magenta), H24a (green). In addition, the C terminus of protein S7 (yellow-green) is shown.

(d) As in (c), but showing the Pct bound complex with the altered position of the mRNA mimic.

Ring I of HygB is involved in nonsequence-specific interactions with the backbone phosphate oxygen atoms of G1494 in addition to base-specific interactions

with G1494 and U1495 (Figure 4c). This explains the observation that binding of HygB protects the N7 of 1494 from attack by DMS (Moazed and Noller, 1987)

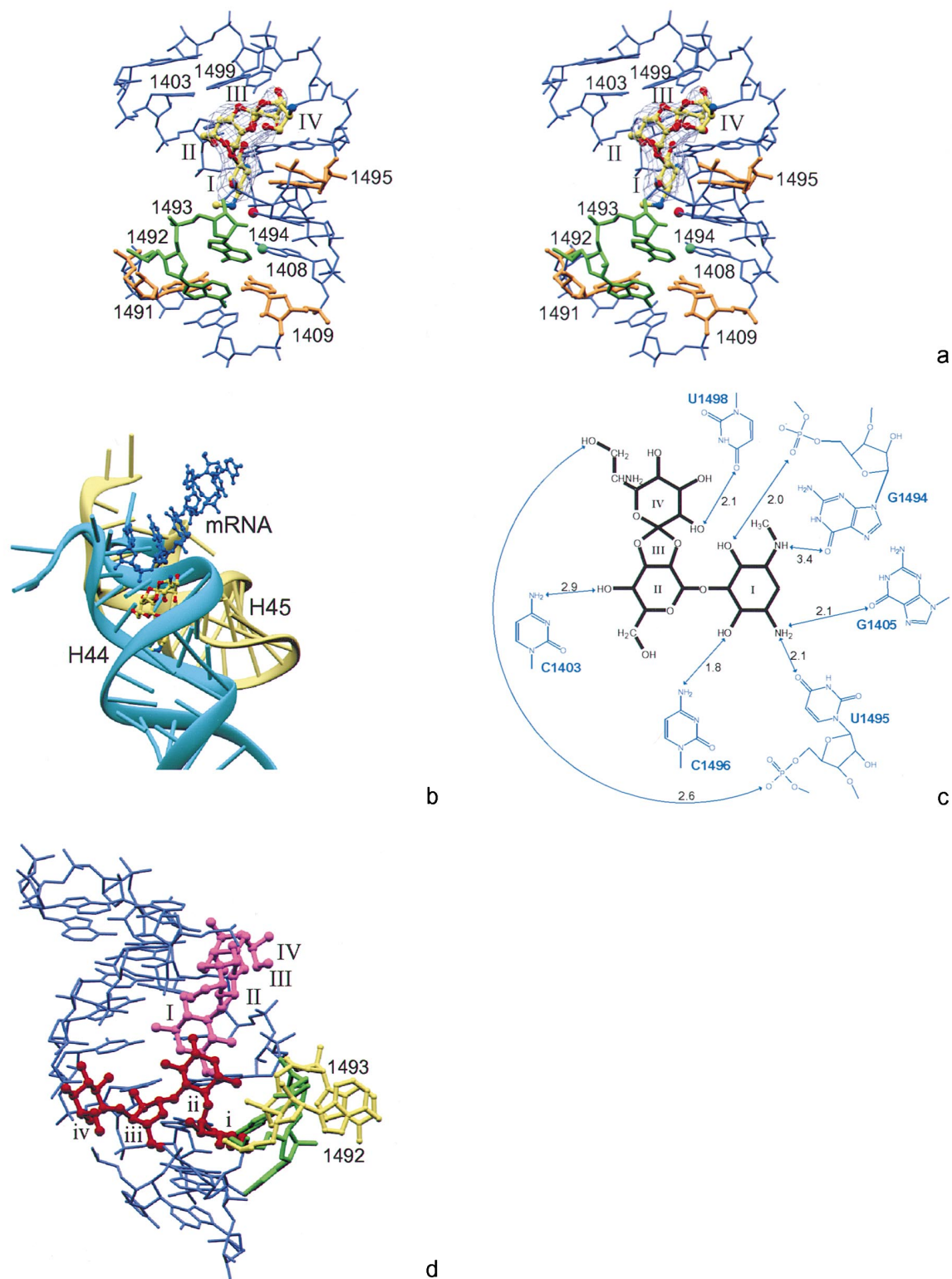


Figure 4. Hygromycin B

(a) Stereo figure of HygB bound to H44 (blue) with rings I, II, III, and IV. Resistance mutations at U1495, G1491, and C1409 (yellow), enhanced modification at A1408 (green sphere), reduced reactivity towards DMS at G1494 (red sphere) are also shown. The proposed decoding center (A1492 and A1493) is shown in green. The initial difference electron density map ( $mF_o - DF_c$ ), calculated before inclusion of HygB in the model, is shown at  $7\sigma$ .

(b) Overview of the HygB binding site along with RNA elements close to the site, H44 (cyan) and H45 (yellow). The mRNA is shown in blue.

(c) Chemical structure diagram of HygB and the proposed interaction with 16S RNA (blue).



since the inside of the major groove at this position is effectively screened by the molecule. The increase in the chemical reactivity of N1 of A1408, which is about 5.5 Å away from the lower tip of HygB, must be due to an indirect effect. In certain eukaryotes, the mutation U1495C is found to completely abolish binding of HygB to the ribosome (Spangler and Blackburn, 1985). This mutation would replace the O4, which is involved in a tight hydrogen bond to the N1 of HygB, with a nitrogen atom and thus remove this apparently crucial interaction. Rings II and III make weak base-specific hydrogen bonds to both C1404 and U1498, but their main role apparently is to position ring IV for