Hydrogen Bonding Stabilizes Globular Proteins*

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ABSTRACT It is clear that intramolecular hydrogen bonds are essential to the structure and stability of globular proteins. It is not clear, however, whether they make a net favorable contribution to this stability. Experimental and theoretical studies are at odds over this important question. Measurements of the change in conformational stability, $\Delta(\Delta G)$, for the mutation of a hydrogen bonded residue to one incapable of hydrogen bonding suggest a stabilization of 1.0 kcal/mol per hydrogen bond. If the $\Delta(\Delta G)$ values are corrected for differences in side-chain hydrophobicity and conformational entropy, then the estimated stabilization becomes 2.2 kcal/mol per hydrogen bond. These and other experimental studies discussed here are consistent and compelling: hydrogen bonding stabilizes globular proteins.

The principle of science, the definition, almost, is the following: the test of all knowledge is experiment. Experiment is the sole judge of scientific "truth." —R. P. Feynman (1963)

INTRODUCTION

To perform their biological functions, most proteins fold to globular conformations that are stabilized by many different noncovalent interactions. The relative importance of these interactions in stabilizing the folded state has been a matter of debate for several decades. Proteins contain both polar and nonpolar groups. When a protein folds, most of the nonpolar side chains (≅80%) are buried in the interior of the protein, shielded from solvent, and tightly packed with other polar and nonpolar groups (Klapper, 1971; Richards, 1977; Lesser and Rose, 1990; Harpaz et al., 1994). This phenomenon, known as the hydrophobic effect, is a major force favoring the folded conformation (Kauzmann, 1959; Tanford, 1962; Dill, 1990; Pace, 1992; Fersht and Serrano, 1993; Honig and Yang, 1995; Lazaridis et al., 1995; Makhatadze and Privalov, 1995; Matthews, 1995; Pace et al., 1996). Many polar side chains (≅65%) and peptide groups (≅70%) are also buried in folding (Lesser and Rose, 1990), and these groups almost invariably form hydrogen bonds with other polar groups (Baker and Hubbard, 1984; Stickle et al., 1992; McDonald and Thornton, 1994). One question being actively debated at present is whether these hydrogen-bonded polar groups make a favorable contribution to globular protein stability. Based on experimental studies, we think they do. Two recent theoretical studies disagree. Honig and Yang (1995) concluded, "The analysis presented here, when combined with the mutation results suggests that, to a first approximation, hydrogen-bonding groups make no contribution to protein stability." And Lazaridis et al. (1995) concluded, "The polar groups contribute little or not at all to protein stability." Recent textbooks have seemingly ignored much recent experimental evidence in coming to similar conclusions (Voet and Voet, 1995; Kyte, 1995). In this article, we first discuss some of the factors that complicate analyses of hydrogen bonding in proteins. Next we discuss recent experimental results that show that hydrogen bonding contributes favorably to protein stability, and finally we consider why the theoretical studies reach a conclusion different from that of the experimental studies.

HYDROGEN BONDING IN PROTEINS

In an unfolded protein, most polar groups are hydrogen bonded to and partially surrounded by water molecules. In a folded protein, most polar groups will form one or more intramolecular hydrogen bonds and will be surrounded by the polar and nonpolar groups that make up the interior of a folded protein. The question that has proved difficult to answer is whether the free energy of the polar groups is lower when they are hydrogen bonded to water in an unfolded protein or to each other in a folded protein.

Intramolecular hydrogen bonds are a ubiquitous feature of protein structure. On average, proteins form 1.1 hydrogen bonds per residue when they fold (Stickle et al., 1992). The >C═O···H-N< hydrogen bond formed by the backbone is most prevalent (68.1%), with >C═O···side chain (10.9%), >N-H···side chain (10.4%), and side chain···side chain hydrogen bonds (10.6%) accounting for the remainder (Stickle et al., 1992). The intrinsic energy of these bonds is not known. It is clear that electrostatic forces provide the major stabilization to hydrogen bonds (Mitchell and Price, 1990). Consequently, hydrogen bond energies will depend on both the dielectric constant and the hydrogen bond geometry. Quantum mechanical calculations suggest that the energy of a hydrogen bond between the carbonyl oxygen of formaldehyde and the amide hydrogen of formamide in vacuo is 6.5 kcal/mol at the optimal geometry and length (2.89 Å),

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*We are delighted to dedicate this paper to Dr. Serge Timasheff in honor of his 70th birthday. His research has provided answers to some of the most difficult questions in protein chemistry. NP thanks him for providing 10 g of β-lactoglobulin for his research in graduate school and is trying to repay him in kind (Lin and Timasheff, 1994).

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and 4.9 kcal/mol for the geometry and length (2.99 Å) of hydrogen bonds in an α-helix (Mitchell and Price, 1990). These values are comparable to the enthalpy change observed for neutral hydrogen bond formation in the vapor phase (Rose and Wolfenden, 1993). The average hydrogen bond length observed in proteins is 3.04 Å (Stickle et al., 1992). Thus, the average hydrogen bond in a folded protein will be longer than the length that leads to optimal strength, and the hydrogen bonds will have a range of angles, not necessarily those giving optimal strength. In addition, the hydrogen bonds will be in a far more complicated environment than the vapor phase, so that additional polar and van der Waals interactions will contribute to the stability. Consequently, at present, we have no reliable estimates of the average strength of intramolecular hydrogen bonds in a folded protein or of intermolecular hydrogen bonds of the same groups to water.

