FOR THE RECORD

Do all backbone polar groups in proteins form hydrogen bonds?

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Abstract

Evidence from proteins and peptides supports the conclusion that intrapeptide hydrogen bonds stabilize the folded form of proteins. Paradoxically, evidence from small molecules supports the opposite conclusion, that intrapeptide hydrogen bonds are less favorable than peptide–water hydrogen bonds. A related issue—often lost in this debate about comparing peptide–peptide to peptide–water hydrogen bonds—involves the energetic cost of an unsatisfied hydrogen bond. Here, experiment and theory agree that breaking a hydrogen bond costs between 5 and 6 kcal/mol. Accordingly, the likelihood of finding an unsatisfied hydrogen bond in a protein is insignificant. This realization establishes a powerful rule for evaluating protein conformations.

Keywords: protein hydrogen bonds; protein stability; hydrogen bond satisfaction; protein folding

The contribution that hydrogen bonds make to protein stability has been an ongoing topic of research since Pauling's seminal models of protein secondary structure (Pauling and Corey 1951; Pauling et al. 1951). Energetic questions about hydrogen-bonding in proteins are usually formulated in terms of a comparison between peptide–water and peptide–peptide hydrogen bonds. Here we examine a related question: Are all hydrogen bond donors and acceptors in proteins satisfied? This question prompted us to reanalyze earlier data and to suggest a hydrogen bonding hypothesis.

In their description of the α-helix, Pauling et al. (1951) asserted that the energy of the peptide N–H···O=C hydrogen bond was of order −8 kcal/mol, and that "such instability would result from the failure to form these bonds that we may be confident of their presence." Pauling's earlier estimate of the total protein hydrogen bond energy was −5 kcal/mol (Mirsky and Pauling 1936). From solution studies of urea dimers, Schellman estimated that an intrapeptide hydrogen bond would be enthalpically favored over a peptide–water hydrogen bond by ∼1.5 kcal/mol (Schellman 1955). These and similar early studies led to the conclusion that the peptide hydrogen bond is a significant factor in stabilizing protein conformations.

This view was to change dramatically following a famous review by Kauzmann (1959), who invoked the thermodynamics of small model compounds to argue that stabilization of the folded state of a protein is due almost exclusively to the hydrophobic effect. Soon after Kauzmann's proposal, Klotz and Franzen (1962) determined that the enthalpy of the interamide hydrogen bond of N-methyl acetamide in water was zero, and concluded that "the intrinsic stability of interpeptide hydrogen bonds in aqueous solution is small." Similarly, hydrogen bonding involving another small molecule, ε-caprolactam, in dilute solution was shown to be negligible (Susi and Ard 1969). Kauzmann's proposal, bolstered by these later studies, led to the widely held view that the hydrophobic effect makes the major energetic contribution to protein stability, with hydrogen bonds contributing little, or perhaps even opposing, the folding process. See Baldwin (2003) for a recent discussion of these issues.

The accumulation of high-resolution X-ray crystal structures of proteins in the 1980s prompted several

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major surveys of hydrogen bonding in proteins (Baker and Hubbard 1984; Stickle et al. 1992; Savage et al. 1993; McDonald and Thornton 1994). These studies concur in finding that most buried polar groups (~90%) in globular proteins are hydrogen-bonded and that most intrapeptide hydrogen bonds are within elements of secondary structure: α-helices, β-sheets, and β-turns.

Concurrent with these surveys, Scholtz et al. (1991) determined that the enthalpy of helix formation for polyalanine in water is favorable by ~1 kcal/mol/hydrogen bond, measured using calorimetry, and this value can be further enhanced by burial and dehydration (Baldwin and Rose 1999; Fernandez et al. 2002). Similar helix formation enthalpies of peptides containing different amino acid residues have recently been reported (Richardson et al. 2005). Makhatadze and Privalov (1993) estimated that the enthalpy of an intrapeptide hydrogen bond buried in the protein interior could be as large as −12 kcal/mol (see also Fig. 1 in Rose and Wolfenden 1993).