interaction with bases in the 1496–1498 region. Ring IV of HygB comes within 4 Å of the second base of the P site bound mRNA codon (Figure 4b). There are only weak interactions with the message, but the antibiotic is in close contact with bases of 16S RNA that do contact the P site mRNA, such as e.g., U1498.

It has been shown that loss of the ability of HygB to bind the 30S is also caused by mutation of either G1491 or C1409, which form a Watson-Crick base pair (De Stasio and Dahlberg, 1990). Neither of these residues is near the binding site of HygB, and the reported loss of binding of the antibiotic must be due to an allosteric effect caused by disruption of the G1491:C1409 base pair, as the authors themselves suggest (De Stasio and Dahlberg, 1990).

HygB binds just above the binding site for paromomycin in H44 (Fourmy et al., 1996; Carter et al., 2000). Interestingly, the 2-deoxystreptamine moiety of paromomycin (ring II), which is structurally similar to ring II found in other aminoglycoside antibiotics including kanamycin and gentamycin, adopts an almost identical orientation to that of ring I of HygB, only about 3 Å further down the helix, or exactly the distance corresponding to one RNA residue (Figure 4d).

## Discussion

### Tetracycline and A Site tRNA Binding

The identification of two binding sites of Tc in distant, functionally important regions of the 30S obviously raises the question as to which site is responsible for the bactericidal effect of the antibiotic. Tc has for a long time been regarded as interfering primarily with binding of aminoacyl tRNA at the ribosomal A site (Maxwell, 1967), and it has been suggested that the interaction with the 890 region of 16S (i.e., the secondary binding site) is not responsible for the observed effect on translation (Epe et al., 1987; Buck and Cooperman, 1990; Oehler et al., 1997). Here we examine the functional implications of both binding sites.

