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Free Energies and Entropies of Water Molecules at the Inhibitor−Protein Interface of DNA Gyrase

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Abstract: Complexes of the antibiotics novobiocin and clorobiocin with DNA gyrase are illustrative of the importance of bound water to binding thermodynamics. Mutants resistant to novobiocin as well as those with a decreased affinity for novobiocin over clorobiocin both involve a less favorable entropy of binding, which more than compensates for a more favorable enthalpy, and additional water molecules at the protein−ligand interface. Free energy, enthalpy, and entropy for these water molecules were calculated by thermodynamic integration computer simulations. The calculations show that addition of the water molecules is entropically unfavorable, with values that are comparable to the measured entropy differences. The free energies and entropies correlate with the change in the number of hydrogen bonds due to the addition of water molecules.

I. Introduction

A recent analysis of crystal structures reveals that over 85% of the structures have at least one water molecule at the protein−ligand interface.[1] These water molecules have significant but not completely understood influences on inhibitor binding thermodynamics. The introduction of ordered (relative to the liquid) water molecules is generally considered to have a significant entropic cost, decreasing the binding affinity of the ligand.[2,3] This influence can be isolated from other factors by considering changes in the ligand or protein that modify the water structure. Differences in binding affinities between different ligands[4−15] or the same ligand with mutated proteins[16−18] have been attributed to the addition of one or more tightly bound water molecules. Both these effects are demonstrated in the DNA gyrase/novobiocin complex.

DNA gyrase is a bacterial enzyme that is a target for several antibiotics.[19] The enzyme from *Escherichia coli* is an A_2B_2 tetramer made up of the two subunits A and B. The antibiotic novobiocin is one in a class of coumarin inhibitors that inhibit gyrase by preventing dimerization of the two B subunits.[20−22] For this system, bound water molecules have been proposed to change the thermodynamics of binding, both by changes in the protein through mutations[16] and by changes in the inhibitor.[14] A mutation of Arg-136 to histidine on the B fragment is one of the naturally occurring mutants resistant to coumarin inhibitors.[23] The B fragment with the R136H mutation has an association constant for novobiocin over an order of magnitude smaller than the wild type (Table 1).[16] The binding has a more favorable enthalpy change, ΔH_a, for the mutant but a much less favorable entropy change, ΔS_a. The structures of R136H and wild type of the 24 kDa N-terminal fragment of the B subunit with novobiocin show that the space created by the absence of the guanidinium group of the arginine residue is occupied by a water

(1) Lu, Y.; Wang, R.; Yang, C. Y.; Wang, S. *J. Chem. Inf. Model.* **2007**, *47*, 668–675.
(2) Dunitz, J. D. *Science* **1994**, *264*, 670.
(3) Ladbury, J. E. *Chem. Biol.* **1996**, *3*, 973–980.
(4) Lam, P.; Jadhav, S.; Evermann, C. J.; Hodge, C. N.; Yu, R.; Bachelor, L. T.; Meek, J. L.; Otto, M. J.; Rayner, M. L.; Wong, N. Y.; Chang, C. H.; Weber, P. C.; Jackson, D. A.; Sharp, T. R.; Erickson-Viitanen, S. *Science* **1994**, *263*, 380–383.
(5) Mikol, V.; Papageorgiou, C.; Borer, X. *J. Med. Chem.* **1995**, *38*, 3361–3367.
(6) Weber, P. C.; Pantoliano, M. W.; Simons, D. M.; Salemme, F. R. *J. Am. Chem. Soc.* **1994**, *116*, 2717–2724.
(7) Vara Prasad, J. V. N.; Lunney, E. A.; Ferguson, D.; Tummino, P. J.; Rubin, J. R.; Reyner, E. L.; Stewart, B. H.; Guttendorf, R. J.; Domagala, J. M.; Suvorov, L. I.; Gulnik, S. V.; Topol, I. A.; Bhat, T. N.; Erickson, J. W. *J. Am. Chem. Soc.* **1995**, *117*, 11070–11074.
(8) Royer, W. E., Jr.; Pardanani, A.; Gibson, Q. H.; Peterson, E. S.; Friedman, J. M. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 14526–14531.
(9) Heiss, C.; Laivenieks, M.; Zeikus, J. G.; Phillips, R. S. *J. Am. Chem. Soc.* **2001**, *123*, 345–346.
(10) Clarke, C.; Woods, R. J.; Gluska, J.; Cooper, A.; Nutley, M. A.; Boons, G. *J. Am. Chem. Soc.* **2001**, *123*, 12238–12247.
(11) Snider, M. J.; Wolfenden, R. *Biochemistry* **2001**, *40*, 11364–11371.
(12) Zechel, D. L.; Boraston, A. B.; Gloster, T.; Boraston, C. M.; Macdonald, J. M.; Tilbrook, D. M. G.; Stick, R. V.; Davies, G. D. *J. Am. Chem. Soc.* **2003**, *125*, 14313–14323.
(13) Gloster, T. M.; Williams, S. J.; Roberts, S.; Tarling, C. A.; Wicki, J.; Withers, S. G.; Davies, G. J. *Chem. Commun.* **2004**, 1794–1795.
(14) Lafitte, D.; Lamour, V.; Tsvetkov, P. O.; Makarov, A. A.; Klich, M.; Moras, P. D. D.; Briand, C.; Gilli, R. *Biochemistry* **2002**, *41*, 7217–7223.
(15) Daranas, A. H.; Shimizu, H.; Homans, S. W. *J. Am. Chem. Soc.* **2004**, *126*, 11870–11876.
(16) Holdgate, G. A.; Tunnicliffe, A.; Ward, W. H. J.; Westin, S. A.; Rosenbrock, G.; Barth, P. T.; Taylor, I. W. F.; Pauptit, R. A.; Timms, D. *Biochemistry* **1997**, *36*, 9663–9673.
(17) Peterson, K. M.; Gopalan, K. V.; Nandy, A.; Srivastava, D. K. *Protein Sci.* **2001**, *10*, 1822–1824.
(18) Connelly, P. R.; Aldape, R. A.; Bruzzese, F. J.; Chambers, S. P.; Fitzgibbon, M. J.; Fleming, M. A.; Itoh, S.; Livingston, D. J.; Navia, M. A.; Thomson, J. A. *Proc. Natl. Acad. Sci. U.S.A.* **19994**, *91*, 1964–1968.
(19) Reese, R. J.; Maxwell, A. *Nucleic Acids Res.* **1991**, *19*, 1399–1405.
(20) Ali, J. A.; Jackson, A. P.; Howells, A. J.; Maxwell, A. *Biochemistry* **1993**, *32*, 2717–2724.
(21) Ali, J. A.; Orphanides, G.; Maxwell, A. *Biochemistry* **1995**, *34*, 9801–9808.
(22) Gilbert, E. J.; Maxwell, A. *Mol. Microbiol.* **1994**, *12*, 365–373.
(23) Contreras, A.; Maxwell, A. *Mol. Microbiol.* **1992**, *6*, 1617–1624.