The observed ubiquity of intrapeptide hydrogen bonds in X-ray structures and the experimental demonstration of their favorable enthalpy of formation in peptides and proteins are consistent with the view that intrapeptide hydrogen bonds are favored over peptide–water hydrogen bonds. In contrast to this view, Honig and colleagues (BenTal et al. 1997) used finite difference Poisson-Boltzmann methods to calculate the energetics of hydrogen bonding of N-methyl acetamide in water and organic solvent. A thermodynamic cycle using these energy values indicates that the formation of N–H···O=C in water and subsequent transfer to a nonpolar solvent is disfavored by several kcal/mol (cf. Fig. 1 in BenTal et al. 1997). The investigators concluded "that the formation and burial of a hydrogen bond opposes protein folding" (BenTal et al. 1997). These conflicting ideas provoked controversy over whether or not such bonds contribute to overall protein stability (Fersht and Serrano 1993; Honig and Yang 1995; Lazaridis et al. 1995; BenTal et al. 1997).

Adding fuel to the fire, Myers and Pace used experimentally determined free energy differences from numerous single-residue polar to apolar mutations to argue that "hydrogen bonds stabilize proteins and that the average net stabilization is −1 to −2 kcal/mol per intramolecular hydrogen bond" (Myers and Pace 1996) with buried residues contributing as much as −3.5 kcal/mol (Shirley et al. 1992). Their conclusion was later corroborated in a study of lysozyme mutants (Takano et al. 1999).

Summarizing this ongoing discussion, the weight of present evidence from peptides and proteins favors the conclusion that an intrapeptide hydrogen bond stabilizes a protein by 1–2 kcal/mol. However, this conclusion has yet to be reconciled with small molecule experiments and calculations, perhaps owing to failure of group additivity (Roseman 1988; Avbelj et al. 2000).

Hydrogen bond satisfaction

An important realization is often overlooked in this comparison between protein–protein and protein–water hydrogen-bonding energy: An "unsatisfied" hydrogen bond donor or acceptor in the interior of a protein will destabilize a protein far more than 1–2 kcal/mol. Both experimental and theoretical studies concur that the enthalpic cost of breaking a hydrogen bond is at least 5–6 kcal/mol (Kresheck and Klotz 1969; Mitchell and Price 1990; 1991; Makhatadze and Privalov 1993; BenTal et al. 1997; Sheu et al. 2003).

Accordingly, unsatisfied buried polar groups are unlikely. The expected Boltzmann-weighted frequency of occurrence of an unsatisfied hydrogen bond can be estimated as

$$P_u = e^{\frac{-\Delta E_{hb}}{RT}} \quad (1)$$

where P_u is the probability of an unsatisfied hydrogen bond conformation relative to the probability of a comparable conformation with hydrogen bond satisfaction, ΔE_{hb} is the energy of a hydrogen bond (~ -5 kcal/mol), R is the gas constant, and T is the temperature. From this very approximate estimate, a conformation with an unsatisfied hydrogen bond donor or acceptor would have a relative probability (P_u) of ~0.02% at room temperature. Although in principle it is conceivable that the local energy penalty of an unsatisfied hydrogen bond could be compensated by the global system energy, this trade-off seems unlikely because proteins are energy mimimized locally (Butterfoss and Hermans 2003). In fact, proteins that do experience energetic deviations of this magnitude would be expected to be partially or even wholly unfolded, as shown in those cases that were studied (Rumbley et al. 2001).

Hydrogen-bonding hypothesis

We hypothesize that *all potential hydrogen bond donors and acceptors in proteins are satisfied a significant fraction of the time, either by intramolecular hydrogen bonds or by hydrogen bonds to solvent water*. In particular, the energetic cost of an unsatisfied, buried hydrogen bond is so steep that the population of nonhydrogen-bonded polar groups in the protein interior will be negligible. Here we define hydrogen bond satisfaction to mean that each donor and acceptor will have at least one hydrogen bond. A carbonyl oxygen may accept two hydrogen bonds, each directed to a lone pair of electrons, and both bonds are assumed to form with water in a fully solvated peptide group. One of these two respective hydrogen bonds is lost upon protein folding for a significant number of protein oxygen acceptors, a result previously referred to as lost hydrogen bonds (Savage et al.

1993). In small molecule crystals both single and double hydrogen bonded sp2 oxygens are frequently observed; completely unsatisfied oxygens are only very rarely observed (Taylor and Kennard 1984). In the work reported here, oxygen atoms with only one hydrogen bond are considered to be satisfied.

The hydrogen-bonding hypothesis implies that polypeptide conformations which are incompatible with complete hydrogen bond satisfaction would not contribute significantly to the population. This hypothesis can provide a powerful criterion for filtering calculated protein and peptide structures.