The unfavorable free energy of removing polar groups from water can be estimated from measurements of the free energy of transfer, ΔG_{tr}. For the transfer of a peptide group (-NH-CH-CO-) from water to the vapor phase, ΔG_{tr} = 12.1 kcal/mol (Makhatadze and Privalov, 1995), and from water to cyclohexane, ΔG_{tr} = 5.3 kcal/mol (Pace, 1995). These results show that ΔG for dehydrating a peptide group is very unfavorable, but that the peptide group can have favorable van der Waals interactions, even in a nonpolar liquid that makes up for a large part of the cost. The calculations of Lazaridis et al. (1995) show that van der Waals interactions between polar and nonpolar groups make a large contribution to protein stability. For the transfer of a peptide group from water to wet *n*-octanol, ΔG_{tr} = 1.1 kcal/mol (Pace, 1995; Wimley et al., 1996). In this case, the peptide group should be extensively hydrogen bonded in both solvents, suggesting that burying a peptide group might be unfavorable, even if it is completely hydrogen bonded. (Both cyclohexane and *n*-octanol are "wet" in the experiments to measure ΔG_{tr}, but the amount of water in the cyclohexane, 2.5 mM, and *n*-octanol, 2.3 M, differs markedly, so that polar solutes should be extensively hydrated in *n*-octanol, but not in cyclohexane.) Roseman (1988) has suggested that $\Delta G_{tr} \approx 0.6$ kcal/mol for the transfer of a hydrogen-bonded peptide group from water to CCl_4 or benzene. The evidence continues to mount, however, that octanol and other nonpolar liquids are poor models for the protein interior because the packing is not as tight, and consequently the van der Waals forces will be less favorable (Harpaz et al., 1994). In addition, the ΔG_{tr} values are based on small model compounds that would represent the upper limit of exposure to solvent. In an unfolded protein, the polar groups should be less exposed to solvent, and the cost of burying the groups would not be as great (Shortle, 1996; Creamer et al., 1995; Tobias et al., 1992).

This discussion points out some of the difficulties encountered in trying to answer the question posed above. We doubt if the question can be answered convincingly by theoretical studies in the near future, but they will be essential for reaching a complete understanding. In contrast, we think experimental studies provide strong evidence that hydrogen bonding contributes favorably to protein stability. The most convincing studies are discussed in the next section.

EXPERIMENTAL STUDIES OF HYDROGEN BONDING

Mutational studies of hydrogen bonding in proteins usually replace one member of a hydrogen bonding pair with a residue incapable of hydrogen bonding. By measuring the difference in stability between the mutant and the wild-type protein, one hopes to gain insight into the contribution of hydrogen bonding to the stability of the protein. Here we consider studies of 89 mutants of six different proteins: RNase T1 (Shirley et al., 1992), barnase (Serrano et al., 1992), BPTI (Yu et al., 1995), arc repressor (Milla et al., 1994), T4 lysozyme (Matthews, 1995), and staphylococcal nuclease (Green et al., 1992; Byrne et al., 1995). The five types of mutations considered are shown in Fig. 1. In the first four types, a polar group is removed and the residue in the mutant protein is smaller than the residue in the wild-type protein. For the fifth type, Thr ⇒ Val mutants, the polar -OH group is replaced by a nonpolar $-CH_3$ group, and the residue in the mutant protein is larger than the residue in the wild-type protein. The Thr ⇒ Val mutants will discussed first.

Results for 12 Thr ⇒ Val mutants are summarized in Table 1. Note that $\Delta(\Delta G)$ is defined so that negative values represent an increase in stability and positive values represent a decrease in stability. For the Thr ⇒ Val mutants in which the Thr is not hydrogen bonded in the wild-type protein, the mutants are stabilized when the -OH group is replaced by a $-CH_3$ group if the -OH group is buried. This is expected because Val is more hydrophobic than Thr (Fig. 1). In contrast, the mutants are destabilized when the -OH group removed is hydrogen bonded in the wild-type protein. To get a more realistic estimate of the contribution of the hydrogen bonds to the $\Delta(\Delta G)$ values, we can correct approximately for the contribution of the difference in hydrophobicity as described in Table 1, and in more detail in Pace (1995). Note now that the $\Delta(\Delta G) = 2.0 \pm 0.7$ kcal/mol per hydrogen bond when the -OH groups are hydrogen bonded in the wild-type protein and 0.1 ± 0.2 kcal/mol when they are not. We conclude that the hydrogen bonds make a favorable contribution to the stability.

What if we consider the reverse mutation, Val ⇒ Thr? For the seven mutants in which the Thr is not hydrogen bonded in the mutant, there is a substantial decrease in the stability (Table 1). In contrast, when the -OH group of the Thr is able to form a hydrogen bond, the decrease in stability is reduced. Again, this suggests that hydrogen bonds make a favorable contribution to stability.

Now we consider the other four types of mutations shown in Fig. 1, where a polar group is removed. For these mutations, the mutants may be left with a cavity because the residue in the mutant is smaller than the residue in the wild-type protein. Results for 52 mutants are summarized in