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Table 1. Thermodynamic Parameters for Binding of Novobiocin and Clorobiocin to the 24 kDa Fragment of DNA Gyrase B Protein for Wild Type and Arg 136 His Mutant

complex	K_a ($\times 10^6$ M^{-1})	ΔG^0 (kcal/mol)	ΔH^0 (kcal/mol)	$T\Delta S^0$ (kcal/mol)	no. of water molecules
WT−clorobiocin[a]	860 ± 220	−12.2 ± 0.1	−9.5 ± 0.6	2.7 ± 0.2	1
WT−novobiocin[b]	23 ± 4	−10.1 ± 0.1	−12.2 ± 0.1	−2.1 ± 0.2	3
R136H−novobiocin[b]	0.83 ± 0.03	−8.1 ± 0.1	−14.3 ± 0.1	−6.1 ± 0.1	4

[a] Reference 14. [b] Reference 16.

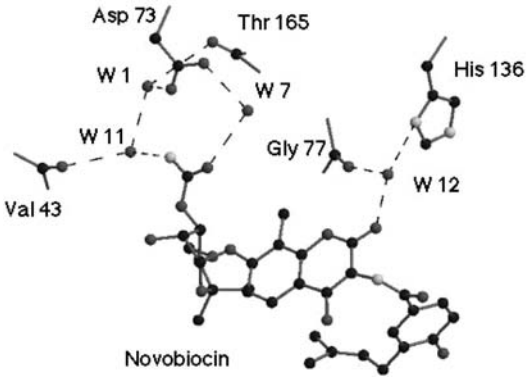

Figure 1. PDB structure 1AJ6 of the complex of novobiocin with the R136H mutant of DNA gyrase.

molecule (water 12 in the 1AJ6 PDB structure and in Figure 1).[16] The large change in K_a is attributed to the presence of this water molecule, which is not present in the wild-type structure. This water molecule is in contact with the solvent.

Water molecules 1 and 11 are located between the carbonate nitrogen attached to the sugar ring on novobiocin and polar atoms (on Val 43 and Asp 73) of the protein. This side of the inhibitor is away from the solvent. For the inhibitor clorobiocin, a pyrrole ring replaces the NH_2 group (Figure 2). The bulkier group fills the space occupied by W 1 and W 11 in the complex with novobiocin.[14] Clorobiocin binds (wild-type) gyrase over an order of magnitude better than novobiocin, with the improved binding due not to enthalpy, which favors novobiocin, but entropy. The entropic contribution, $-T\Delta S$, is 4.8 kcal/mol more favorable for clorobiocin than novobiocin (see Table 1). The large thermodynamic changes between clorobiocin and novobiocin, as well as those between the wild-type and mutant proteins, appear to be largely due to only a few water molecules.[14,16]

