Hydrogen Bonding Increases Packing Density in the Protein Interior

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ABSTRACT **The contribution of hydrogen bonds and the burial of polar groups to protein stability is a controversial subject. Theoretical studies suggest that burying polar groups in the protein interior makes an unfavorable contribution to the stability, but experimental studies show that burying polar groups, especially those that are hydrogen bonded, contributes favorably to protein stability. Understanding the factors that are not properly accounted for by the theoretical models would improve the models so that they more accurately describe experimental results. It has been suggested that hydrogen bonds may contribute to protein stability, in part, by increasing packing density in the protein interior, and thereby increasing the contribution of van der Waals interactions to protein stability. To investigate the influence of hydrogen bonds on packing density, we analyzed 687 crystal structures and determined the volume of buried polar groups as a function of their extent of hydrogen bonding. Our findings show that peptide groups and polar side chains that form hydrogen bonds occupy a smaller volume than the same groups when they do not form hydrogen bonds. For example, peptide groups in which both polar groups are hydrogen bonded occupy a volume, on average, 5.2 Å^3 less than a peptide group that is not hydrogen bonded. Proteins 2006;63:278–282.**

Key words: van der Waals interactions; protein folding; protein stability; hydrogen bonds

INTRODUCTION

For over 40 years, the burial of nonpolar groups—the hydrophobic effect—was thought to be the dominant force stabilizing globular proteins.[1,2] More recently, experimental studies have suggested that polar group burial contributes more to protein stability than nonpolar group burial.[3] It now appears that van der Waals forces make a major contribution to protein stability, and these dispersion forces are important to both polar[3,4] and nonpolar[5,6] group burial. These forces depend on the polarizability of the groups involved and on how tightly groups are packed in folded and unfolded proteins.[7] This article examines how tightly polar groups are packed in folded proteins and shows how this depends on hydrogen bonding.

When a protein folds to its native structure, the packing density of atoms is about the same as that of close packed spheres and that observed in crystals of small organic molecules.[8–12] As a consequence of this tight packing, the van der Waals forces are much greater in the native state than in the denatured state. Although the interior of a protein is well packed, the packing is not uniform. In an early study, Kuntz found that polar regions had a higher packing density (1.55 g/cc) than hydrophobic regions (0.93 g/cc).[13] Later, a rough correlation between the packing density and the polarity of a region was observed.[14] More recently, it was shown that the tightest packing was observed in the hydrogen-bonded backbone regions of the protein.[15] Others have shown that small cavities and other packing irregularities are common in the interior of proteins,[16–18] and the packing of surface groups is similar to buried groups when surface waters were included in the calculations.[19] To better understand protein folding and stability, it is important to understand how differences in packing between proteins and in different areas of the same protein effect the stability.

Increased packing in the interior of the protein increases the van der Waals interactions between atoms. Both experimental and computational studies have shown van der Waals interactions play an important role in protein stability. Studies of leucine to alanine substitutions in T4 lysozyme have shown a relationship between the size of the cavity formed and the loss of protein stability.[20] Matthews found cavity formation decreased stability by 24 cal/mol/Å^3. Sneddon and Tobias[21] concluded that the change in stability in two isoleucine to valine substitutions was the result of reduced packing in RNase T1. Other work also indicates that packing and van der Waals interactions are important to protein stability.[22–24] Additionally, polar

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groups contribute significantly to the overall van der Waals interactions. Laziridis et al.[25] calculated the van der Waals contribution to the stability of folded RNase A in a vacuum and found that the contribution of polar–polar interactions was −140 kcal/mol, of nonpolar–nonpolar interactions was −148 kcal/mol, and of polar–nonpolar interactions was −366 kcal/mol.[25]

Recently, Honig[26] suggested hydrogen bonds might stabilize proteins indirectly by increasing the local packing of the protein interior.[26] Previous theoretical calculations had concluded that it was more energetically favorable for the polar groups to be hydrated by water in the unfolded protein than buried in the protein interior, even if they formed intramolecular hydrogen bonds.[27] However, experimental work has shown that removing buried polar groups that form hydrogen bonds reduces protein stability.[28–33] Although it is difficult to identify all the factors that contribute to the overall change in stability from experimental results, theoretical calculations are limited to the factors that are explicitly included. The discrepancy between the experimental and theoretical results suggests that the theoretical models fail to include significant contributions to protein stability. To better understand the contribution of polar group burial and hydrogen bonding to protein stability, it is necessary to identify the factors that contribute significantly to the experimental results, but were not included in the theoretical models.