Reconciliation of hydrogen bond satisfaction with database surveys

If the Protein Data Bank (PDB) represents an equilibrium distribution of protein conformations (Shortle 2003), one would expect to find approximately one unsatisfied hydrogen bond per every 5000 polar groups (0.02%), not one in 10 as found in previous surveys (McDonald and Thornton 1994). To test our hypothesis, we analyzed the subset of "highly unsatisfied" protein main-chain donors and acceptors from McDonald and Thornton (1994) for which structure factors are available and electron density maps can be calculated (Kleywegt et al. 2004). In every instance, the apparent lack of satisfaction could be rationalized (Table 1). Reasons vary: Often, the group in question is occluded by a side chain with low electron density, where an alternate side-chain rotamer allows solvent access. In some cases, another experimentally indistinguishable side-chain rotamer of GLN, ASP, or THR would have provided a hydrogen bond partner for the atom in question. In several cases, a crystallographic water is situated nearby, although accessibility calculations score the donor/acceptor atom as solvent-inaccessible (see Savage et al. 1993 and Sadasivan et al. 1998).

Inspection of individual static X-ray crystallographic structures, even when combined with electron density maps, may be inadequate to evaluate hydrogen bond satisfaction. For example, the ribonuclease structure 7rsa from the PDB (Berman et al. 2000) has an unsatisfied main-chain N–H donor in residue GLU49. However, comparison of 17 available, independent X-ray crystal structures of this same molecule offers a more complete picture of how this ostensibly unsatisfied group may, in fact, interact with water. In four structures, the group is buried and unsatisfied, but it is solvent-accessible in the remaining 13 structures. The unsatisfied examples are 1fs3, 4rat, 7rsa, and 8rat; satisfied are 1bel, 1rat, 1rbx, 1rha, 1rhb, 2rat, 3rat, 3rn3, 5rat, 5rsa, 6rat, 7rat, and 9rat. This variation in solvent accessibilities for the same molecule, as represented by multiple, independent crystal structures, can be interpreted to mean that in solution, conformational variation would permit water access to the group in question a significant fraction of time. Indeed, the actual conformational fluctuations experienced by the protein in solution are likely to be even larger than those represented by a population of crystal structures, and therefore, this group should probably not be classified as unsatisfied.

It follows from these observations that most unsatisfied hydrogen bond donors and acceptors seen in database surveys are artifacts that arise from limitations in identifying hydrogen bonds by applying geometric criteria to static structures. In general, instances in which main-chain polar groups ostensibly lack hydrogen bond partners are an unavoidable consequence of basing the analysis on a time-averaged crystal structure.

Hydrogen bond satisfaction and simulations

Given the high energetic cost of an unsatisfied main-chain polar group, almost all such groups would be expected to participate in hydrogen bonds in the conformational microstates modeled by simulations. Molecular dynamics studies, which simulate time-dependent trajectories for individual molecules, should be useful for quantifying this prediction but, in fact, have provided contradictory results.

We find that molecular dynamics trajectories of small globular proteins in explicit solvent using the CHARMM force field result in conformations with *more* unsatisfied main-chain donors and acceptors than the respective crystal structures in the majority of the conformations sampled during simulation. Two simulations were performed at 300 K: one using the CHARMM22 force field, isobaric periodic boundary conditions (Berendsen et al. 1984), with a primitive cell of $62 \times 62 \times 62$ Å, particle-mesh Ewald electrostatics (Darden et al. 1993), and the program NAMD (Kale et al. 1999). Bonds to hydrogen atoms were constrained with the SHAKE algorithm (Rychaert et al. 1977). The second simulation was with the CHARMM27 (MacKerell et al. 1998) force field, spherical water solvation with at least five layers of water surrounding the protein and Coulombic electrostatics with a dielectric constant of 1 and the program CHARMM (Brooks et al. 1983). Both simulations included the TIP3P water model (Jorgensen et al. 1983). After minimization, heating, and equilibration, configurations were sampled every 0.1 psec for 100 psec. Hydrogen bond satisfaction was calculated with HBPLUS using the relaxed criteria described by McDonald and Thornton to obtain "highly unsatisfied" donors and acceptors (McDonald and Thornton 1994), except that alternative ASN, GLN, and HIS orientations were explored. Explicit water was