There are exceptions to the "less water, better binding" heuristic suggested by these studies, in which compounds with more water molecules at the interface bind with greater affinity.[15] In addition, several computational studies using free energy perturbation[24] and inhomogeneous fluid solvent theory[25−27] have shown that the entropy of the bound water molecules varies considerably and in some cases can be greater than that of bulk water. For the related, but distinct, problem of water displacement from the binding site by the ligand, inhomogeneous fluid solvent theory also finds that the entropy of water molecules in the empty binding site can vary considerably.[28] This variability makes assessing the role of water difficult. A number of studies have evaluated the importance of water molecules using protein−ligand docking and scoring models, with a general, but not universal, consensus that the inclusion of water improves accuracy.[29−38] The variability of the entropic contribution from the bound water presents a challenge for empirical scoring models.[31,34] For example, the GOLD scoring model adds a constant entropic penalty term (of 0.5 kcal/mol) for each bound water.[34]

The entropic contribution of the bound water molecules appears to be the key to understanding the binding thermodynamics for the novobiocin/clorobiocin−DNA gyrase complex. In this study we use computational methods to calculate the entropy change, as well as the free energy change, for adding water molecules to the three sites, which change for the various complexes (W1, W11, and W12). A number of studies have calculated free energies for water molecules at protein−ligand interfaces[39−42] and in protein cavities.[24,43−46] These studies have not calculated the entropy change, except for our own study, which calculated free energy changes as a function of temperature to extract the entropy and enthalpy changes.[24] In this study, we will use the same approach. This calculates the exact thermodynamics, depending only on the quality of the potential energy function used.

(24) Olano, L. R.; Rick, S. W. *J. Am. Chem. Soc.* **2004**, *126*, 7991–8000.
(25) Li, Z.; Lazardis, T. *J. Am. Chem. Soc.* **2003**, *125*, 6636–6637.
(26) Li, Z.; Lazardis, T. *J. Phys. Chem. B* **2005**, *109*, 662–670.
(27) Li, Z.; Lazardis, T. *J. Phys. Chem. B* **2006**, *110*, 1464–1475.
(28) Abel, R.; Young, T.; Farid, R.; Berne, B. J.; Friesnder, R. A. *J. Am. Chem. Soc.* **2008**, *130*, 2817–2831.
(29) Birch, L.; Murrary, C. W.; Hartshorn, M. K.; Tickle, I. J.; Verdonk, M. L. *J. Comput.-Aided Mol. Des.* **2002**, *16*, 855–869.
(30) Wang, R.; Lu, Y.; Wang, S. *J. Med. Chem.* **2003**, *46*, 2287–2303.
(31) Fornabaio, M.; Spyrakis, F.; Mozzarelli, A.; Cozzini, P.; Abraham, D. J.; Kellogg, G. E. *J. Med. Chem.* **2004**, *47*, 4507–4516.
(32) Friesner, R. A.; Banks, J. L.; Murphy, R. B.; Halgren, T. A.; Klicic, J. J.; Mainz, D. T.; Repasky, M. P.; Knoll, E. H.; Shelley, M.; Perry, J. K.; Shaw, D. E.; Francis, P.; Shenkin, P. S. *J. Med. Chem.* **2004**, *47*, 1739–1749.
(33) Lloyd, D. G.; García-Sosa, A. T.; Alberts, I. L.; Todorov, N. P.; Mancera, R. L. *J. Comput.-Aided Mol. Design* **2004**, *18*, 89–100.
(34) Verdonk, M. L.; Chessari, G.; Cole, J. C.; Hartshorn, M. J.; Murray, C. W.; Nissink, J.; Taylor, R. D.; Taylor, R. *J. Med. Chem.* **2005**, *48*, 6504–6515.
(35) García-Sosa, A. T.; Firth-Clark, S.; Mancera, R. L. *J. Chem. Inf. Model.* **2005**, *45*, 634–633.
(36) de Graaf, C.; Pospisil, P.; Pos, W.; Folkers, G.; Vermeulen, N. *J. Med. Chem.* **2005**, *48*, 2308–2318.
(37) de Graaf, C.; Oostenbrink, C.; Keizers, P.; van der Wijst, T.; Jongejan, A.; Vermeulen, N. *J. Med. Chem.* **2006**, *49*, 2417–2430.
(38) Roberts, B. C.; Mancera, R. L. *J. Chem. Inf. Model.* **2008**, *48*, 397–408.
(39) Helms, V.; Wade, R. C. *Biophys. J.* **1995**, *69*, 810–824.
(40) Hamelberg, D.; McCammon, J. A. *J. Am. Chem. Soc.* **2004**, *126*, 7683–7689.
(41) Lu, Y.; Yang, C.; Wang, S. *J. Am. Chem. Soc.* **2006**, *128*, 11830–11839.
(42) Barillari, C.; Taylor, J.; Viner, R.; Essex, J. W. *J. Am. Chem. Soc.* **2007**, *129*, 2577–2587.
(43) Wade, R. C.; Mazor, M. H.; McCammon, J. A.; Quiocho, F. A. *J. Am. Chem. Soc.* **1990**, *112*, 7057–7059.
(44) Wade, R. C.; Mazor, M. H.; McCammon, J. A.; Quiocho, F. A. *Biopolymers* **1991**, *31*, 919–931.
(45) Zhang, L.; Hermans, J. *Proteins: Struct., Funct., Genet.* **1996**, *24*, 433–438.
(46) Roux, B.; Nina, M.; Pomès, R.; Smith, J. C. *Biophys. J.* **1996**, *71*, 670–681.