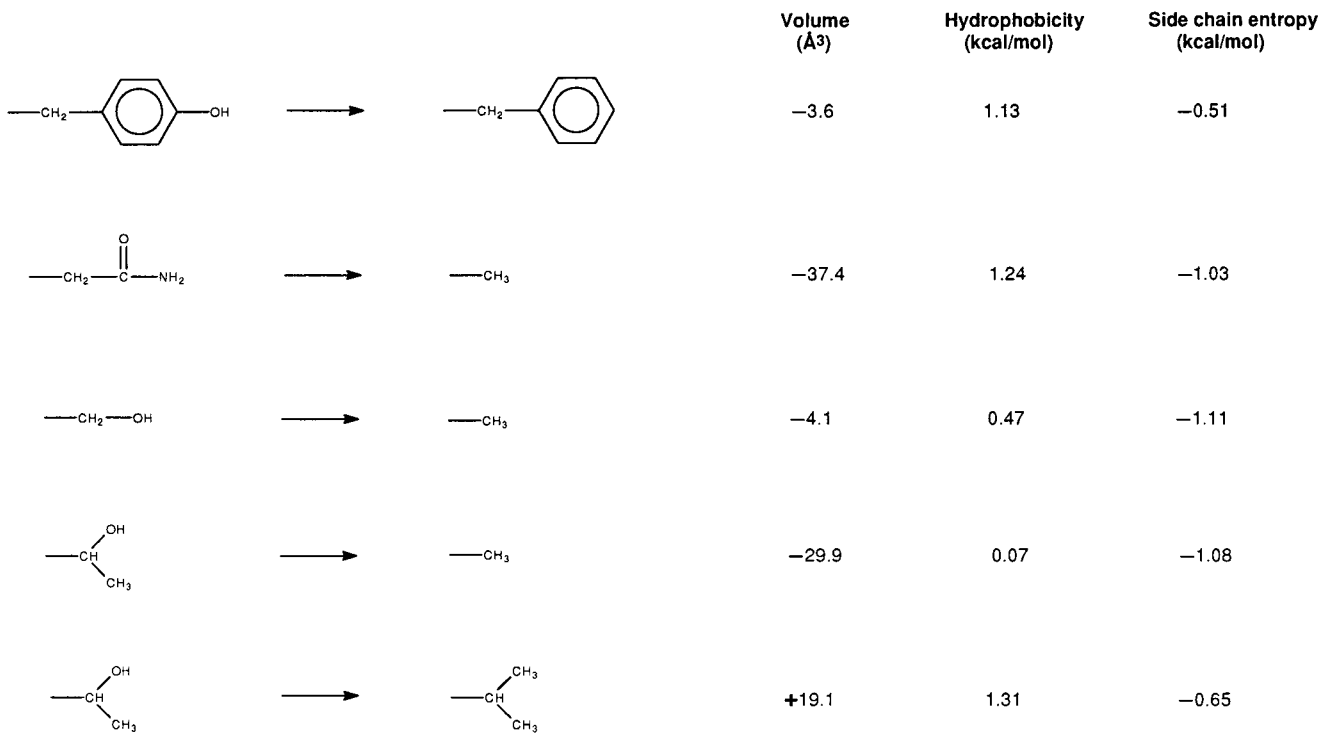

FIGURE 1 Differences in structure, volume, hydrophobicity, and side-chain conformational entropy ($T\Delta S$) for the following mutations: Tyr ⇒ Phe, Asn ⇒ Ala, Ser ⇒ Ala, Thr ⇒ Ala, and Thr ⇒ Val. The differences in residue volume are from Harpaz et al. (1994). The differences in hydrophobicity are from Pace (1995) and are based on the *n*-octanol hydrophobicity scale of Flauchere and Pliska (1983). The differences in conformational entropy are based on the mean $T\Delta S_{conf}$ values at 300 K given by Doig and Sternberg (1995).

Table 2. Note that the polar groups are on average 82% buried, and that the hydrophobicity of the residue in the mutant is always greater than that of the wild-type residue (Fig. 1). As a consequence, if only the hydrophobic effect were operative, we would expect the mutants to be more stable and the $\Delta(\Delta G)$ values to be negative.

In addition, because the cost of fixing the shorter side chains in the mutants will be less than that of fixing the longer wild-type side chains (Lee et al., 1994; Doig and Sternberg, 1995), the differences in conformational entropy will also lead to more stable mutants and negative $\Delta(\Delta G)$ values. (See, for example, the discussion by Hammen et al., 1995.) However, the mutants are less stable than the wild-type proteins by an average of 1.0 kcal/mol per hydrogen bond (Table 2), and if the information in Fig. 1 is used to correct for the differences in side chain hydrophobicity and conformational entropy, the average $\Delta(\Delta G) = 2.2$ kcal/mol per hydrogen bond. For 16 mutants of these same types where the polar group is not hydrogen bonded in wild-type protein, the average measured $\Delta(\Delta G) = 0.0 \pm 0.6$ kcal/mol. Non-hydrogen-bonded polar groups apparently contribute little to stability. The polar groups in these mutants are, however, only 33% buried on average, so they do not provide a direct comparison with the mutants in Table 2. Again, we think these data strongly support the idea that hydrogen bonds make a favorable contribution to protein stability.

The standard deviations in Table 2 are large. This is not surprising, because each mutation is unique and a variety of factors such as hydrogen bond type and geometry in the wild-type protein and the size of the cavity in the mutant will contribute to the measured $\Delta(\Delta G)$ values. We can make a rough estimate of the possible contribution of cavity size to the $\Delta(\Delta G)$ values. Based on studies of hydrophobic mutants, Eriksson et al. (1992) find that cavities contribute 24 cal/Å^3 to the $\Delta(\Delta G)$ values. The potential size of the cavities in the mutants ranges from 4 to 37 Å^3 (Fig. 1), which corresponds to a maximum contribution to the $\Delta(\Delta G)$ values of 0.1 to 0.9 kcal/mol. Applying the maximum correction would still leave the corrected $\Delta(\Delta G)$ values in Table 2 ranging from 1.6 to 2.2 kcal/mol. Cavity size probably contributes to the measured $\Delta(\Delta G)$ values. However, the fact that the corrected $\Delta(\Delta G)$ values for the four types of mutations in Table 2 do not differ significantly, even though two of them can leave much larger cavities than the other two, suggests that it is not a large contribution. We will postpone a discussion of another key question, i.e., what happens to the remaining member of the hydrogen bonding pair, until the next section.