It is straightforward to rationalize the effect of Tc at the A site. The tRNA in the A site can be modeled by a superposition of the 7.8 Å structure of the 70S ribosome complex (Cate et al., 1999) onto our 30S structure as we have described previously (Carter et al., 2000). The

antibiotic is located between H34 in the head of the 30S and the modeled A site tRNA, but on the other side of the tRNA anticodon stem loop relative to the codon:anticodon interaction (Figure 2b) (Carter et al., 2000). In the superposition, Tc just touches the A site tRNA and is consistent with prevention of A site tRNA binding by direct steric hindrance. This is especially true in the presence of the 50S since the head of the 30S then moves down further toward the body (Cate et al., 1999), so the clash of tRNA with tetracycline would be even more serious. At the same time, because Tc is on the opposite side of tRNA from the codon:anticodon interaction, it is also possible that the initial presentation of the EF-Tu ternary complex and the first step in decoding can proceed even in the presence of tetracycline since tRNA, when bound to EF-Tu, is known to have a quite different orientation in the 30S from that of A site tRNA (Stark et al., 1997). This would be consistent with the observation that GTP hydrolysis by EF-Tu proceeds even in the presence of tetracycline (Gordon, 1969), and with kinetic experiments that suggest that tetracycline only affects the rate of binding of EF-Tu ternary complex to the ribosome, but not the final level of binding (Semenkov Yu et al., 1982).

The structure, in combination with the available biochemical data, immediately suggests a possible mechanism for tetracycline action: the initial binding of a ternary complex of EF-Tu, aa-tRNA, and GTP to the ribosome is not affected by Tc because the angle of approach of the tRNA when bound to EF-Tu is sufficiently different from that of free A site tRNA so as to avoid a steric clash with the bound antibiotic. Since Tc is located on the other side of the tRNA with respect to the codon, there would be no interference with the codon:anticodon interaction, and decoding would be allowed to proceed. Successful decoding would lead to GTP hydrolysis on EF-Tu, and the subsequent release of the factor from the ribosome. The rotation of tRNA into the A site that follows would lead to a steric clash with tetracycline and its ejection from the ribosome. This model is consistent with structural as well as biochemical data on Tc binding, and represents a very effective mode of killing bacterial cells because it acts catalytically. Each time a ternary complex arrives at the ribosome, GTP is hydrolyzed by EF-Tu in an unproductive way because the tRNA is subsequently ejected and no peptidyl transfer occurs. So apart from preventing protein synthesis, the binding of tetracycline to the ribosome is also energetically very expensive for the cell.

### A Possible Second Mode of Tetracycline Action

Most existing biochemical data suggest that Tc only affects translation by its action in the A site. However, the location of the second site on H27, which is supported by previous biochemical data, suggests that it too may play a role. H27 and its surroundings have been implicated in conformational changes associated with a transition from an error-prone *ram* state to a hyperac-

(d) The superimposed structures of HygB (magenta; this work) and paromomycin (red; from Carter et al, 2000) in the 30S subunit. Two positions of the proposed decoding center (A1492 and A1493) are shown, the HygB bound state (green) and paromomycin bound state (yellow). The ring names for paromomycin are shown with lowercase letters (i, ii, iii, iv) for clarity.

curate restrictive state (Allen and Noller, 1989; Lodmell and Dahlberg, 1997); in particular, H27 has been proposed to switch between two base-pairing schemes during this transition. (Lodmell and Dahlberg, 1997) (Figures 2d and 2e). Tc binds H27 in a manner that suggests it might act to preferentially stabilize the conformation of H27 observed in the crystal, which corresponds to the *ram* state. Thus, Tc could also disrupt 30S function by stabilizing the *ram* state in a manner analogous to that proposed for streptomycin (Carter et al., 2000).

Some support for this model comes from studies on the action of colicin E3, which cleaves 16S RNA between nucleotides 1493 and 1494. Both streptomycin and tetracycline block cleavage by colicin E3 in streptomycin-sensitive cells, but not in resistant cells (Dahlberg et al., 1973). This finding is compatible with the hypothesis that colicin E3 cleavage requires the restrictive state of the 30S. In this view, both streptomycin and tetracycline stabilize the *ram* state and prevent transition to the restrictive state, thus preventing cleavage, while in streptomycin-resistant cells, such a transition would not be prevented by streptomycin. If this secondary effect of tetracycline is real, the two essentially independent modes (blocking A site tRNA and inhibiting the transition to the restrictive state) could act synergistically. Clearly, further experiments will be needed to ascertain whether the secondary site is a physiologically relevant site or a nonspecific one.