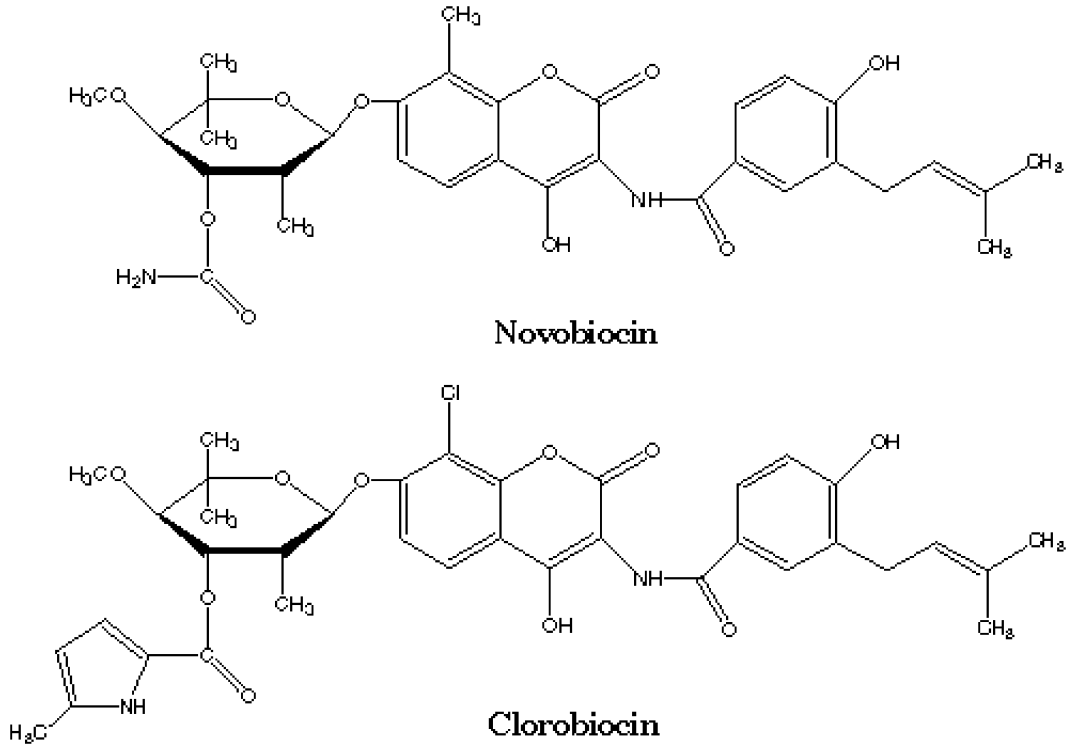

Figure 2. Chemical structures of DNA gyrase inhibitors.

II. Methods

Free Energy Calculations. The free energy calculations were done in one of two ways, depending on the proximity of the water molecules to the bulk solvent. These two approaches, the alchemical "double decoupling method", in which interactions are turned on while a restraining potential is turned off, and a potential of mean force (PMF) approach, in which the molecule is made to move from the binding site, have both been applied to many binding free energy calculations, as reviewed recently.[47] The free energy calculations for water molecules in the 1 and 11 positions are done with the double decoupling method, as described previously, except thermodynamic integration was used rather than free energy perturbation theory.[24] In this method, interactions are turned off between the one bound water molecule and all other atoms in the system. The noninteracting molecule is localized in the site with a harmonic potential with a force constant, k_{harm}, equal to 3 kcal mol^{-1} Å^{-2}. A correction for the harmonic restraint equal to $-kT \ln [\rho(\pi kT/k_{harm})^{3/2}]$ is added to the calculated free energy.[45,46,48] This method is similar to the methods developed for other free energy calculations[45,46,48] except for the addition of a short-range interaction to keep other water molecules out of that position. This interaction is of the form $\varepsilon(r_{Ox}/\sigma)^{-12}$, where r_{Ox} is the distance between the position the water molecule is being added, r_x, and the oxygen atoms on all other water molecules. The parameters σ and ε are set equal to 2.0 Å and 0.143 kcal/mol, respectively. To calculate the free energy of adding a water molecule to this position relative to the bulk liquid, the free energy of adding a water molecule to the liquid must be calculated. The free energy for this process is -7.04 ± 0.04 kcal/mol and the entropy change $T\Delta S$ is -4.03 ± 0.04 kcal/mol. For the water molecule in site 12, which is in contact with solvent water molecules, the PMF approach was used. In this approach, the interactions of the specified bound water are not turned off, but rather the water molecule is reversibly forced out of the bound water position by use of the potential $E_\lambda = \lambda_i 4\varepsilon[(r_{ix}^2 + (1-\lambda)\delta)/\sigma^2]^{-6}$, where the sum is over all water molecule oxygen atoms and λ is a free energy variable, varying from 0 to 1. This method uses separated-shifted scaling method, adding the term $(1-\lambda)\delta$ to avoid singularities as r_{ix} goes to zero.[49] The parameter δ was set equal to 7.0 Å^2 and σ and ε have the same values as given above. It was hoped that this approach would be more efficient for calculating hydration free energy changes for water molecules that can move to the solvent easily, but this turned out not to be the case. It was better to turn off the interactions in the protein/ligand site and then, in a separate calculation, turn them on again in the liquid, rather than to force the molecule to exit to the solvent. Free energy calculations for all the water sites were done at three temperatures, so the entropy could be calculated from the temperature dependence, by use of $\Delta S = -[\Delta G(T+\Delta T) - \Delta G(T-\Delta T)]/(2\Delta T)$. The enthalpy change can be found from $\Delta H = \Delta G + T\Delta S$.

