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What determines the strength of noncovalent association of ligands to proteins in aqueous solution?

(molecular dynamics/free energy calculation/avidin/α-chymotrypsin)

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ABSTRACT **Free energy perturbation methods using molecular dynamics have been used to calculate the absolute free energy of association of two ligand–protein complexes. The calculations reproduce the significantly more negative free energy of association of biotin to streptavidin, compared to *N*-L-acetyltryptophanamide/α-chymotrypsin. This difference in free energy of association is due to van der Waals/dispersion effects in the nearly ideally preformed cavity that streptavidin presents to biotin, which involves four tryptophan residues.**

One of the exciting developments in computer modeling of complex molecules in solution has been the capability to calculate relative free energies of association of these molecules and to relate these values to experiment (1, 2). This development has been catalyzed by methodological advances (3, 4) and increased computer capabilities. In favorable cases, relative free energies of association within 1 kcal/mol (1 cal = 4.184 J) of experiment have been achieved (2). In such cases, the calculations could be of use in experimental ligand design. However, inaccuracies in molecular mechanical force fields and representation of the system and, even more importantly, limitations in one's ability to completely sample the relevant regions of conformational space, have restricted the number of systems to which such free energy calculations could be applied to give chemical accuracy (5, 6).

Nonetheless, such free energy calculations can be very valuable and interesting even when such accuracy is not achieved, because mechanistic insight into noncovalent association in general and protein–ligand design in particular can be extracted from them (7, 8). The fundamental question asked here is: Why do some protein ligand systems have a significantly higher binding affinity than others? Below, we describe the calculation of the absolute free energy of the association of biotin with streptavidin ($K_{assoc} = 10^{14}$) (9) and *N*-L-acetyltryptophanamide (NATA) to α-chymotrypsin ($K_{assoc} = 5 \times 10^3$) and show that the reason for the greater affinity of biotin for streptavidin is the van der Waals energy, both because of the hydrophobic effect of water and because of the near ideal cavity in streptavidin for binding biotin, with four tryptophan residues surrounding this cavity.

One can start with the standard free energy cycle (Fig. 1) (10), in which $\Delta\Delta G_{bind}$ is either the difference between the horizontal processes, usually measured experimentally, or the difference in the mutational vertical processes that can be simulated with molecular dynamics/free energy calculations. We have shown that by using this cycle with ligand = biotin and ligand′ = thiobiotin or iminobiotin we can calculate the relative free energies of association to streptavidin in excellent agreement with experiment (9). However, if ligand′ is a molecule consisting only of dummy atoms, one can use the cycle to calculate the absolute free energy of association of ligand. This, as noted by Jorgensen *et al.* (11) in their study of the association of two methane molecules, requires one to mutate methane to nothing in water and when associated with another methane molecule. Such absolute free energy calculations are straightforward in principle, but will they lead to reasonable answers for large and complex ligands in water and in the binding sites of proteins?

Merz (12) has shown that this approach works for CO_2 binding to carbonic anhydrase, Miyamoto and co-worker (13) have found excellent agreement for binding K^+ to a calixspherand, Jorgensen and co-worker (14) have had success with this approach for nucleic acid bases in organic solvents, and Lee *et al.* (15) have calculated the absolute free energy of association of phosphorylcholine analogs to an immunoglobulin, but no one has carried out such a large and dramatic change as in the biotin–streptavidin association studied by Miyamoto and Kollman (9). In that paper, either all of biotin or all of biotin but the terminal CO_2^- group were mutated to dummy atoms both in water and in the protein; in either case, a ΔG for association in the range of −20 kcal/mol could be calculated, in good agreement with experiment. Given the approximations in that study (neglect of any changes in intramolecular energies of biotin free and bound and underestimate of translational/rotational entropy losses due to the use of hydrogen bond restraints) and the difficulty in precisely estimating the magnitude of the errors, we turned to another ligand–protein complex with much lower affinity, with a ligand of size comparable to biotin to use as a control. The results of that calculation, presented here along with the results of the biotin–streptavidin calculation, enable fundamental insights into the nature of ligand–protein associations.