Table 1. *Reconciling apparent main-chain donor/acceptor lack of satisfaction from the database*

PDB code[a]	Donor/acceptor	Rationalization[b]
1ake	PRO9A O	Occluding side chain (ILE116) has poor density[c]
	GLN28A N	Potential MET21 SD bond[d]
	LYS157A N	Occluding side chain (LYS157) has poor density[c]
1cob	ASP81A O	Potential ARG77 NH1/NH2 bond[d]
	ASN137A N	Potential THR135 OG1 bond[d]
1snc	LEU108 O	Potential THR41 OG1 bond[e]
1ubq	ILE36 N	Potential ILE30 O bond (4.26 Å)[e]
2aza	CYS3A N	Occluding side chain (GLN2) has poor density[c]
	GLU4A N	Occluding side chain (GLN2) has poor density[c]
2cyp	TYR71 O	Occluding side chain (PHE77) has poor density[c]
	GLN86 N	Occluding side chain (GLN86) has poor density[c]
	PHE91 O	Occluding side chain (LYS29) has poor density[c]
	PRO122 O	Potential GLN117 OE1/NE2 bond[d]
	LEU161 O	Potential ASN272 OD1/ND2 bond[d]
	GLU188 N	Potential GLN222 OE1/NE2 bond[d]
	LEU238 O	Potential LEU245 N bond[e]
2hmz	VAL21A N	Occluding side chain (ILE20) has poor density[c]
	HIS43A N	Potential ASN40 OD1/ND2 bond[d]
2scp	ASN85A N	Potential MET82 O bond (4.06 Å)[e]
4bp2	ASN71 O	Potential GLN4 OE1/NE2 bond[d]
5cyt	VAL20R O	Potential HOH22 bond
6xia	TRP15 N	Potential ASN56 OD1/OD2 bond[d]
	GLY21 N	Potential TRP19 ring bond, potential HOH6 bond
	PHE93 N	Potential ASN91 OD1/ND2 bond[d]
	GLY138 O	Potential ARG187 N bond (4.05 Å)[e]
	LYS148 N	Potential GLY145 O bond (4.01 Å)[e]
	ARG187 N	Potential GLY138 O bond (see above)
	ASN214 O	Potential GLU180 OE1/OE2 bond[d]

[a] PDB identification code for structural models identified as having unsatisfied main-chain hydrogen bond donors and acceptors (McDonald and Thornton 1994) and for which satisfactory electron density maps could be calculated by the Uppsala electron density server (Kleywegt et al. 2004).
[b] The apparently unsatisfied donor or acceptor was classified as satisfied upon inspection of the PDB structure together with solvent molecules and the electron density map. In each case an explanation is noted and further described by one of the respective footnotes below.
[c] The side chain of a residue occluding the hydrogen bond donor or acceptor is in a region of poor electron density, and an alternate rotamer would allow solvent access to the unsatisfied group.
[d] A different rotamer of the occluding side chain would enable a potential intrapeptide hydrogen bond.
[e] Distance or orientation between N and O is slightly outside threshold criteria, but these atoms would interact weakly nevertheless.

included in the analysis; both buried and accessible unsatisfied groups are found and included in the analysis.

The distributions of unsatisfied hydrogen bonds during equilibrated simulations of an 82-residue globular protein are shown in Figure 1; similar results are obtained with other proteins. Two sets of simulation conditions were investigated, and they differed in solvation systems, versions of the force field, and methods of electrostatic calculations. Both give rise to two or more unsatisfied main chain donors or acceptors in 90% of the conformations during the trajectory.

In fact, molecular mechanics force fields are known to be deficient at reproducing a satisfactory hydrogen-bonding potential with regard to orientation parameters (Lii and Allinger 1998; Grzybowski et al. 2000; Fabiola et al. 2002; Morozov et al. 2004), although the hydrogen bond electrostatic interaction energy for partial charges calculated using the CHARMM force field is consistent with a value of −5 kcal/mol (Grzybowski et al. 2000; Buck and Karplus 2001; Morozov et al. 2004). The nature of hydrogen bonds formed during molecular dynamics simulations has been previously characterized (Buck and Karplus 2001), but the preliminary results in Figure 1 suggest the need for a more comprehensive investigation into this topic.