DNA Gyrase/Novobiocin Structure. All calculations were done with the R136H mutant of the 24 kDa B subunit fragment of DNA gyrase from *Escherichia coli* by use of the 1aj6 structure for the gyrase/novobiocin complex.[16] In this structure, there are two loop regions, residues 83−85 and 105−111, that are unresolved. These regions were reconstructed from the coordinates of the 1kij structure on the 43 kDa fragment of DNA gyrase from *Thermus thermophilus* in complex with novobiocin.[50]

(47) Deng, Y.; Roux, B. *J. Phys. Chem. B* **2009**, *113*, 2234–2246.
(48) Gilson, M. K.; Given, J. A.; Bush, B. L.; McCammon, J. A. *Biophys. J.* **1997**, *72*, 1047–1069.
(49) Zacharias, M.; Straatsma, T. P.; McCammon, J. A. *J. Chem. Phys.* **1994**, *100*, 9025–9031.
(50) Lamour, V.; Hoermann, L.; Jeltsch, J.; Oudet, P.; Moras, D. *J. Biol. Chem.* **2002**, *277*, 18947–18953.

Protonation states of various groups must also be assigned. Novobiocin is an acid with a pK_a equal to 4.3,[51] so the acidic proton, on the phenolic oxygen connected to the coumarin double ring, is taken to be absent. In addition to the mutant histidine at position 136, the 24 kDa subunit of *E. coli* DNA gyrase has 11 histidine residues. On the basis of the pK_a calculations for the wild type of Schechner et al.,[52] we assigned the following protonation states (residue number, protonation state): 37 HID, 38 HIP, 55 HIE, 64 HIE, 83 HIE, 99 HIP, 116 HIP, 141 HIE, 147 HIP, 215 HIE, and 217 HIP, where HID is the Nδ tautomer, HIE is the Nε tautomer, and HIP is doubly protonated. This gives a charge for DNA gyrase equal to −5 and novobiocin adds another negative charge. Due to the overall negative charge, including the nearby novobiocin negative charge, we took the histidine 137 residue to be the doubly protonated positively charged form. In addition, in the 1aj6 structure, there are oxygen atoms (the main-chain oxygen on Arg 76 for the Nδ atom and the Oγ on Thr 80 for the Nε atom) close to both nitrogens on the histidine ring. Also, given that it replaces an arginine residue with a +1 charge, a positively charge histidine seemed the simplest assumption to make. Isothermal titration calorimetry (ITC) measurements with different buffers find that the enthalpy of binding, ΔH^0 for the wild type/novobiocin complex is independent of the buffer.[16] The two buffers used (20 mM phosphate buffer with pH 7.4 and 69 mM Tris-HCl at pH 7.4) have enthalpies of ionization that are different by 10.5 kcal/mol, so if binding involved proton movement, it would have different ΔH^0 values for the two buffers. For R136H mutant/novobiocin binding, ΔH^0 is different for the two buffers by 3.2 kcal/mol (the values in Table 1 are with the Tris buffer) so any proton changes upon binding must involve a partially ionized group.[16] For all these reasons—the overall negative charge, the nearby hydrogen-bond acceptors, and the fact that histidine replaces the positively charged arginine residue—the simplest assumption is that His-136 is doubly protonated and remains so during binding.