METHODS

We used the free energy perturbation method to calculate the binding free energy of complexation. This method uses an easily derived equation from classical statistical mechanics to relate the free energy difference between two states to the ensemble average of the potential energy difference between the states. The details have already been presented in the literature (5, 6). As shown in previous studies (11–13), this approach can be related to the thermodynamic cycle in Fig. 1 in the case of the binding free energy calculation of the protein–ligand complex. ΔG_{solv} is the difference in free energy upon mutating a ligand in water. ΔG_{prot} is the change in free energy for the same mutation with the ligand bound to a protein. If the ligand′ consists of dummy atoms, which have no van der Waals or electrostatic interactions with their environment, $\Delta G_{bind'}$ becomes zero. The experimentally relevant free energy difference $\Delta G_{bind} - \Delta G_{bind'}$, can be related to the calculated free energy difference $\Delta G_{solv} - \Delta G_{prot}$, since

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Abbreviation: NATA, *N*-L-acetyltryptophanamide.

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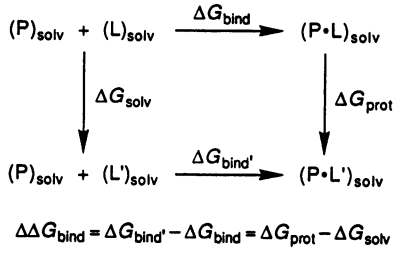

FIG. 1. Thermodynamic cycle for complex formation of a ligand (L) with a protein (P). Binding free energy of complexation of a ligand with a protein ΔG_{bind} (horizontal arrow) can be measured by experiment but is difficult to study by computational approaches. On the other hand, the processes represented by the vertical arrows, which, although not physically measurable, are straightforward to simulate. ΔG_{solv} is the difference in free energy upon mutating the ligand in water. ΔG_{prot} is the change in free energy for the same mutation with the ligand bound to the protein. The experimentally relevant free energy difference $\Delta G_{bind} - \Delta G_{bind'}$ can be related to the calculated free energy difference $\Delta G_{solv} - \Delta G_{prot}$, since the free energy is a state function. ΔG_{solv} and ΔG_{prot} are calculated by the molecular dynamics/free energy perturbation method.

the free energy is a state function. The ΔG_{solv} and ΔG_{prot} are calculated by the molecular dynamics/free energy perturbation method implemented in the AMBER package (16–18).

In the aqueous simulation, the perturbation in both directions (forward, ligand → ligand'; reverse, ligand' → ligand) were carried out. In the solvated protein, the perturbations in the reverse direction were not performed since it was difficult for water molecules to come out of the binding pocket once they moved in. The entire ligand was treated as the perturbation group and no intraligand free energy changes were considered in the calculation. The separate perturbations of the two components of the nonbonded interaction (i.e., the perturbation of electrostatic and hydrogen-bonding parameters followed by that of van der Waals parameters) were carried out to allow the role of the different contributions to the ligand–protein interactions to be identified. A constant dielectric of 1.0 and a residue-based nonbonded cutoff of 10 Å were used in all calculations. An integration time step of 2 fs was used since the SHAKE algorithm was applied to all bonds (19). The temperature was maintained at 298 K by coupling to a temperature bath. In the aqueous simulation, periodic boundary conditions were applied with constant pressure of 1 atm. The complete protocol of the simulations for the biotin–streptavidin system are presented in ref. 9, but the essence is summarized here.

The mutation of biotin to nothing in a periodic box of ≈500 TIP3P (20) water molecules was carried out using periodic boundary conditions and running the calculations both forward and backward with 6-ps equilibration in each direction. To mutate biotin to nothing when bound to streptavidin (21), we created an 18-Å spherical shell of waters around the center of mass of biotin. After removing waters within 2 Å of a protein atom, the molecular dynamics of biotin, any protein residue containing an atom within 12 Å of any atom of biotin and all the waters proceeded, using the "cap" option (22) to keep the water molecules from drifting away. We carried out the mutation in the protein only in the forward direction with a number of different protocols. As more fully expressed in ref. 9, we found that the most reliable protocol involved restraining the five hydrogen bonds that the ureido group formed with the protein and the two that the $-CO_2^-$ formed to hydrogen-bond distances of 2.8 Å, with a harmonic force constant of 5 kcal/mol·Å^2. This ensured that the ligand remained near its x-ray crystallographically determined position as it disappeared. The free energy calculations with this protocol were carried out with both models of the side chain, although in the simulation when the entire biotin was mutated to nothing, protocols with or without restraints with 36 and 48 ps, respectively, for the electrostatic and van der Waals perturbation (see Table 1) and protocols of 100 and 108 ps with restraints on the hydrogen bonds all led to similar $\Delta\Delta G_{bind}$ of 20–22 kcal/mol for biotin disappearance. Longer simulations (100 and 108 ps, respectively, for the electrostatic and van der Waals perturbations) without restraints or those run in the reverse direction led to significantly small $\Delta\Delta G_{bind}$ values, but the dominance of the van der Waals over the electrostatic term remained (9).