Another experimental study shows convincingly that hydrogen bonding makes a favorable contribution to protein stability. Huyghues-Despointes et al. (1995) have measured the contribution of an $(i, i + 4)$ interaction between Gln and Asp to the stability of the α-helical form of a peptide. They use NMR to show that the Asp carboxyl group forms a specific hydrogen bond with the amide group of Gln in the helical peptide. This interaction stabilizes the α-helix by 0.4

TABLE 1 Δ(ΔG) values for Val ⇒ Thr and Thr ⇒ Val mutants

Mutants	% Buried*	Δ(ΔG) (kcal/mol)# Measured	Corrected
Thr ⇒ Val mutants			
Thr -OH not hydrogen bonded in wild type			
T13V§	0	0.13	0.13
T82V§	8	−0.27	−0.17
T33V§	47	−0.60	0.02
T41V§	100	−1.00	0.31
Thr -OH hydrogen bonded in wild-type			
8 mutants¶	90 ± 19	1.0 ± 0.7	2.0 ± 0.7
Val ⇒ Thr mutants			
Thr -OH not hydrogen bonded in mutant			
6 Mutants (Staph nuclease)§			2.5 ± 0.9
Val 149 ⇒ Thr (T4 lysozyme)‖			2.8
Thr -OH hydrogen bonded in mutant			
Val 75 ⇒ Thr (T4 lysozyme)‖			1.3
Val 87 ⇒ Thr (T4 lysozyme)‖			1.6

*Percentage buried for the -OH group was determined using the method of Lee and Richards (Richards, 1977), as implemented by Lesser and Rose (1990).
#Δ(ΔG) = ΔG(wild-type) − ΔG(mutant). ΔG is defined for unfolding so that positive Δ(ΔG) values show that the mutant is less stable than wild-type protein. The values are per hydrogen bond for groups that form more than one hydrogen bond. The measured values are from the references given below. The corrected value = measured value + (fraction -OH group buried) × 1.31. This is an approximate correction for the difference in hydrophobicity between the Val and Thr side chains (Fig. 1). No correction was made for differences in conformational entropy.
§Byrne et al. (1995).
¶Green et al. (1992); Byrne et al. (1995); Serrano et al. (1992); Matthews (1995).
‖Blaber et al. (1993).

TABLE 2 Δ(ΔG) values for 52 hydrogen bonding mutants

Mutant	Number	% Buried*	Δ(ΔG) (kcal/mol)# Measured	Corrected
Tyr ⇒ Phe	13	86 ± 19	0.6 ± 0.8	2.0 ± 0.9
Asn ⇒ Ala	18	80 ± 21	1.1 ± 0.8	2.3 ± 1.2
Ser ⇒ Ala	10	77 ± 25	1.1 ± 1.5	2.3 ± 1.6
Thr ⇒ Ala	11	79 ± 27	1.0 ± 0.9	2.1 ± 0.9
Total	52	81 ± 22	1.0 ± 1.0	2.2 ± 1.1

*The accessibility (% buried) of the polar group in the side chain in the wild-type protein was calculated using the method of Lee and Richards (Richards, 1977), as implemented by Lesser and Rose (1990).
#Δ(ΔG) = ΔG(wild-type) − ΔG(mutant). ΔG is defined for unfolding so that positive Δ(ΔG) values show that the mutant is less stable than the wild-type protein. The values are per hydrogen bond. The measured Δ(ΔG) values are from the following references: RNase T1 (Shirley et al., 1992); barnase (Serrano et al., 1992); staphylococcal nuclease (Green et al., 1992; Byrne et al., 1995) BPTI (Yu et al., 1995); arc repressor (Milla et al., 1994); T4 lysozyme (Matthews, 1995). The corrected Δ(ΔG) values = [measured value + (fraction polar group buried) × (difference in hydrophobicity given in Fig. 1) − (difference in $T\Delta S_{conf}$ given in Fig. 1)]/(number of hydrogen bonds removed by the mutation). This is an approximate correction for the difference in hydrophobicity and conformational entropy between the wild type and mutant side chains. The number of hydrogen bonds removed was determined using the program HBOND, kindly provided by L. Presta (Stickle et al., 1992).

kcal/mol when Asp is uncharged and by 1.0 kcal/mol when Asp is charged. Thus the free energy must be lower with the polar groups hydrogen bonded to each other rather than water, even though they are surrounded by 55 M water. They show also that Gln and Asp occur in protein helices spaced $(i, i + 4)$ far more frequently than would be expected by random occurrence, and that the majority of these are hydrogen bonded. This demonstrates that a hydrogen bonding interaction between side chains can contribute favorably to the stability of an α-helix and, therefore, of a protein. The free energy of this hydrogen bond is somewhat less than the protein hydrogen bonds in Table 1, but there are possible explanations for this. First, the side chains in an α-helix are more mobile than those in a folded protein, so fixing them in a geometry favorable for interaction will entail a greater loss in conformational entropy. Second, the lower dielectric constant of the protein interior should result in an enthalpically stronger bond than the solvent-exposed hydrogen bonds of side chains in an α-helix.

Other evidence from studies of the helix-to-coil transition of peptides supports a favorable contribution of backbone hydrogen bonding to stability. Munoz and Serrano (1995) developed an algorithm and parameter set that successfully predicts the helical content of 423 peptides. The best fit value for the contribution of backbone hydrogen bonds to α-helix stability was 0.8 kcal/mol per hydrogen bond. This is consistent with measurements indicating that ΔH for helix formation in a 50-residue peptide containing mainly alanine is about −1 kcal/mol per residue (Scholtz et al., 1991). Munoz and Serrano (1995) point out that "the final values of the parameters used do not significantly differ in absolute terms from those extracted from mutagenesis studies in proteins. This indicates that the same physico-chemical principles stand for both systems."

Many other recent studies of proteins (Cordes et al., 1996; Byrne et al., 1995; Yu et al., 1995; Makhatadze and Privalov, 1995; Hammen et al., 1995; Thorson et al., 1995; Marqusee and Sauer, 1994; Zhukovsky et al., 1994; Yamada et al., 1994; Kaarsholm et al., 1993) and peptides (Haberman and Murphy, 1996; Petukhov et al., 1996; Forood et al., 1993, 1994; Scholtz et al., 1993; Bruch et al., 1991) also suggest that polar groups in general and hydrogen bonding in particular contribute favorably to protein stability. In contrast, few recent experimental studies have suggested the converse (Eberhardt and Raines, 1994). There are unique difficulties, however, in interpreting studies of intermolecular hydrogen bond formation such as this one (see Dill, 1990, for a discussion).