Simulation Details. All protein molecular dynamics simulations were performed with the Amber7 suite of programs.[53] Charges for novobiocin were generated from a RESP[54] charge-fitting procedure with input from Hartree−Fock calculations at the 6-31G* level by use of the Gaussian03 program.[55] Additional parameters were generated with the gaff parameter set.[56] The charges and gaff parameter type for each atom are given in Supporting Information. The TIP4P-Ew model was used for water.[57] Charge neutrality of the system was created by adding five sodium ions, by use of the Amber 99 parameter set.[58] The system contain 7891 water molecules. Simulations were run in the T, P, N ensemble at a pressure of 1 atm and temperatures of 283, 298, and 313 K. All bonds containing hydrogen atoms were constrained with SHAKE, a 1 fs time step was used, and long-range electrostatics were treated with particle mesh Ewald. For the water molecules at positions 1 and 11, 15 λ values were used, each simulated, on average, for 500 ps. For water molecule at site 12, using the different method, 17 λ values were used, each simulated, on average, for 1200 ps.

Table 2. Free Energies for Addition of a Water Molecule to Various Positions at Different Temperatures

	ΔG (kcal/mol)		
	at 283 K	at 298 K	at 313 K
W1	−15.12 ± 0.08	−14.73 ± 0.10	−14.44 ± 0.09
W11	−10.26 ± 0.11	−9.87 ± 0.14	−9.67 ± 0.15
W11′	−7.76 ± 0.12	−7.42 ± 0.14	−7.32 ± 0.11
W12	−2.88 ± 0.14	−2.81 ± 0.14	−2.76 ± 0.12

III. Results

Tables 2 and 3 give the calculated free energy changes for addition of water molecules to the positions labeled in Figure 1. The value for W11 is calculated twice, once with the water at position W1 and once without. The value without the W1 water is listed as W11′, and this value plus that for W1 gives the free energy for addition of two water molecules to the empty cavity. Table 2 gives the results for free energy calculations at the three temperatures. To the values for W1, W11, and W11′ sites, the harmonic restraint correction[45,46,48] has been added. To get the hydration free energy (the difference between the free energy of a water molecule in the bulk liquid and in the specified site) for these three sites, the free energy of a water molecule in the liquid has to be subtracted. For the water in position W12, the method used finds the free energy difference between that site and bulk water directly. For all water molecules, the free energy of hydration is negative, indicating that water is stable in that position, the entropy change is negative, and the enthalpy change is negative. One possible exception is W11′ water, for which ΔG, ΔH, and ΔS are about zero, and so this water is stable only if there is a neighboring water at position W1. The large enthalpic change, which outweighs the unfavorable entropy change, leads to the stability of the water molecules in these positions.

The average number of hydrogen bonds each water molecule makes to the protein, the inhibitor, or other water molecules from our simulations (at the end point of the free energy calculation when the water is fully interacting with the rest of the system) is given in Table 3. The Mancera and Buckingham[59] definition of a hydrogen bond is used, in which a hydrogen bond is taken to exist if the oxygen−oxygen distance is less than 3.6 Å and the angle between the O−H vector on the hydrogen-bonding donor and the O−O vector is between 130° and 180°. With these criteria, the water at W1 forms 2.2 hydrogen bonds on average. One hydrogen bond is made to the water at W11 (W11 acts as the hydrogen-bond donor) and another is made to the Oδ atom of Asp-73 (W1 is the donor). Another hydrogen bond is made a fraction of the time (0.2) to the Thr-165 O atom. The water at W11 makes about 3 hydrogen bonds. In addition to the hydrogen bond to the W1 water, it makes a hydrogen bond to the Val-43 O atom (W11 is the donor) and the another with one of the amide hydrogens connected to the noviose sugar of the novobiocin molecule. The W12 water forms about 3 hydrogen bonds: one with the Gly-77 O atom, one with the

(51) *The Merck Index*, 12th ed.; Budavari, S., Rathway, N. J., Eds.; Merck & Co.: Rahway, NJ, 1996; p 1155, no. 6818.
(52) Schechner, M. A.; Dejaegere, A. P.; Stote, R. H. *Int. J. Quantum Chem.* **2004**, *97*, 747–763.
(53) Case, D. A.; et al. AMBER 7; University of California, San Francisco, 2002.
(54) Bayley, C. I.; Cieplak, P.; Cornell, W. D.; Kollman, P. A. *J. Phys. Chem.* **1993**, *97*, 10269–10280.
(55) Frisch, M. J.; et al. Gaussian03; Gaussian Inc.: Pittsburgh, PA, 2003.
(56) Wang, J.; Wolf, R. M.; Caldwell, J. M.; Kollman, P. A.; Case, D. A. *J. Comput. Chem.* **2004**, *25*, 1157–1174.
(57) Horn, H. W.; Swope, W. C.; Pitera, J. W.; Madera, J. D.; Dick, T. J.; Hura, G. L.; Head-Gordon, T. *J. Chem. Phys.* **2004**, *120*, 9665–9678.
(58) Wang, J.; Cieplak, P.; Kollman, P. A. *J. Comput. Chem.* **2000**, *21*, 1049–1074.
(59) Mancera, R. L.; Buckingham, A. D. *J. Phys. Chem.* **1995**, *99*, 14632–14640.