NATA was created from standard residues in the AMBER data base, with the exception that the terminal NH_2 group retained the amide N charge and distributed the remaining charge equally on the two terminal hydrogens to retain neutrality. The backbone geometry of this molecule was taken from the P1 leucine of an inhibitor bound to α-chymotrypsin found in ref. no. 1CHO (23) from the Brookhaven Protein Data Bank. The χ_1 and χ_2 angles of the side chain were manually adjusted to visually optimally fit the tryptophan in the P1 pocket. After solvating the ligand–protein complex with an 18-Å shell of waters centered on the ligand molecule, the system was minimized with hydrogen-bond distance restraints between CO of the tryptophan and the backbone NH of Gly-196 and the NH of tryptophan and the CO of Ser-214. Then molecular dynamics and free energy perturbation calculations were carried out exactly as done in biotin–streptavidin (9). The simulation of NATA in water was carried out exactly as for biotin itself (9).

RESULTS AND DISCUSSION

In Table 1 we present the free energies of disappearance of biotin, partial disappearance of biotin, and disappearance of NATA in their respective binding sites in the proteins and in water. As one can see, the agreement with experiment for the disappearance of NATA is comparable to that for biotin; in

Table 1. Results of free energy calculations

	ΔG_{solv},* kcal/mol			ΔG_{prot},† kcal/mol			$\Delta\Delta G_{bind}$, kcal/mol			
							Calc.			
Perturbation	Elec	VdW	Total	Elec	VdW	Total	Elec	VdW	Total	Exp.‡
Biotin → CTM§	14.1 ± 1.3	0.5 ± 0.3	14.5 ± 1.1	20.3	15.2	35.5	6.2	14.7	21.0	18.3
Biotin → nothing	97.9 ± 1.6	−4.1 ± 0.5	93.8 ± 2.1	101.0	14.0	115.0	3.0	18.2	21.2	18.3
NATA → nothing	15.3 ± 0.4	−1.8 ± 0.4	13.5 ± 0.1	22.2	0.4	22.6	6.9	2.2	9.1	5.2

*Errors represent half the hysteresis between forward and reverse simulations. Electrostatic free energy (Elec) and van der Waals energy (VdW) were determined by the 100-ps simulations, respectively. Total free energy (Total) is the sum of Elec and VdW.

†Elec and VdW were determined by the 36- and 48-ps simulations, respectively, and internal distance restraints were applied on the hydrogen bonds between the ligand and the protein residues.

‡Experimentally determined from the dissociation constants of the biotin complex with streptavidin (24) and the NATA complex with α-chymotrypsin (25).

§Partial disappearance into the C-terminal moiety (CTM) $-CH_2CH_2CH_2CO_2^-$ (see Fig. 2A and ref. 9).

each case, the calculations lead to larger free energies for dissociation than were found by experiment.

As more fully discussed in ref. 9, the approximations and errors inherent in these calculations are as follows: (*i*) incomplete hydration, (*ii*) incomplete conformational adjustment of the protein in the unbound state, (*iii*) neglect of conformational flexibility of the carboxyl side chain of biotin when unbound, and (*iv*) application of hydrogen-bond restraints. All would tend to lead to an overestimate of $\Delta\Delta G_{bind}$, consistent with the results of our calculations.

We summarize the points made in ref. 9 here. (*i*) The conformational differences between apostreptavidin and liganded streptavidin in the crystal structures are modest and include ordering of two surface loops and formation of a salt bridge between adjacent loops (21). The large deviation of one loop in the binding site found experimentally was observed in the protein structure after the perturbation calculation from the biotin complex to the uncomplexed protein (9). (*ii*) The disappearance of the ligand also might result in waters not fully solvating the cavity. As shown by Wade *et al.* (26), some protein cavities are of a nature that the free energy price is unfavorable to place waters in them. As we have noted (9), the number of waters (five or six) found in the streptavidin cavity as biotin disappears is similar to that found crystallographically (24). (*iii*) The neglect of conformational flexibility of the biotin side chain when unbound is likely to lead to an overestimate of the dissociation free energy of 1–2 kcal/mol (9). (*iv*) The effect of applying the hydrogen-bond restraints is likely to lead to an overestimate of the free energy of dissociation in the range of a few kcal/mol (14).