THEORETICAL STUDIES OF HYDROGEN BONDING

Theoretical studies on the energetics of polyalanine α-helix formation led to the following conclusion (Yang and Honig, 1995): "Hydrogen bond formation is found to contribute

little to helix stability because the internal hydrogen bonding energy is largely canceled by the large free energy cost associated with removing polar groups from water." They estimate that dehydration of a peptide group costs 4.9 kcal/mol and that formation of a backbone hydrogen bond gains only 3.5 kcal/mol in the coil to α-helix transition. This may be true for their model α-helix, but we doubt that the desolvation of a peptide group in the folding of a protein will be this unfavorable. As noted above, for transfer of a peptide group from water to cyclohexane, ΔG_{tr} = 5.3 kcal/mol. Surely transfer from water to the interior of a protein will be more favorable because the packing will be better and interactions with other polar groups will be possible. For example, for a Val ⇒ Thr substitution, a $\Delta(\Delta G)$ = 6.6 kcal/mol is predicted based on ΔG_{tr} values measured in cyclohexane (Pace, 1995). However, $\Delta(\Delta G)$ is only 2.5 kcal/mol for the completely buried, non-hydrogen-bonded Val ⇒ Thr mutants in Table 1. Thus, the energetic cost of placing an -OH group at a location designed for a $-CH_3$ group is surprisingly low, and the results for the non-hydrogen-bonded Thr ⇒ Val mutants suggest that it is considerably less when the -OH group is placed at a location designed for an -OH group. These results may also reflect the fact that polar groups in an unfolded protein will be less accessible to solvent than those in the model compounds used to measure the ΔG_{tr} values or in the widely used models for the unfolded state (Creamer et al., 1995). This and other evidence (Byrne et al. 1995; Blaber et al., 1993; Hellinga et al., 1992; Dao-Pin et al., 1991) suggest that the cost of burying polar groups is less than previously thought, and considerably less than predicted from ΔG_{tr} values for cyclohexane. This idea is supported by theoretical calculations (see table 9 in Lazaridis et al., 1995).

With regard to mutational studies of hydrogen bonding such as those discussed above, Yang and Honig (1995) state: "The net effect of a hydrogen bond cannot be evaluated when only one member of a hydrogen bonding pair has been removed because one is then left with a protein with an unsatisfied buried polar group. Thus existing mutation experiments do not suggest that hydrogen bonds contribute to protein stability." As discussed previously (Shirley et al., 1992; Pace, 1995), there are three possible fates for the remaining polar group: 1) It may form a new intramolecular hydrogen bond, in which case $\Delta(\Delta G) \approx 0$ would be expected; 2) it may form an intermolecular hydrogen bond with water, in which case the impact of the remaining polar group will be minimized, so that the $\Delta(\Delta G)$ value will better reflect the cost of removing an intramolecular hydrogen bond; or 3) it may be left unpaired, so that a more positive $\Delta(\Delta G)$ would be expected. Honig and Yang (1995) assume the third possibility for all of the hydrogen bonding mutants, but this is certainly not the case. For example, in one of the first studies of hydrogen bonding using mutagenesis, Alber et al. (1987) show that the -OH group of Thr 157 is hydrogen bonded to the -OH group of Thr 155 and the >N-H group of Asp 159 in wild-type T4 lysozyme. In the Thr 157 ⇒ Gly mutant, a water replaces the -OH of Thr and forms the same two hydrogen bonds. Unfortunately, crystal structures are not available for most of the mutants, and we are left to guess at the hydrogen bonding status of the remaining partner. Even if the crystal structure is known, it is frequently difficult to assess the hydrogen bonding of a polar group to water. Some polar groups that are accessible to water often do not immobilize a water molecule sufficiently so that it is observed in the crystal structure (Savage et al., 1993; Levitt and Park, 1993; Karplus and Faerman, 1994). For example, Zhang and Matthews (1994) compared 10 different crystal forms of T4 lysozyme, including 18 crystallographically independent molecules, and found that no solvent binding site was occupied in all 18 lysozyme molecules. Failure to observe a water molecule near a polar group in a protein crystal structure does not necessarily indicate a lack of hydration.

Probably some of the mutants fall into each of the three categories noted above, and this is one reason for the large standard deviations in Table 2. The accessibilities of the polar groups included in Table 2 range from 24% to 100% buried (14 of 52 are 100% buried), with an average equal to 81% buried, and, as shown in Fig. 1, the potential cavity size in the mutants ranges from 4 to 37 $Å^3$. Surely the more accessible the polar group removed and the larger the potential cavity, the more likely the remaining polar group is to be hydrated. For the mutants in Table 2, the average corrected $\Delta(\Delta G)$ = 2.3 ± 1.1 kcal/mol for the 14 100% buried polar groups and 2.0 ± 1.2 kcal/mol for the 14 most exposed polar groups (24% to 70% buried). Thus, the corrected $\Delta(\Delta G)$ values do not depend to a great extent on the mutation considered or on the accessibility of the hydrogen bond to water. This suggests that cavity size and the hydrogen bonding status of the remaining polar group are not key factors contributing to the $\Delta(\Delta G)$ values. These observations may, in part, be another reflection of the fact that it is less costly to bury non-hydrogen-bonded polar groups than generally thought. It is certain that all of the hydrogen bonding mutants will not be left with an unpaired polar group in the mutant proteins, and when they are, the energetic cost is probably less than the calculations of Honig and Yang (1995) suggest. Consequently, we disagree with Honig and Yang and think that mutational studies do suggest that hydrogen bonds contribute to protein stability.

To avoid the problems of cavities and unpaired partners, Thorson et al. (1995) used a different approach. By substituting non-natural amino acids, they were able to replace one member of a hydrogen bonding pair with an isosteric group with weak hydrogen bonding character. Based on their results they concluded: "These results support the notion that intramolecular hydrogen bonds in folded proteins make a net favorable contribution of 1–2 kcal/mol to protein stability relative to the corresponding hydrogen bonds to solvent in the unfolded protein."