Table 3. Thermodynamic Properties for Transfer of a Water Molecule from Solvent to Various Positions

	ΔG (kcal/mol)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)	no. of H bonds	change in H bonds
W1	-7.7 ± 0.1	-10.5 ± 1.2	-2.8 ± 1.2	2.2 ± 0.1	2.2 ± 0.1
W11	-2.8 ± 0.1	-4.7 ± 1.9	-1.9 ± 1.9	2.9 ± 0.1	1.8 ± 0.1
W11′	-0.4 ± 0.1	-0.1 ± 1.6	-0.3 ± 1.6	1.4 ± 0.2	0.8 ± 0.4
W1 + W11′	-8.1 ± 0.2	-10.6 ± 2.0	-3.1 ± 2.0	3.6 ± 0.2	3.0 ± 0.4
W12	-2.8 ± 0.1	-3.9 ± 1.8	-1.2 ± 1.8	2.7 ± 0.1	1.9 ± 0.1

phenolic oxygen on the coumarin double ring on the novobiocin molecule (which is taken to be unprotonated; see Methods), both of these as a donor, and a third (made a fraction 0.7 of the time) with a solvent water molecule as an acceptor. A hydrogen bond with the Nδ atom on His-136 is rarely made. These hydrogen bonds are indicated in Figure 1.

The formation of hydrogen bonds with the water molecules is consistent with the negative ΔH. Less consistent is the fact that W11 and W12 form the most hydrogen bonds, about 3, but have a less favorable ΔG than W1, which forms less hydrogen bonds. More strongly correlated to the free energy is the change in the number of hydrogen bonds, or the number of hydrogen bonds that form as the water is added minus the number that are lost. To do this, we identified the atoms that formed hydrogen bonds to the specific water molecule (those atoms are mentioned in the previous paragraph) and calculated the number of hydrogen bonds those atoms form with and without the water in that position. In all cases, the hydrogen bonds change by fractional amount, rather than through the formation of new contacts that occur only when the water molecule is absent. For example, one of the W12 water's hydrogen-bond partners, the Gly-77 O atom, decreases the number of hydrogen bonds it forms upon addition of water by 0.4 ± 0.2, mostly due to a decrease in the fraction of time a hydrogen bond is made with the His-136 Nδ atom (from 0.8 ± 0.1 without water to 0.5 ± 0.1 with water). The novobiocin phenolic oxygen atom does not changes its hydrogen-bond structure noticeably. The third significant hydrogen-bond partner with the water at site W12 is a solvating water molecule. A water molecule closest to the position of W12 decreases its number of hydrogen bonds to other water molecules, not counting W12, from 2.8 ± 0.3 to 2.4 ± 0.1, upon addition of W12. This means that the gain of hydrogen bonds between W12 and its closest neighbor (made a fraction 0.7 of the time) is partially offset (by 0.4) by a loss in hydrogen bonds with other neighboring water molecules. The net effect is that addition of the W12 water leads to an increase in hydrogen bonds by only 1.9 rather than 2.7.

The hydrogen-bond neighbors of water W11 also change the number of hydrogen bonds with other atoms as this water is added. W11's neighbor Val-43 O shows a decrease in the fraction of time a hydrogen bond is made to other protein atoms (a hydrogen bond to the Asn-46 N−H changes from 0.46 ± 0.08 to 0.10 ± 0.04 and to the Ala-47 N−H changes from 0.5 ± 0.1 to 0.19 ± 0.07). The amide N atom on novobiocin, which also hydrogen-bonds to W11, does not show any appreciable change in hydrogen bonds upon addition of W11. The third hydrogen-bond partner of W11 is W1. This water shows a decrease of 0.5 ± 0.3 in hydrogen bonds with other atoms as W11 is added. This decrease is mostly due to loss in a hydrogen bond with the Thr-165 O atom, which changes from 0.4 ± 0.2 to 0.09 ± 0.04. This all leads to a net change in hydrogen bonds equal to 1.8 ± 0.1. When W11 is added without the water W1 there (in the change labeled W11′), a similar analysis reveals that the hydrogen-bonded neighbors lose 0.6 ± 0.3 as the 1.4 ± 0.2 hydrogen bonds are made to give a net change in hydrogen bonds equal to 0.8 ± 0.4 hydrogen bonds. For the W1 water, no hydrogen bonds are lost as that water is added, so that the change in hydrogen bonds is simply 2.2 ± 0.1.