In addition, by mutating the biotin charge model from 6-31G* electrostatic potential derived charges, which tend to overestimate polarity, to STO-3G electrostatic potential derived charges, which tend to underestimate polarity, the electrostatic free energies are changed by ≈15 kcal/mol both in the binding site and in solution (9). Thus, it is likely that any unusual polarization of the ureido group of biotin, invoked (21) to explain the tight binding of biotin to avidin/streptavidin is also operative in solution and would not be a special effect of biotin binding. Thus, in summary, we feel our calculated results (Table 1) are reasonable and supportable given that we reproduced them with five different protocols (9).

We emphasize, as noted in ref. 9, that our goal was to determine whether one would calculate a $\Delta\Delta G_{bind}$ in the range of 20 kcal/mol. As noted previously (1), the most severe limitation in free energy calculation is sampling conformational space. It is not just a matter of sampling longer, but also sampling in the correct region of conformational space. In the calculations of the absolute free energy of binding of biotin (9), the goal was to determine what protocol, if any, could lead to a $\Delta\Delta G_{bind}$ of the order of 20 kcal/mol, not to calculate $\Delta\Delta G_{bind}$ within ±1 kcal/mol, which is a realistic goal of relative free energy calculations (2). We found values of 20–22 kcal/mol for five independent protocols (9). But given the uncertainties noted above, it was important to study a completely different protein–ligand association.

The dramatic difference in free energy of association of biotin–streptavidin compared to NATA–α-chymotrypsin is reproduced by the calculation (Table 1). Even more interestingly, it is the van der Waals energy that largely differentiates the two ligand–protein associations. In each case, the electrostatic energy is 3–7 kcal/mol more favorable in the protein than in water, but the van der Waals energy is tremendously more favorable in biotin–streptavidin than in NATA–α-chymotrypsin.

Although the individual free energy components are not independent of path, in contrast to the free energy itself, we have presented a well-defined path here (first disappearance of charges and then disappearance of van der Waals energies). By constraining the hydrogen-bond distances, we also reduce the coupling between electrostatic and van der Waals effects. Thus, we feel our interpretation of these free energies reasonable and useful. Given the two β-sheet hydrogen bonds in the NATA–α-chymotrypsin complex, the compatibility of our calculated electrostatic free energies for NATA–α-chymotrypsin to that determined by Tobias *et al.* (27) for a model β-sheet is also worthy of note.

A referee has noted that the NATA–α-chymotrypsin system may not be a good control for the biotin–streptavidin calculations, because the former is a model and the latter is a well-characterized structure. This is a fair criticism, but we chose NATA–α-chymotrypsin because it was a ligand about the same size and chemical nature as biotin, with much weaker affinity to a protein on which we had significant modeling experience and results that connected well with experiments (28). Only time will tell how generally useful our approach will be on other protein–ligand systems, but we feel the data presented here offer considerable encouragement.