To better understand the broader impact of hydrogen bonding on protein stability, we have explored the effect of hydrogen bonding on local packing interactions. We used Voronoi polyhedra[34] as described by Richards[11] to calculate the atom volumes of the proteins. This Voronoi volume assigns to an atom all space that is closer to the atom center than any other atom center. Because the location of hydrogen atoms are not generally reported in crystallographic coordinates, we utilized a unified atom set where the volume of the hydrogen is included in the volume of the atom to which it is covalently attached. Using the Voronoi volume, we calculated the atom and residue volumes for 687 X-ray structures with better than 1.8-Å resolution. These atom and residue volumes were then classified based on their hydrogen bonding and extent of burial. We then compared the volumes of hydrogen-bonded groups to those that do not form hydrogen bonds to gain an understanding of how hydrogen bonds influence packing density. Our results show that hydrogen bonding significantly increases the packing density.

RESULTS AND DISCUSSION

Surface and Buried Residues

We calculated the volumes occupied by the amino acid residues in a sample of 687 folded proteins. For these volume calculations, ligands and waters reported in the structure file were included. Several previous studies have done similar calculations.[10–12,17,35] For each residue, we calculated the backbone (—NH—CH—CO—) and side-chain volumes separately. The backbone and side chains were further subdivided based on their contact with solvent. If any atom in the backbone was in contact with the molecular surface, that backbone was classified as surface. If no atom in the backbone was in contact with the molecular surface, that backbone was classified as buried. The same definitions were applied to the side chains for each residue. Consequently, our buried backbone groups and side chains are completely buried. Our sample of 687 proteins contains 149,263 residues, an average of 217 residues per protein. For the backbone groups, 46.5% were buried and 53.5% were on the surface. The average volumes of the buried backbones for each type of residue are shown in Table I. The amino acids with the most buried backbone groups were Ile with 72%, Val with 69%, Cys with 68%, and Leu with 67%; and those with the least buried backbone groups were Asp with 34%, Arg with 31%, Glu with 25%, and Lys with 18%.

Polar and charged side chains are much less frequently buried than backbone groups. The average volumes for the buried polar and charged side chains are shown in Table II. Only 2.6% of the side chains capable of forming hydrogen bonds are completely buried, compared to 46.5% for the backbone groups. The charged side chains are only rarely completely buried: Lys (0.22%), Arg (0.31%), Glu (0.72%), and Asp (1.41%). The uncharged side chains capable of forming hydrogen bonds are also generally not completely buried: Gln (1.49%), His (2.44%), Asn (2.56%), Tyr (3.82%), Trp (4.85%), Thr (5.78%), and Ser (6.13%). These results are similar to those of Harpaz et al.,[12] who analyzed the completely buried groups in 108 globular proteins. They found that the buried surface was composed of 40% nonpolar side chains, 31% peptide groups, 19% α-carbons, 7% polar side chains, and 2% charged side chains. The important point for this paper is that the polar surface buried when a protein folds is mainly peptide groups.

Hydrogen Bonding by the Peptide Groups

Each peptide group can potentially form three hydrogen bonds: one by the amide hydrogen, and two by the carbonyl oxygen.[36] For this article, we will count these groups as hydrogen bonded if they form at least one hydrogen bond. In other words, if the carbonyl oxygen of the peptide group forms just one of the two possible hydrogen bonds it will be counted as hydrogen bonded. There are 69,410 completely buried peptide groups in our sample of 687 proteins (Table I). Of these, 3898 or 5.6% form no hydrogen bonds, 19,510 or 27.5% form one hydrogen bond, and 46,362 or 66.8% form two hydrogen bonds. Taking into account the proline residues that have only one group capable of hydrogen bonding, 95.4% of the amide hydrogens and carbonyl oxygens of the completely buried peptide groups are hydrogen bonded. This corresponds to 188 hydrogen bonds per protein. Because the average protein in the sample contains 217 residues, this amounts to about 0.88 hydrogen bonds per residue. The hydrogen bonding analysis by Stickle et al. found an average of 1.08 hydrogen bonds per residue in a sample of 42 globular proteins.[37] Their analysis considered both the peptide groups and the side chains and buried and surface residues. Combining their results with ours leads to the conclusion that about 80% of

TABLE I. Differences in Volume Associated with Hydrogen Bonding for the Buried Backbone Groups