These hydrogen-bond changes are shown in Table 3 and show a stronger correlation with ΔG than simply the number of hydrogen bonds made. This analysis helps to explain why the W1 position has more favorable ΔG than the W11 and W12 positions, because while water molecules at both those positions make more hydrogen bonds with their neighbors, they also disrupt more hydrogen bonds, leading to a smaller change in hydrogen bonds than at the W1 position. (In addition, the water at position W1 makes a hydrogen bond with the negatively charged Asp-73 Oδ atom, so this hydrogen bond is stronger than average, with a more favorable ΔH.) Both the entropic contribution to the free energy, $T\Delta S$, which increases, and the enthalpy, which decreases, show a correlation with the change in hydrogen bonds.

IV. Conclusion

The calculations find that addition of water molecules to the protein−ligand interface is entropically unfavorable. The resulting $T\Delta S$ values are -1.1 ± 1.8 kcal/mol for the water at position W12 and -3.1 ± 2.0 kcal/mol for the addition of two water molecules at sites W1 and W11 (see Figure 1). These can be compared to the difference in the entropy changes for the binding of novobiocin or clorobiocin to the wild type or R136H mutant of DNA gyrase. The R136H mutation introduces the W12 water to the novobiocin/gyrase interface and results in a decrease in $T\Delta S$ equal to 4.0 ± 0.2 kcal/mol. Our calculations suggest that 1 kcal/mol of this is due to the ordered water. The binding of clorobiocin, which eliminates the W1 and W 11 water molecules, results in a decrease in $T\Delta S$ equal to 4.8 ± 0.2 kcal/mol, of which our calculations would suggest that 3.1 kcal/mol is due to the two water molecules. Taken together, our results indicate that a sizable fraction, but not all, of the entropic differences in the binding of ligands involving different numbers of water molecules is directly due to the water molecules.

The range of values of the entropy changes shows that not all water molecules would have the same entropic penalty to the binding thermodynamics of ligands. This variability is in agreement with earlier theoretical studies using free energy perturbation[24] and inhomogeneous fluid solvent theory[25−27] as well as conclusions drawn from experimental data on protein stability.[60] The water molecules considered in this study form different numbers of hydrogen bonds with neighboring atoms, including other water molecules, the protein, and the inhibitor. However, the free energy, entropy, and enthalpy changes correlate more strongly with the *change* in hydrogen bonds made as the water molecule is added, rather than the number of hydrogen bonds the water makes (see Table 3). The number of

(60) Takano, K.; Funahashi, J.; Yamagata, Y.; Fujii, S.; Yutani, K. *J. Mol. Biol.* **1997**, *274*, 132–142.

hydrogen bonds changes by an amount different from that directly involving the added water because the added water disrupts the local hydrogen bonds formed in its absence. This disruption is due to hydrogen bonds, made both with and without the water, that are made less often when the water is there. The presence of the water gives the neighboring atoms another hydrogen-bond partner and this appears to decrease the probability that other hydrogen bonds are made. This change in the local hydrogen-bond structure has previously been reported for water molecules in protein interiors[24] and indicates that the entropy change for localizing a water molecule at a specific site, with a loss of conformational freedom, is partially offset by a gain in conformation freedom of the nearby atoms involved in hydrogen bonds.

For the design of drugs, having a water molecule with more than two hydrogen bonds is entropically unfavorable and it would be better to modify the ligand to eliminate the water or to reduce the number of hydrogen bonds that water can make. A fit to our data suggests that $T\Delta S$ should decrease by an amount of 1.7 kcal/mol for each hydrogen bond made by addition of the water molecule. This is very close to the value of 1.6 kcal/mol by Cooper,[61] an estimate based on the loss of degrees of freedom of a bound water for each hydrogen bond made. Although there is no reason for the entropy change to have a linear dependence on the number of hydrogen bonds, each hydrogen bond will limit rotational and translational freedom, consistent with a decrease in entropy. Analyses of crystal structure *B* factors for bound waters in protein interiors[62] and at protein−ligand interfaces[1] reveal that the water molecules become more localized as hydrogen bonds are made, but the decrease is not linear. The decrease is large as each of the first three hydrogen bonds is made, and adding the fourth has little or no effect on the *B* factor, suggesting that there is little entropic penalty for forming the fourth hydrogen bond. For the range of hydrogen bonds made by water molecules in the present study (1−2), $T\Delta S$ changes from about 0 to −2 kcal/mol. The hydrogen-bond numbers of these three water molecules are fairly typical of water molecules found at protein/ligand interfaces. The analysis of crystal structures by Wang and co-workers[1] reveals that water molecules at protein/ligand interfaces form on average 3 hydrogen bonds, with 2 being almost equally likely. The entropies, as well as the free energies, of the water molecules in this study may be therefore fairly representative of water molecules commonly found in protein/inhibitor complexes, but the values for specific molecules will depend on their local environment.

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Supporting Information Available: Charges and gaff parameter type for each atom. This material is available free of charge via the Internet at http://pubs.acs.org.

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(61) Cooper, A. *Biophys. Chem.* **2005**, *115*, 89–97.

(62) Park, S.; Saven, J. G. *Proteins: Struct., Funct., Bioinf.* **2005**, *60*, 450–463.