How is the dominance of the van der Waals contribution to the biotin binding free energy (Table 1) compatible with one's preconceived notion that the van der Waals forces ought to be similar in both protein and water? This can be understood when one realizes that the ethane is actually more soluble in water than methane, and propane is only 0.2 kcal/mol in free energy less soluble (29). How is the greater solubility of ethane than methane in water compatible with the hydrophobic effect? It is a matter of reference state, with ethane partitioning more favorably into water relative to the gas phase. Free energy calculations (4) have been able to reproduce these relative free energies to within ±0.3 kcal/mol without adjustable parameters, with exchange repulsion (A/R^{12}) and dispersion attraction ($1/R^6$) (i.e., the standard van der Waals interaction terms) determining the relative free energy of solvation of these hydrocarbons in water. As discussed in ref. 4, the solvation free energy due to van der Waals effects is a balance between cavity repulsion effects, which are positive and come from the repulsive part of the van der Waals energy, and dispersion effects, which are negative and come from the attractive part of the van der Waals energy (4). As shown by Singh and co-workers (30, 31), the magnitude of the repulsive effect is unique to water compared to nonaqueous solvents and is fundamentally a manifestation of the hydrophobic effect. In their perturbation calculation from Me_4C to CH_4, they found that the van der Waals energy decreased monotonically in MeOH and dimethyl sulfoxide, while the free energy initially increased and then decreased in water. A physical picture of this, compatible with the classic picture of the hydrophobic effect, is that water molecules surrounding the growing nonpolar group are willing to experience some small exchange repulsion in order to maintain their hydrogen-bonded network. For methane → propane, this exchange repulsion nearly exactly cancels the dispersion attraction and methane and propane have approximately equal water/gas-phase partition coefficients. Similarly, the van der Waals contribution to the free energy of solvation of biotin or NATA are small and (for disappearing the entire molecules) favorable for the disappearance. On the other hand, in the streptavidin binding site the loss of dispersion attraction makes the disappearance of biotin unfavorable by ≈15 kcal/mol.

In an ideally formed protein cavity, one has paid the free energy price upon synthesis of the protein to leave a hole just the correct size for the requisite ligand; thus, when the ligand binds, one can take advantage of dispersion attraction without any payment of exchange repulsion (Fig. 2*A*). It helps, in the case of streptavidin, to have four tryptophan ligands to increase the density of close atoms in the site and thus to increase the favorable dispersion attraction. In contrast, in