	Neither polar group hydrogen bonded			One polar group hydrogen bonded				Both polar groups hydrogen bonded			
Res	Vol Å^3	SE $\sigma/\sqrt{N}$	N	Vol Å^3	ΔV Å^3	SE $\sigma/\sqrt{N}$	N	Vol Å^3	ΔV Å^3	SE $\sigma/\sqrt{N}$	N
ALA	58.4	0.7	159	58.8	0.5	0.3	1599	53.4	−4.9	0.1	4608
ARG	57.6	1.2	74	56.9	−0.7	0.4	605	52.4	−5.2	0.1	1397
ASN	59.4	0.9	138	56.9	−2.5	0.3	838	52.2	−7.2	0.1	1595
ASP	60.7	1.0	155	57.1	−3.7	0.3	933	52.6	−8.2	0.1	2023
CYS	56.6	1.1	59	57.1	0.5	0.4	445	52.7	−3.9	0.1	974
GLN	58.0	1.3	49	58.2	0.2	0.6	490	52.3	−5.7	0.1	1356
GLU	58.7	1.3	63	57.8	−0.9	0.4	689	52.6	−6.2	0.1	1596
GLY	71.3	1.0	177	72.7	1.3	0.4	1635	65.7	−5.6	0.1	2579
HIS	55.4	0.4	79	55.5	0.1	0.4	452	52.1	−3.3	0.1	1076
ILE	57.4	0.7	200	55.4	−2.0	0.3	1153	51.3	−6.0	<0.1	4576
LEU	56.8	0.6	158	57.3	0.5	0.2	2055	52.0	−4.8	<0.1	6224
LYS	56.0	1.0	39	58.0	2.0	0.5	459	52.5	−3.6	0.1	1026
MET	56.9	1.2	49	56.3	−0.6	0.4	421	52.0	−4.9	0.1	1369
PHE	57.5	0.8	151	56.7	−0.8	0.3	891	52.3	−5.2	0.1	2720
PRO	54.0	0.3	1519	48.5	−5.5	0.1	1750			NA	
SER	58.0	0.6	178	58.0	0.0	0.3	1176	53.4	−4.6	0.1	2082
THR	57.7	0.7	239	56.9	−0.8	0.3	1122	52.2	−5.5	0.1	2561
TRP	57.0	0.9	64	55.2	−1.9	0.5	327	51.8	−5.2	0.1	855
TYR	56.5	0.8	126	56.1	−0.4	0.3	699	52.0	−4.5	0.1	2150
VAL	56.3	0.5	222	55.4	−0.9	0.2	1411	51.7	−4.6	<0.1	5595
			3898		−0.90		19150		−5.23		46362

Res = residue; Vol = volume; SE = standard error; N = number; $\Delta V = V_{\text{Hbond}} - V_{\text{No H Bonds}}$; NA = not applicable; the last entry in the N column is the sum of the individual entries. The last entry in the ΔV column is the weighted average of the individual entries.

TABLE II. Differences in Volume Associated with Hydrogen Bonding for the Buried Polar and Charged Side Chains

	No hydrogen bonds			One or more hydrogen bonds			
Res	Vol Å^3	SE $\sigma/\text{Å}N$	N	Vol Å^3	ΔV Å^3	SE $\sigma/\text{Å}N$	N
ARG			0	139.7	ND	1.0	21
ASN	75.1	1.2	11	73.6	−1.5	0.3	163
ASP	71.8	2.3	2	63.9	−7.9	0.3	125
GLN	98.4	1.7	8	97.0	−1.4	0.5	75
GLU	91.0	2.5	4	88.7	−2.3	0.5	63
HIS	102.7	0.3	2	105.3	2.6	0.6	84
LYS			0	112.8	ND	1.1	19
SER	42.2	0.8	25	41.2	−1.0	0.1	527
THR	66.9	0.7	33	67.2	0.4	0.2	465
TRP	178.6	1.4	20	178.1	−0.6	0.7	85
TYR	145.3	1.1	22	144.4	−0.8	0.4	180
			127		−1.02		1807

Res = residue; Vol = volume; SE = standard error; N = number; $\Delta V = V_{\text{Hbond}} - V_{\text{No H Bonds}}$; ND = not determined; The last entry in the N column is the sum of the individual entries. The last entry in the ΔV column is the weighted average of the individual entries.

the intramolecular hydrogen bonds in folded proteins are formed by the completely buried peptide groups that we are analyzing in this article. This is still probably an underestimate of the number of hydrogen bonds. For example, 51 of the buried peptide groups are not counted as hydrogen bonded by the normal criteria, but 26 of these have a polar group within 3.5 Å of a crystallographic water molecule. Thus, as many others have pointed out, very few of the buried peptide groups are not hydrogen bonded and, as Fleming and Rose recently pointed out, those that appear to be are generally satisfied in other ways.[38]