#### Pactamycin Binding Suggests Disruption of the Shine-Dalgarno:Anti-Shine-Dalgarno Interaction in Prokaryotes

The two crucial bases involved in Pct binding, G693 and C795, are universally conserved in all kingdoms; they lie at the tips of the stem loops H23b and H24a, which are also universally conserved (Gutell, 1996). This implies that the mode of binding of Pct is the same in all species. Not surprisingly, resistance to Pct is caused by mutations in any of three bases, A694G, C795U, or C796U.

It has been suggested that Pct could function by locking the tips of helices 23 and 25 together, and thus restrict the flexibility of the subunit during translation (Mankin, 1997). Pct does not bind to helix 25, but this idea could be true for the helices 23b and 24a instead since Pct is effectively sandwiched between these two stem loops (Figures 3a and 3d). Furthermore, the platform of the 30S is known to change conformation during subunit association (Lata et al., 1996). However, the antibiotic may also act simply by displacing E site mRNA as observed, which could have the consequence of preventing proper E site interactions and/or movement of mRNA during translation.

In bacteria, this displacement of the message would have additional consequences for initiation, as observed. The displacement would prevent the interaction between the Shine-Dalgarno sequence of mRNA and the anti-Shine-Dalgarno region at the 3' end of 16S RNA, which would occur immediately past the E site codon. It is thus likely that movement of the last base of the E site triplet by 12.5 Å seriously hampers or perhaps even abolishes Shine-Dalgarno:anti-Shine-Dalgarno interaction during initiation in prokaryotes.

It is also interesting to note that the parts of 16S RNA responsible for its binding (H23b and H24a) also contain bases that are protected by initiation factor 3 (IF3) (Moazed et al., 1995). This suggests that Pct might interfere with the function of IF3 during initiation in a way that prevents the release of IF3 from the 30S and thus the formation of 70S ribosomes, which is another observed phenotype for Pct.

#### Hygromycin B Restricts the Movement of Helix 44

HygB is known to inhibit translocation by sequestering tRNA in the ribosomal A site (Cabanas et al., 1978a). The part of H44 to which HygB binds has recently been implicated in movements during translocation (Frank and Agrawal, 2000), which suggests that HygB could restrict or inhibit a conformational change that is crucial for the movement of this helix during translocation. This, in turn, would prevent movement of the A site bound tRNA into the P site with an overall net effect of sequestering tRNA in the A site. The observation that binding of HygB causes increased affinity for tRNA at the A site (Eustice and Wilhelm, 1984a) indicates that it could also alter the balance between *ram* and restrictive states as has been suggested for streptomycin (Carter et al., 2000). This is consistent with the finding that transition between the two states affects bases at the very top of H44, i.e., at the site of HygB binding (Lodmell and Dahlberg, 1997).

#### Conclusion

The atomic structures of ribosomal subunits have ushered in a new era for understanding the structural basis of antibiotic action on the ribosome. The work presented here provides a structural explanation for the antibiotic action of tetracycline, pactamycin, and hygromycin B on the bacterial 30S subunit. They also provide insights into the universal process of translation, in a manner analogous to the way classical inhibitors shed light on enzyme mechanisms. Interestingly, these antibiotics act at different sites and in different ways not only from each other, but also from the group of three previously studied, thus illustrating the great diversity of antibiotic binding sites and modes of action on the ribosome. The structures rationalize much previous biochemical and genetic data, and pave the way for the design of modified or novel antibacterial drugs that have the potential of overcoming microbial resistance.