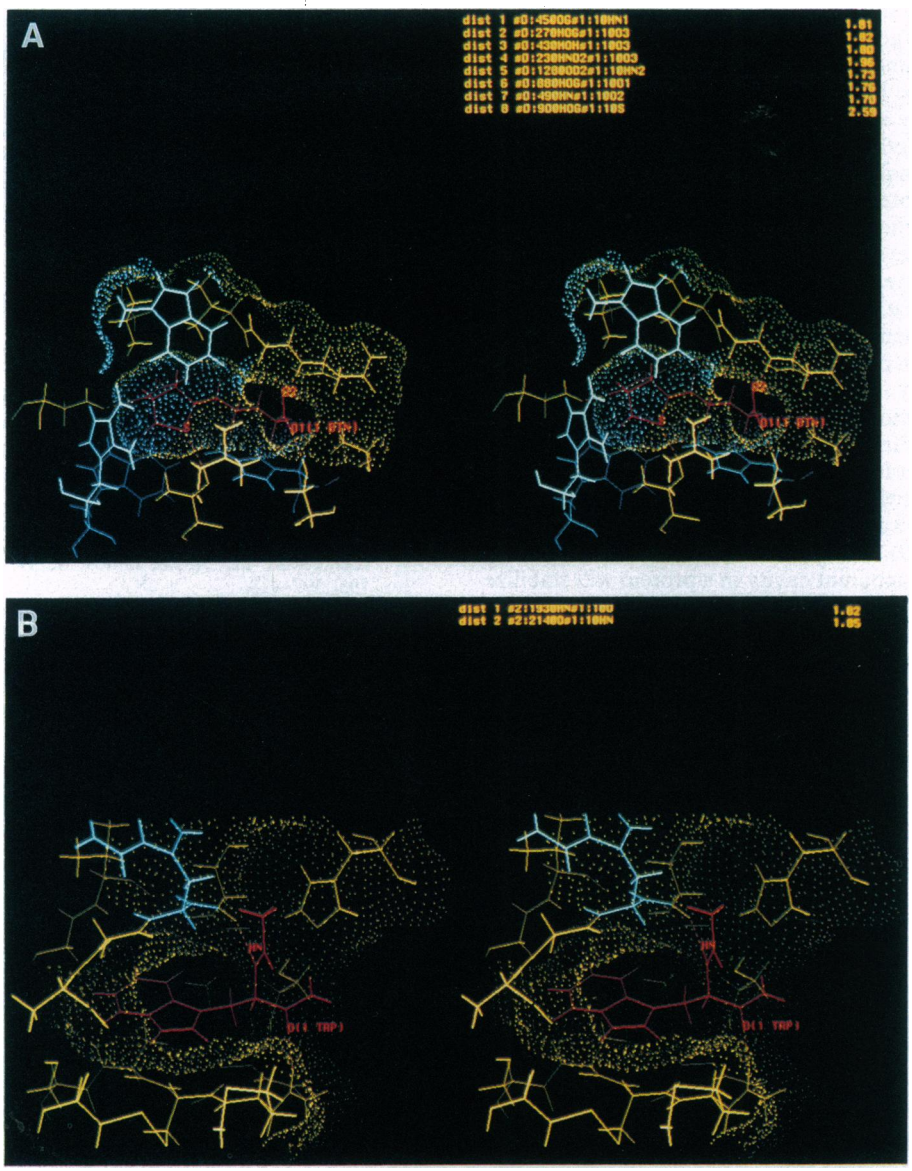

FIG. 2. Stereoviews of binding sites of the energy-minimized ligand–protein complexes. Ligand is red, and protein is yellow, with tryptophan residues highlighted in blue. Solvent-accessible surface of the protein is represented by dots. Hydrogen bonds between the ligand and the protein are shown in dashed lines and their distances are listed in the top right corner of the figures. (*A*) Biotin–streptavidin complex. (*B*) NATA–α-chymotrypsin complex.

the NATA–α-chymotrypsin complex (Fig. 2*B*), only the side-chain tryptophan is buried by the protein surface, with less efficient packing of the protein atoms surrounding the cavity. Eighty-three atoms of α-chymotrypsin have short contacts (<4 Å) to the ligand, while the corresponding number of atoms is 128 in the streptavidin–biotin complex. Two tryptophans (Trp-79 and Trp-108) in the binding pocket have the most van der Waals contacts with biotin, suggesting that there would be a significant decrease in affinity in mutating those to smaller residues.

In many studies of protein stability and recognition, emphasis has been placed on electrostatic effects. Electrostatic effects are much longer range and have the potential to give much larger free energy effects than van der Waals energies (32), but there is often cancelation between electrostatic effects in water and protein that reduces the magnitude that one can achieve for electrostatic contributions to ligand binding and catalysis. In the two cases presented here, the electrostatic term does contribute favorably to binding. We would like to stress, as has been noted before (33, 34), that the same preorganization effects are the key in electrostatic recognition as in the van der Waals recognition found in biotin–avidin.

One example of this is the binding of alkali cations to many cyclic polyether ionophores (13), which is favorable in aqueous solution, despite the fact that the ether groups in the ionophore typically have a much less favorable alignment with the cation than do water molecules in aqueous solution. In fact, the K^+ interaction energy with a calixspherand (13) is in the range of −80 kcal/mol, whereas the K^+ interaction with water molecules in aqueous solution is about −140 kcal/mol. On the other hand, the water–water energy increases by about +70 kcal/mol when K^+ is solvated by water, but the extra strain in the ionophore when K^+ binds is very small. Thus, the ionophore has paid the free energy price to align its dipoles when it is synthesized and, therefore, it doesn't have to pay the large price that water does upon interaction with the cation. The interesting implication of this is that one cannot easily relate structure to free energy; i.e., the water dipoles are aligned much more favorably with K^+ in solution than was found in the ionophores, but the net free energy favors association.

Similarly, the oxyanion hole in the serine proteases does not have to form as good hydrogen bonds as found in water to an oxyanion, as long as little reorganization price needs to be paid (35, 36). The NH groups in the oxyanion hole have been oriented by the structure of the protein to point in the same direction, something they would be unlikely to do if not tied down. This enables them to stabilize the oxyanion formed in serine protease catalysis.

What are the implications of these results for ligand and protein design? One should be able to use computational methods to determine dynamically averaged cavity sizes to determine ligands that will experience mainly dispersion, not cavity repulsion, when bound to proteins. Second, one could actually seek areas in proteins with prealigned dipoles, which interact unfavorably with each other (e.g., the two NH groups in the oxyanion hole), to take advantage of this in ligand design (37). Finally, it is clear that filling up cavities with nonpolar groups is very important in protein stability, as has been demonstrated by the large stability decreases found in mutating large hydrophobic groups to small in barnase (38) and T4 lysozyme (39). It is not always clear whether adding a larger group to an apparent cavity in a protein will stabilize it, because of the subtle balance between repulsion and dispersion effects (40, 41). However, the use of nonnatural amino acids to more precisely fill cavities without experiencing extra van der Waals repulsion should enable one to design more stable proteins (42).

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