Hydrogen bond satisfaction and the unfolded state of proteins

The hydrogen-bonding hypothesis can be used as a powerful criterion to filter unlikely conformations in

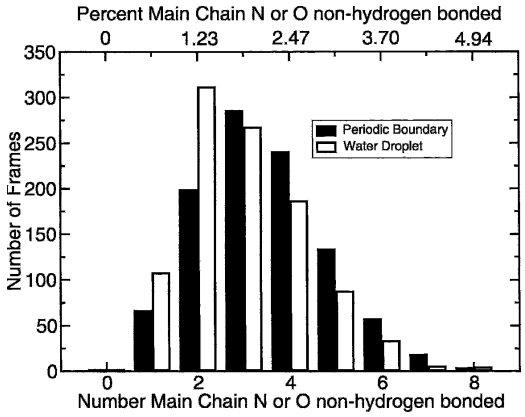

Figure 1. Unsatisfied main chain hydrogen bonds during molecular dynamics simulations. Data are shown for molecular dynamics simulations of the 82 residue syntenin PDZ2 domain (1r6j) (162 main chain donors and acceptors and two PRO residues). The X-ray crystal structure of this protein (Kang et al. 2004) has one internal main chain unsatisfied hydrogen bond donor, LEU233 N.

the unfolded state, as demonstrated by a simple computational experiment. Adopting penta-alanine as a model system (Hummer et al. 2001; Margulis et al. 2002; Mu et al. 2005), we generated a population of sterically allowed structures of Ala_5 using Monte Carlo backbone torsion angle sampling; these conformations were scored for hydrogen bond satisfaction. In this exercise, the entire backbone torsional space ($-180 \leq \phi,\psi \leq +180$) was sampled at random for each residue, resulting in 51,727 sterically allowed peptide conformations in 10^7 attempts. Intrapeptide hydrogen bonds were identified using criteria similar to those described by Kortemme et al. (2003) ($\psi \geq 90°$, $\theta \geq 110°$, O–N distance $\leq$4.5 Å), while peptide-solvent hydrogen bonds were identified by probing five different positions within the cone of approach around either the N–H or C=O vectors with a pseudo-water oxygen atom, as described previously (Fleming et al. 2005). The peptide was classified as hydrogen-bonded to water when a water oxygen was sterically allowed in an orientation compatible with hydrogen bonding. This latter method can discriminate between conformations that can form strong hydrogen bonds with water and those that cannot, regardless of the possible presence of accessible N–H or C=O surface. Thus, our method is more stringent than the criteria of McDonald and Thornton (1994), where any accessible surface was scored as a successful solvent–peptide hydrogen bond. More stringent criteria are appropriate when culling unlikely structures from simulations, where atom positions do not represent averages, as they do in crystal structures. Of the 51,727 sterically allowed penta-alanine conformers, 28,558 were found to be hydrogen-bond satisfied, i.e., 45% of the sterically allowed population could be rejected as energetically infeasible. The absolute rejection rate will depend on polypeptide chain length, but hydrogen-bond satisfaction is a useful metric of energetic feasibility at any chain length.

We have focused on satisfaction of main chain polar groups, ignoring side chain donors and acceptors. However, the argument can be extended to side chain groups as well. Hydrogen bond satisfaction can also be an effective criterion for the evaluation of protein structures determined by NMR (Lipsitz et al. 2002) and X-ray crystallography (Savage et al. 1993; Hooft et al. 1996; Fabiola et al. 2002) in addition to its use in assessing the unfolded population (Lindorff-Larsen et al. 2004).

Summary

Protein hydrogen bonds are ubiquitous, directional, and largely local, partitioning the polypeptide chain into α- and 3_{10}-helices, β-sheet, and β-turns. Together, these hydrogen-bonded backbone structures account for at least 75% of the conformation, on average, with remaining residues participating in both additional intramolecular hydrogen bonding and hydrogen bonding to water.

Unsatisfied backbone polar groups are energetically expensive, to the degree that they almost never occur. Previous database surveys found that ~10% of these groups fail to form hydrogen bonds, either internally or with water. However, prompted by the hydrogen-bonding hypothesis, we argue that these exceptions can be rationalized convincingly. In retrospect, Pauling's instincts about the importance of hydrogen bonds in protein conformation seem well justified.

The difference between ~90% and ~100% hydrogen bond satisfaction is tantamount to the difference between a statistical *trend* and a *rule*. We suggest that this rule can serve as a powerful filter for assessing the merit of experimental structures and the validity of simulated conformations.

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