#### Experimental Procedures

##### Crystallization and Data Collection

Purification and crystallization of 30S subunits from the thermophilic bacterium *Thermus thermophilus* was carried out as described previously (Wimberly et al., 2000). Large, single crystals were soaked in 80 μM of either tetracycline (4-[dimethylamino]-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-2-naphthacene-1-carboxamide, obtained from ICN), pactamycin (2-hydroxy-6-methylbenzoic acid [5-[[3-acetylphenyl]amino]-4-amino-3-[[[(dimethylamino)-carbonyl]amino]-1,2-dihydroxy-3-(1-hydroxyethyl)-2-methylcyclopentyl]methyl ester, obtained from the US National Cancer Institute, Bethesda, MD), or hygromycin B (O-6-amino-6-deoxy-L-glycero-D-galacto-heptopyranosylidene-(1→2-3)-O-β-D-talopyranosyl-(1→5)-2-deoxy-N<sup>3</sup>-methyl-D-streptamine, obtained from ICN) before flash-freezing in liquid N<sub>2</sub>. For comparison, the minimum inhibitory concentrations of these antibiotics is 0.3–3 μM for tetracy-

cline (Ross et al., 1998), 8–80  $\mu$ M for pactamycin (Cohen et al., 1969a), and around 6  $\mu$ M for hygromycin B (Cabanas et al., 1978a). Diffraction data extending to between 3.3 and 3.4 Å were collected at beamline ID14-4 at the European Synchrotron Radiation Facility (ESRF), Grenoble, France.

#### Structure Determination and Refinement

Diffraction images were integrated and scaled using the HKL2000 package (Otwinowski and Minor, 1997) and structure factors were calculated with the program TRUNCATE (Collaborative Computational Project, 1994). All crystals belonged to the tetragonal space-group P4<sub>1</sub>2<sub>1</sub>2 with cell dimensions  $a = b = 401.158$  Å,  $c = 176.944$  Å (Tc),  $a = b = 401.719$  Å,  $c = 177.002$  Å (Pct), and  $a = b = 402.063$  Å,  $c = 175.263$  Å (HygB). The refined 3 Å structure of the native 30S subunit (PDB accession code 1FJF) was used as the starting model for further refinement in CNS (Brunger et al., 1998). Initially, the model was subjected to rigid-body refinement against each antibiotic data set using individual proteins and the primary domains of the 16S (the 5', central, 3' major, and 3' minor domains) as separate rigid objects to accommodate small structural rearrangements as well as differences in unit cell. This procedure was followed by positional and grouped B factor refinement according to the standard refinement scheme provided by CNS where 5% of the reflections were set aside for cross-validation. Care was taken to ensure that these reflections were the same 5% that had been used in the original refinement of the native 30S structure. In the case of Pct, the six residue mRNA occupying the P and E sites of the 30S was left out of the refinement to produce an omit map showing the new position of the message in the presence of the antibiotic. For Tc, the position of the Mg<sup>2+</sup> ion involved in binding at the primary binding site was derived from the structure of [Mg-tetracycline]<sup>+</sup> bound to the tet-repressor (PDB accession code 2TRT) (Hinrichs et al., 1994). In all cases, both  $\sigma_A$ -weighted  $mF_o - DF_c$  and  $2mF_o - DF_c$  difference maps (Read, 1986) showed good density at the antibiotic binding sites that allowed an unambiguous placement of the ligand molecules within the 30S structure. Tc was modeled into the difference density using a known small molecule crystal structure of the drug (CSD entry TETCYH10 [Stezowski, 1976]), whereas HygB and Pct were modeled by combining chemical structure information with three-dimensional geometry and energy minimization. After modeling of the antibiotic molecules into the difference density, a final round of refinement was carried out that included the ligands.

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#### Protein Data Bank ID Codes

Coordinates have been deposited in the RCSB protein data bank with accession codes 1HNW (Tc), 1HNX (Pct), and 1HNZ (HygB). Prior to release, coordinates are available for academic users upon request from D. E. B. (ding@mrc-lmb.cam.ac.uk).