Communication

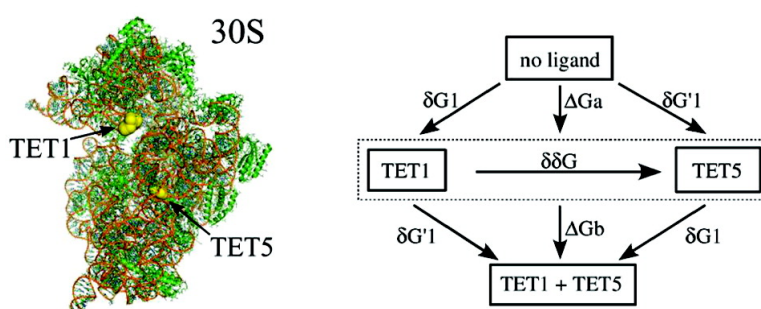# Molecular Dynamics Simulations of the 30S Ribosomal Subunit Reveal a Preferred Tetracycline Binding Site

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Molecular Dynamics Simulations of the 30S Ribosomal Subunit Reveal a Preferred Tetracycline Binding Site

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Tetracycline (Tc) and its analogues are important antibiotics affecting both gram-positive and gram-negative bacteria.[1,2] Tc inhibits protein synthesis by binding to the 30S subunit of the bacterial ribosome, preventing tRNA binding to the A-site.[2,3] Clinical use has led to several resistance mechanisms, including export of Tc from the cell and mutations in the bacterial rRNA. Much effort is being made to develop new Tcs that can evade resistance while effectively inhibiting protein synthesis.

Extensive biochemical and genetic studies[4−7] have given results that are somewhat difficult to interpret. Crystal structures of Tc bound to the *Thermus thermophilus* 30S subunit were determined by two groups.[8,9] Though nonpathogenic, *Thermus* is considered a valid model organism to understand ribosomal antibiotics.[10−12] Both groups found the same primary site (called TET1), with the strongest Tc electron density, close to the A-site, consistent with an inhibitory role. A Tc-bound Mg^{2+} ion was inferred from the electron density. Both groups reported a secondary Tc-binding site, called TET5. No Mg^{2+} ion was observed, but this is not conclusive, since electron density[8] was weaker in this region. One of the groups reported four additional Tc sites.[9] Both groups discussed whether multiple Tc binding sites can work cooperatively, and suggested that the primary binding site alone could explain Tc's bacteriostatic action.

We have done molecular dynamics (MD) simulations of 30S ribosomal subunits to characterize Tc binding and help resolve the ambiguity regarding the number and strength of Tc binding sites. We compared the binding free energies of Tc to the TET1, primary site and the TET5, secondary site. Other secondary sites were not considered, because they were unseen by one group[8] and are thus expected to have affinities lower than TET5. We benefit from a recent, high quality Tc force field[13] and from the maturity of current free energy methods.[14] We use structures obtained by MD, along with a well-established, continuum electrostatic (CE) method for the free energies.[14,15] The method is first parametrized and tested by computing the TET1 binding free energy differences between Tc and its analogues minocycline (Mc) and doxycycline (Dc), giving good agreement with experiment.[18,19] We also computed the free energy to introduce an Mg^{2+} cation in the TET1 and TET5 sites, using a more rigorous method, where the Mg^{2+} is gradually introduced during an MD simulation.[13,14e] CE gave good agreement with the more rigorous method; both methods predict that Mg^{2+} is pre-bound in each site in the absence of Tc. The free energy calculations then show that TET1 binding is indeed stronger than TET5 binding, as suggested (but not proven) by the weaker TET5 electron density,[8] resolving the previous ambiguity.

The simulations were done as follows. The starting point was the crystal structure of the 30S subunit with Tc bound to the TET1 and TET5 sites.[8] Simulations were done separately for both sites, including RNA and protein within a 26 Å-radius sphere centered on the Tc site, along with 1650−1850 water molecules and 80−100 sodium and choride counterions. Building models from the

entire 70S ribosome,[3] instead of the 30S subunit, would presumably not affect our results significantly, since the closest atoms of the 50S subunit are over 24 Å from the centers of the TET1 and TET5 spheres used here. The medium outside each 26 Å sphere was modeled as a dielectric continuum with the dielectric constant of bulk water, $\epsilon = 80$. Protein, RNA, water, and ions were described by standard force fields;[16] the ligands were described by a force field developed recently.[13] For each site, we considered eight structural models, including neutral and zwitterionic Tc tautomers, with two possible orientations of the Tc acetamide group. For each site and model, MD was run for 5 nanoseconds. We also computed the free energy to introduce an Mg^{2+} cation in each site, using the rigorous, MD free-energy simulation method[13,14e] (see Supporting Information for more details). The MD structures were then used to compute the contribution of electrostatic interactions to the ligand/ribosome binding free energy. Nonelectrostatic contributions are expected to cancel approximately when the different models and binding sites are compared[14] and are neglected. Protein, RNA, and Tc (or Mc or Dc) were treated as one homogeneous dielectric medium, with a dielectric ϵ, and solvent as another, with a dielectric of 80. The free energy of the bound and unbound states was obtained by solving numerically the Poisson−Boltzmann equation of continuum electrostatics.[17] The unbound structures were obtained by removing Tc (or Mc or Dc or Mg^{2+}) from the bound structures. The dielectric ϵ was set to 8, to reproduce the experimental binding free energy differences between Tc, Mc, and Dc (see above and Supporting Information).