Our understanding of the hydration of proteins and its contribution to protein stability is still in its infancy. Experimentalists and theoreticians agree on this point. Lazaridis et al. (1995) state: "It is questionable whether a simple solvation model that provides accurate results can be found." Makhatadze and Privalov (1995) are more pessimistic:

"Water is a mysterious liquid. All attempts regarding its quantitative theoretical description have so far failed. As for the hydration effects, they are doubly mysterious." It is especially interesting that even the experimentalists cannot agree on whether hydrophobic cavities in proteins contain water or not (Buckle et al., 1996; Ernst et al., 1995; Matthews et al., 1995). The environment of water molecules in proteins can differ markedly. In RNase T1, one water molecule is completely buried, forms six hydrogen bonds, and has a low temperature factor (12.9 $Å^2$) (Malin et al., 1991). In contrast, a water molecule in a hydrophobic cavity (31 $Å^3$) of the Ile 76 $\Rightarrow$ Ala mutant of barnase is also completely buried, but forms only a single hydrogen bond, and has a high temperature factor (36.5 $Å^2$) (Buckle et al., 1996). Surely these two water molecules do not contribute equally to stability, but little is known about the contribution of hydration to the conformational stability of proteins. To learn more will require both experimental studies of hydration such as those of Connelly et al. (1994) and Bhat et al. (1994), and theoretical studies of hydration, such as those of Williams et al. (1994) and Zhang and Hermans (1996). Tanford (1980) wrote: "Life as we know it originated in water and could not exist in its absence." It is not surprising, then, that "mysterious" water is so important for both the stability and folding of proteins.

CONCLUSIONS

In 1987, Fersht reviewed mutational studies of the interaction of tRNA synthetase with its substrates and concluded that the net stabilization energy provided by uncharged hydrogen bonds was 0.5 to 1.8 kcal/mol. Most experimental studies since then have reached a similar conclusion. Some of the most convincing were discussed above. We conclude that hydrogen bonds stabilize proteins and that the average net stabilization is 1 to 2 kcal/mol per intramolecular hydrogen bond. We also find that water to cyclohexane ΔG_{tr} values overestimate the cost of burying polar groups in proteins. This probably results from the tighter packing of the groups in the protein interior, leading to favorable van der Waals interactions, and the less-than-full exposure of polar groups to solvent in unfolded proteins.

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REFERENCES

Alber, T., S. Dao-Pin, K. Wilson, J. A. Wozniak, S. P. Cook, and B. W. Matthews. 1987. Contributions of hydrogen bonds of Thr 157 to the thermodynamic stability of phage T4 lysozyme. *Nature.* 330:41–46.

Baker, E. N., and R. E. Hubbard. 1984. Hydrogen bonding in globular proteins. *Prog. Biophys. Mol. Biol.* 44:97–197.

Bhat, T. N., G. A. Bentley, G. Boulot, M. I. Greene, D. Tello, W. Dall'Acqua, H. Souchon, F. P. Schwartz, R. A. Mariuzza, and R. J. Poljak. 1994. Bound water molecules and conformational stabilization help mediate an antigen-antibody association. *Proc. Natl. Acad. Sci. USA.* 91:1089–1093.

Blaber, M., J. D. Lindstrom, N. Gassner, J. Xu, D. W. Heinz, and B. W. Matthews. 1993. The energetic cost and the structural consequences of burying a hydroxyl group within the core of a protein determined from Ala to Ser and Val to Thr substitutions in T4 lysozyme. *Biochemistry.* 32:11363–11373.

Bruch, M. D., M. M. Dhingra, and L. M. Gierasch. 1991. Side chain-backbone hydrogen bonding contributes to helix stability in peptides derived from an alpha-helical region of carboxypeptidase A. *Proteins Struct. Funct. Genet.* 10:130–139.

Buckle, A. M., P. Cramer, and A. R. Fersht. 1996. Structural and energetic responses to cavity-creating mutations in hydrophobic cores: observation of a buried water molecule and the hydrophilic nature of such hydrophobic cavities. *Biochemistry.* 35:4298–4305

Byrne, M. P., R. L. Manuel, L. G. Lowe, and W. E. Stites. 1995. Energetic contribution of side chain hydrogen bonding to the stability of staphylococcal nuclease. *Biochemistry.* 34:13949–13960.

Connelly, P. R., R. A. Aldape, F. J. Bruzzese, S. P. Chambers, M. J. Fitzgibbon, M. A. Fleming, S. Itah, D. J. Livingston, M. A. Navia, J. A. Thomson, and K. P. Wilson. 1994. Enthalpy of hydrogen bond formation in a protein-ligand binding reaction. *Proc. Natl. Acad. Sci. USA.* 91: 1964–1968.

Cordes, M. H. J., A. R. Davidson, and R. T. Sauer. 1996. Sequence space, folding and protein design. *Curr. Opin. Struct. Biol.* 6:3–10.

Creamer, T. P., R. Srinivasan, and G. D. Rose. 1995. Modeling unfolded states of peptides and proteins. *Biochemistry.* 34:16245–16250.

Dao-Pin, S., D. E. Anderson, W. A. Baase, F. W. Dahlquist, and B. W. Matthews. 1991. Structural and thermodynamic consequences of burying a charged residue within the hydrophobic core of T4 lysozyme. *Biochemistry.* 30:11521–11529.

Dill, K. A. 1990. Dominant forces in protein folding. *Biochemistry.* 29: 7133–7155.

Doig, A. J., and M. J. E. Sternberg. 1995. Side-chain conformational entropy in protein folding. *Protein Sci.* 4:2247–2251.

Eberhardt, E. S., and R. T. Raines. 1994. Amide-amide and amide-water hydrogen bonds: implications for protein folding and stability. *J. Am. Chem. Soc.* 116:2149–2150.