Hydrogen Bonding by the Side Chains

The side chains capable of hydrogen bonding are shown in Table II. They can form from one (Trp) to five (Arg) hydrogen bonds.[36] For this article, we will count the side chain as hydrogen bonded if it forms one or more hydrogen bonds. There are only 1934 completely buried side chains

capable of forming hydrogen bonds (Table II). This is a surprisingly small number compared to the number of buried peptide groups (69,410) and it emphasizes the fact that the buried groups in a folded protein are mainly backbone peptide groups and nonpolar side chains. If the total surface buried is considered, proteins bury 82% of their peptide groups and 83% of their nonpolar side chains (Ala, Val, Ile, Leu, Met, Phe, Trp, and Cys) when they fold.[39] Of the 1934 side chains that are completely buried, 93.4% form at least one hydrogen bond. For comparison, 95.4% of the polar groups of the buried peptide groups are hydrogen bonded. Again, this is just a lower limit on the number of groups that are hydrogen bonded since we have not considered hydrogen bonds to water molecules.

Effect of Hydrogen Bonding on Backbone Volumes

To understand how hydrogen bonding influences packing in a protein, we compared the volume of buried residues that form intramolecular hydrogen bonds with those that are not hydrogen bonded. Table I shows the volumes calculated for the buried backbone groups. Note that the groups are subdivided based on whether one of the two polar groups is hydrogen bonded, or both groups are hydrogen bonded. Because the nitrogen of the proline backbone cannot form a hydrogen bond, we classify proline residues as hydrogen bonded when the carbonyl oxygen forms one or two hydrogen bonds. The results show that forming a hydrogen bond by either the amide hydrogen or the carbonyl oxygen of the peptide group decreases the average volume by 0.90 $Å^3$, and that when both of the groups are hydrogen bonded the average volume decreases by 5.2 $Å^3$. Thus, it is clear that peptide groups are more tightly packed when they are hydrogen bonded and, consequently, that van der Waals interactions are likely to contribute to their stability.

Effect of Hydrogen Bonding on Side-Chain Volumes

In Table II, we list the side chains that can form hydrogen bonds. The buried composition is interesting: 28.5% Ser, 25.8% Thr, 10.4% Tyr, 9.0% Asn, 6.6% Asp, 5.4% Trp, 4.5% His, 4.3% Gln, 3.5% Glu, 1.1% Arg, and 0.9% Lys. Clearly, some of these side chains are much more likely to be completely buried than others. In particular, Arg and Lys side chains are significantly less likely to be buried than any of the other polar or charged side chains. One consequence is that there are no completely buried Lys or Arg side chains that are not hydrogen bonded and they could not be included in our analysis. For the other side chains, we find that the volume is 1.0 $Å^3$ less when they are hydrogen bonded than when they are not.

Concluding Remarks

The volumes occupied by the hydrogen bonded backbone groups is significantly smaller than the volumes occupied by the nonhydrogen bonded backbone groups. Similarly, the volume is smaller for polar and charged side chains when they form at least one hydrogen bond. This shows, not surprisingly, that the packing density in the interior of proteins is greatest in the regions that are hydrogen bonded. We previously suggested that the hydrogen bonding and van der Waals interactions of peptide groups in the tightly packed interior of a folded protein are more favorable than similar interactions with water in the unfolded protein.[3] The results reported here suggest that dipole—induced dipole interactions and van der Waals interactions will be strongest in the hydrogen bonded regions in the interior of the protein.

MATERIALS AND METHODS

Packing Densities

We used the atomic radii determined by Chothia,[10] to calculate the total volume using Richards' Method B[11] implemented in the code-mbg library.[12,40,41] For these volume calculations, ligands and waters reported in the structure file were included. Inclusion of these atoms avoids misallocation of empty space to protein atom volumes, which would result in larger volumes.

Protein Data Set

We used PISCES[42] to generate a nonredundant set of high-resolution protein structures. The criteria we used to select structures were: (1) X-ray structures with resolution better than 1.8 Å, (2) less than 50% amino acid sequence identity, and (3) have between 80 and 1000 amino acids. From this set, we eliminated all structures that contained gaps and missing atoms, which results in 687 structures for analysis. The pdb codes and chain identifiers are available as supplementary material.

Classification

Atoms were classified based on burial and hydrogen bonding. To determine the classification of the atoms of the amino acids, a Connolly molecular surface[43] and atomic contacts were calculated using the code-mbg library.[12,40,41] Atom burial was divided into two groups: surface, atoms that contact the molecular surface; and buried, atoms that do not contact the molecular surface. Backbone groups and side chains were classified based on the most exposed atom. For example, a side chain with one surface atom is classified as surface, and only if the backbone group or side chain had no surface atoms was it classified as buried. The hydrogen bonding of the polar groups was determined using HBPLUS.[44]

Supplementary Material

A text file containing the pdb codes and chain identifiers, pdbcode.txt, is available as supplementary material.

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