The free energy calculations indicate that Tc binds to the primary site TET1 in its zwitterionic tautomer. The co-bound Mg^{2+} interacts directly with three ribosomal phosphate groups (Figure 1A). The MD structure agrees with the crystal structure, with an rms deviation for atomic positions of just 1.5 Å for Tc and its immediate neighbors. Experimental, Tc-ribosome, intergroup distances are well-reproduced. In the secondary site TET5, the zwitterionic tautomer is also preferred, and Mg^{2+} is predicted to be co-bound. This is not inconsistent with the experimental electron density data, which is moderately precise.[8] The Tc is more shifted (by 2.3 Å) with respect to the crystal structure.[8] In this site, the co-bound Mg^{2+} interacts directly with just one ribosomal phosphate group (Figure 1B), completing its coordination sphere with two water molecules. Binding to the TET5 secondary site is thus weaker, with a binding free energy difference $\delta\delta G$ of 3 ± 2 kcal/mol relative to the TET1, primary site. This is consistent (Figure 1C) with the weaker, observed, electron density in TET5.[8] The continuum electrostatic treatment of the ribosome and the surrounding solution[17] includes one adjustable parameter: the dielectric constant ϵ used for the protein and RNA. Our choice of $\epsilon = 8$ yields good agreement with the experimental binding free energy differences between Tc, Mc, and Dc[18,19] (see Supporting Information for details). It also yields agreement with the more rigorous, MD free energy simulations for the Mg^{2+} binding free energies to TET1 and TET5.
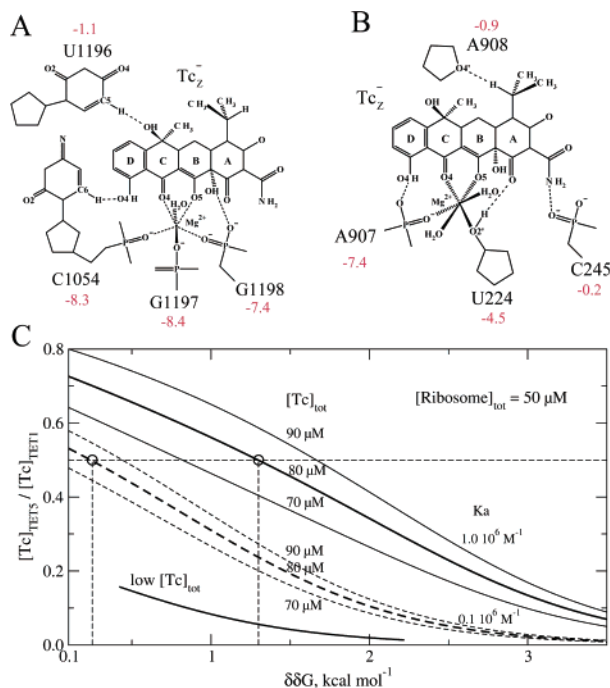
Figure 1. The Tc binding sites TET1 (A) and TET5 (B); selected group contributions to the binding free energies in kcal/mol. (C) Relative occupancy $[Tc]_{TET5}/[Tc]_{TET1}$ of TET5 and TET1 vs the computed binding free energy difference $\delta\delta G$ between sites. In the crystal cell,[8] total ribosome and Tc concentrations are 50 and 80 μM. With an association constant $K_a = 10^6$ M^{-1}, TET1 is fully occupied, while TET5 half-occupancy (qualitatively consistent with the X-ray data) occurs for $\delta\delta G \approx 1.3$ kcal/mol. The "low [Tc]" curve crudely mimics physiological conditions: [ribosome] = [Tc] = 1 μM ($\sim$1000 ribosomes per bacterial cell, $\sim$1 g of Tc in the bloodstream); TET5 is then largely unoccupied.

Varying ϵ between 4 and 16 changes $\delta\delta G$ by just ± 1 kcal/mol. The remaining uncertainty in $\delta\delta G$ is due to the finite MD simulation length.

Considering the experimental association constant K_a for a single Tc binding to the 30S subunit,[18,20] the experimental Tc and ribosome concentrations in the crystal cell,[8] and the TET1/TET5 binding free energy difference $\delta\delta G$, we can compute the occupancies of the TET1 and TET5 sites (see Supporting Information and Figure 1C). With $K_a = 10^6$ M^{-1} and $\delta\delta G = 1$ kcal/mol, we find a TET1 occupancy of $\approx$100% and a TET5 occupancy of about 57%, which appears compatible with the experimental electron density maps.[8] Taking into account the K_a uncertainty, the moderate X-ray resolution, and differences between the crystalline and solution conditions, the computed range for $\delta\delta G$ appears consistent with the X-ray data; the true $\delta\delta G$ value is likely to be in the lower part of the computed range, closer to 1–2 than to 4–5 kcal/mol. With more physiological concentrations, [Ribosome] = [Tc] = 1 μM, TET5 is largely unoccupied (Figure 1C).

In summary, we have presented evidence for the existence of a single, predominant binding site for Tc on the ribosome, suggesting that other reported binding sites are weaker and not highly occupied at physiological Tc concentrations. This lends support to the view that allosteric coupling between binding sites is not important or at least not crucial for the function of tetracycline. Our simulations complement the X-ray data, giving a more detailed picture of Tc binding, including thermodynamic information such as individual residue free energy contributions. The continuum electrostatic model has many caveats[14,15] and is only semiquantitative, so that additional, experimental tests are needed. Nevertheless, protein, RNA, water, and ions were described by well-tested force fields;[16] the Tc/Mc/Dc force field was developed in the same painstaking way.[13] The simulations were long, and 16 different structural models were compared. The results are robust with respect to the details of the Poisson−Boltzmann treatment, with a large range of parameter choices all leading to the same qualitative picture: the primary site is computed to have a larger Tc affinity than the putative secondary site TET5.

Supporting Information Available: Full ref 16a; details of simulation methods and results. This material is available free of charge via the Internet at http://pubs.acs.org.

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