Eriksson, A. E., W. A. Baase, X-J. Zhang, D. W. Heinz, M. Blaber, E. P. Baldwin, and B. W. Matthews. 1992. The response of a protein structure to cavity-creating mutations and its relationship to the hydrophobic effect. *Science.* 255:178–183.

Ernst, J. A., R. T. Clubb, H-X. Zhou, A. M. Gronenborn, and G. M. Clore. 1995. Demonstration of positionally disordered water within a protein hydrophobic cavity by NMR. *Science.* 267:1813–1817.

Fersht, A. R. 1987. The hydrogen bond in molecular recognition. *Trends Biochem. Sci.* 12:301–304.

Fersht, A. R., and L. Serrano. 1993. Principles of protein stability derived from protein engineering experiments. *Curr. Opin. Struct. Biol.* 3:75–83.

Flauchere, J. L., and V. E. Pliska. 1983. Hydrophobic parameters of amino-acid side chains from the partitioning of *N*-acetyl-amino-acid amides. *Eur. J. Med. Chem.* 18:369–375.

Forood, B., E. J. Feliciano, and K. P. Nambiar. 1993. Stabilization of alpha helical structures in short peptides via end capping. *Proc. Natl. Acad. Sci. USA.* 90:838–842.

Forood, B., H. K. Reddy, and K. P. Nambiar. 1994. Extraordinary helicity in short peptides via end capping design. *J. Am. Chem. Soc.* 116: 6935–6936.

Green, S. M., A. K. Meeker, and D. Shortle. 1992. Contributions of the polar, uncharged amino acids to the stability of staphylococcal nuclease: evidence for mutational effects on the free energy of the denatured state. *Biochemistry.* 31:5717–5728.

Haberman, S. M., and K. P. Murphy. 1996. Energetics of hydrogen bonding in proteins: a model compound study. *Protein Sci.* 5:1229–1239.

Hammen, P. K., J. M. Scholtz, J. W. Anderson, E. B. Waygood, and R. E. Klevit. 1995. Investigation of a side-chain–side-chain hydrogen bond by

mutagenesis, thermodynamics, and NMR spectroscopy. *Protein Sci.* 4:936–944.

Harpaz, Y., M. Gerstein, and C. Chothia. 1994. Volume changes in protein folding. *Structure.* 2:641–649.

Hellinga, H. W., R. Wynn, and F. M. Richards. 1992. The hydrophobic core of *Escherichia coli* thioredoxin shows a high tolerance to nonconservative single amino acid substitutions. *Biochemistry.* 31:11203–11209.

Honig, B., and A. S. Yang. 1995. Free energy balance in protein folding. *Adv. Protein Chem.* 46:27–58.

Huyghues-Despointes, B. M. P., T. M. Klingler, and R. L. Baldwin. 1995. Measuring the strength of side-chain hydrogen bonds in peptide helices: the Gln-Asp (i, i + 4) interaction. *Biochemistry.* 34:13267–13271.

Kaarsholm, N. C., K. Norris, R. J. Jorgensen, J. Mikkelsen, S. Ludvigsen, O. H. Olsen, A. R. Sorensen, and S. Havelund. 1993. Engineering stability of the insulin monomer fold with application to structure-activity relationships. *Biochemistry.* 32:10773–10778.

Karplus, A. P., and C. Faerman. 1994. Ordered water in macromolecular structure. *Curr. Opin. Struct. Biol.* 4:770–776.

Kauzmann, W. 1959. Some factors in the interpretation of protein denaturation. *Adv. Protein Chem.* 14:1–63.

Klapper, M. H. 1971. On the nature of the protein interior. *Biochim. Biophys. Acta.* 229:557–566.

Kyte, J. 1995. Structure in Protein Chemistry. Garland Publishing, New York and London.

Lazaridis, T., G. Archontis, and M. Karplus. 1995. Enthalpic contribution to protein stability: insights from atom-based calculations and statistical mechanics. *Adv. Protein Chem.* 47:231–306.

Lee, K. H., D. Xie, E. Freire, and L. M. Amzel. 1994. Estimation of changes in side-chain configurational entropy in binding and folding—general methods and application to helix formation. *Proteins Struct. Funct. Genet.* 20:68–84.

Lesser, G. J., and G. D. Rose. 1990. Hydrophobicity of amino acid subgroups in proteins. *Proteins Struct. Funct. Genet.* 8:6–13.

Levitt, M., and B. H. Park. 1993. Water: now you see it, now you don't. *Structure.* 1:223–226.

Lin, T-Y., and S. N. Timasheff. 1994. Why do some organisms use a urea-methylamine mixture as osmolyte? Thermodynamic compensation of urea and trimethylamine N-oxide interactions with protein. *Biochemistry.* 33:12695–12701.

Makhatadze, G. I., and P. L. Privalov. 1995. Energetics of protein structure. *Adv. Protein Chem.* 47:307–425.

Malin, R., P. Zielenkiewicz, and W. Saenger. 1991. Structurally conserved water molecules in ribonuclease T1. *J. Biol. Chem.* 266:4848–4852.

Marqusee, S., and R. T. Sauer. 1994. Contributions of a hydrogen bond/salt bridge network to the stability of secondary and tertiary structure in λ repressor. *Protein Sci.* 3:2217–2225.

Matthews, B. W. 1995. Studies on protein stability with T4 lysozyme. *Adv. Protein Chem.* 46:249–278.

Matthews, B. W., A. G. Morton, and F. W. Dahlquist. 1995. Technical comments: use of NMR to detect water within nonpolar protein cavities (response by J. A. Ernst, R. T. Clubb, H-X. Zhou, A. M. Gronenborn and G. M. Clore). *Science.* 270:1847–1849.

McDonald, I. K., and J. M. Thornton. 1994. Satisfying hydrogen bonding potential in proteins. *J. Mol. Biol.* 238:777–793.

Milla, M. E., B. M. Brown, and R. T. Sauer. 1994. Protein stability effects of a complete set of alanine substitutions in arc repressor. *Nature Struct. Biol.* 1:518–523.

Mitchell, J. B. O., and S. L. Price. 1990. The nature of the N-H-O-C hydrogen bond: an intermolecular perturbation theory study of the formamide/formaldehyde complex. *J. Comp. Chem.* 11:1217–1233.

Munoz, V., and L. Serrano. 1995. Elucidating the folding problem of helical peptides using empirical parameters. II. Helix macrodipole effects and rational modification of the helical content of natural peptides. *J. Mol. Biol.* 245:275–296.

Pace, C. N. 1992. Contribution of the hydrophobic effect to globular protein stability. *J. Mol. Biol.* 226:29–35.

Pace, C. N. 1995. Evaluating contribution of hydrogen bonding and hydrophobic bonding to protein folding. *Methods Enzymol.* 259:538–554.

Pace, C. N., B. A. Shirley, M. McNutt, and K. Gajiwala. 1996. Forces contributing to the conformational stability of proteins. *FASEB J.* 10:75–83.

Petukhov, M., N. Yumoto, S. Murase, R. Onmura, and S. Yoshikawa. 1996. Factors that affect the stabilization of α-helices in short peptides by a capping box. *Biochemistry.* 35:387–397.

Richards, F. M. 1977. Areas, volumes, packing, and protein structure. *Annu. Rev. Biophys. Bioeng.* 6:151–176.

Rose, G. D., and R. Wolfenden. 1993. Hydrogen bonding, hydrophobicity, packing, and protein folding. *Annu. Rev. Biophys. Biomol. Struct.* 22: 381–415.

Roseman, M. A. 1988. Hydrophobicity of the peptide C=O···H-N hydrogen bonded group. *J. Mol. Biol.* 201:621–623.

Savage, H. J., C. J. Elliott, C. M. Freeman, and J. L. Finney. 1993. Lost hydrogen bonds and buried surface area: rationalising stability in globular proteins. *J. Chem. Soc. Faraday Trans.* 89:2609–2617.

Scholtz, J. M., S. Marqusee, R. L. Baldwin, E. J. York, J. M. Stewart, M. Santoro, and D. W. Bolen. 1991. Calorimetric determination of the enthalpy change for the α-helix to coil transition of an alanine peptide in water. *Proc. Natl. Acad. Sci. USA.* 88:2854–2858.

Scholtz, J. M., H. Qian, V. H. Robbins, and R. L. Baldwin. 1993. The energetics of ion-pair and hydrogen-bonding interactions in a helical peptide. *Biochemistry.* 32:9668–9676.

Serrano, L., J. T. Kellis, P. C. A. Matouschek, and A. R. Fersht. 1992. The folding of an enzyme. II. Substructure of barnase and the contribution of different interactions to protein stability. *J. Mol. Biol.* 224:783–804.

Shirley, B. A., R. Stanssens, U. Hahn, and C. N. Pace. 1992. Contribution of hydrogen bonding to the conformational stability of ribonuclease T1. *Biochemistry.* 31:725–732.

Shortle, D. 1996. The denatured state (the other half of the folding equation) and its role in protein stability. *FASEB J.* 10:27–34.

Stickle, D. F., L. G. Presta, K. A. Dill, and G. D. Rose. 1992. Hydrogen bonding in globular proteins. *J. Mol. Biol.* 226:1143–1159.

Tanford, C. 1962. Contribution of hydrophobic interactions to the stability of the globular conformation of proteins. *J. Am. Chem. Soc.* 84: 4240–4247.

Tanford, C. 1980. The Hydrophobic Effect: Formation of Micelles and Biological Membranes. John Wiley and Sons, New York.

Thorson, J. S., E. Chapman, and P. G. Schultz. 1995. Analysis of hydrogen bonding strengths in proteins using unnatural amino acids. *J. Am. Chem. Soc.* 117:9361–9362.

Tobias, D. J., S. F. Sneddon, and C. L. Brooks III. 1992. Stability of a model β-sheet in water. *J. Mol. Biol.* 227:1244–1252.

Voet, D., and J. G. Voet. 1995. Biochemistry. John Wiley and Sons, New York.

Williams, M. A., J. M. Goodfellow, and J. M. Thornton. 1994. Buried waters and internal cavities in monomeric proteins. *Protein Sci.* 3:1224–1235.

Wimley, W. C., T. P. Creamer, and S. H. White. 1996. Solvation energies of amino acid sidechains and backbone in a family of host-guest pentapeptides. *Biochemistry.* 35:5109–5124.

Yamada, H., E. Kanaya, Y. Ueno, M. Ikehara, H. Nakamura, and M. Kikkuchi. 1994. Contribution of a hydrogen bond to the thermal stability of the mutant human lysozyme C77/95S. *Biol. Pharm. Bull.* 17:612–616.

Yang, A. S., and B. Honig. 1995. Free energy determinants of secondary structure formation. I. Alpha helices. *J. Mol. Biol.* 252:351–365.

Yu, M. H., J. S. Weissman, and P. S. Kim. 1995. Contribution of individual side-chains to the stability of BPTI examined by alanine-scanning mutagenesis. *J. Mol. Biol.* 249:388–397.

Zhang, L., and J. Hermans. 1996. Hydrophobicity of cavities in proteins. *Proteins Struct. Funct. Genet.* 24:433–438.

Zhang, X-J., and B. W. Matthews. 1994. Conservation of solvent-binding sites in 10 crystal forms of T4 lysozyme. *Protein Sci.* 3:1031–1039.

Zhukovsky, E. A., M. G. Mulkerrin, and L. G. Presta. 1994. Contribution to global protein stabilizaton of the N-capping box in human growth hormone. *Biochemistry.* 33:9856–9864.