Polar Group Burial Contributes More to Protein Stability than Nonpolar Group Burial†

C. Nick Pace*

Departments of Medical Biochemistry and Genetics and Biochemistry and Biophysics and Center for Advanced Biomolecular Research, Texas A&M University, College Station, Texas 77843-1114

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ABSTRACT: On the basis of studies of Asn to Ala mutants, the gain in stability from burying amide groups that are hydrogen bonded to peptide groups is 80 cal/(mol Å^3). On the basis of similar studies of Leu to Ala and Ile to Val mutants, the gain in stability from burying $-CH_2-$ groups is 50 cal/(mol Å^3). Thus, the burial of an amide group contributes more to protein stability than the burial of an equivalent volume of $-CH_2-$ groups. Applying these results to folded proteins leads to the surprising conclusion that peptide group burial makes a larger contribution to protein stability than nonpolar side chain burial. Several studies have shown that the desolvation penalty for burying peptide groups is considerably smaller than generally thought. This suggests that the hydrogen bonding and van der Waals interactions of peptide groups in the tightly packed interior of folded protein are more favorable than similar interactions with water in the unfolded protein.

When proteins fold, 83% of the nonpolar side chains (Ala, Val, Leu, Ile, Met, Phe, Trp, Cys), 82% of the peptide groups (−CO−NH−), 63% of the polar side chains (Asn, Gln, Ser, Thr, Tyr), and 54% of the charged side chains (Asp, Glu, His, Arg, Lys) are buried in the interior of the molecule out of contact with water (*1*). Since about 1960, the prevailing view has been that proteins are stabilized mainly by the hydrophobic effect through the burial of the nonpolar side chains (*2*). For example, in 1990 Dill (*3*) wrote an influential review and concluded: "More than 30 years after Kauzmann's insightful hypothesis, there is now strong accumulated evidence that hydrophobicity is the dominant force of protein folding ...". In a previous analysis of mutant proteins designed to investigate the forces stabilizing proteins, we suggested that hydrogen bonding and the hydrophobic effect make large but comparable contributions to protein stability (*4*). Here we examine this question a different way by comparing the stability gained by burying amide groups with the stability gained by burying $-CH_2-$ groups but reach a similar conclusion.

Contribution of Buried Amide Groups to Protein Stability. We previously discussed studies of eight Asn to Ala mutants in which the Asn side chain is hydrogen bonded to a peptide group in the wild-type protein (*5*). A summary of these results is given in Table 1. The amide groups under consideration are almost completely buried and form 2.8 ± 0.7 intramolecular hydrogen bonds to their peptide group partners. When the amide group is removed, the average decrease in stability is 2.9 kcal/mol. What factors contribute to this decrease in stability? There is a difference in conformational entropy between the Asn and Ala side chains that would favor the mutant by about 1 kcal/mol (*6*, *7*). Thus, correcting for the conformational entropy difference would make the stability decreases even larger. Since only one of these mutations is in a helix and it is at a buried site, differences in helix propensity will not contribute to the observed Δ(Δ*G*) values. Making an Asn to Ala mutation can potentially leave a 37.4 Å^3 cavity in the mutant (*8*). For hydrophobic mutants (*9*), the cost of cavity formation is 22 cal/(mol Å^3). So, if a cavity of 37.4 Å^3 were left in the mutant protein, it would decrease the stability of the mutant by about 0.8 kcal/mol [37.4 Å^3 × 22 cal/(mol Å^3)]. The mutant proteins might change their conformation to fill the cavity, but they will do so only if it leads to a lower free energy, and this would reduce the Δ(Δ*G*) values. Thus, the large decrease in stability results mainly because the hydrogen bonding and van der Waals

Table 1: Average Δ(Δ*G*) Values for Eight Asn to Ala, Leu to Ala, and Ile to Val Mutants

mutation	Δ*V*[d] (Å^3)	buried[e] (%)	Δ(Δ*G*)[f] (kcal/mol)	Δ(Δ*G*)[g] [cal/(mol Å^3)]
Asn to Ala[a]	37.4	95 ± 5	−2.9 ± 1.4	−78
Leu to Ala[b]	74.5	99 ± 4	−3.6 ± 0.9	−48
Ile to Val[c]	25.8	100	−1.3 ± 0.4	−50

[a] The data for the Asn to Ala mutants are from Table 5 of ref *5*. All of the Asn amide groups are hydrogen bonded to peptide groups. The number of intramolecular hydrogen bonds they form ranges from 2 to 4 with an average = 2.8 ± 0.7. [b] The data for the Leu to Ala mutants are from Table 4 in ref *30*. [c] The data for the Ile to Val mutants are from Table 1 in ref *4*. [d] Δ*V* is the difference in volume between the side chain in the wild-type protein and the side chain in the mutant. The Δ*V* values are based on the volumes of the side chain when buried in the interior of a protein and are from Harpaz et al. (*8*). [e] The percent buried for the side chain in the wild-type proteins was calculated as described by Lee and Richards (*31*). [f] These are the observed Δ(Δ*G*) values from the references cited in footnotes *a*−*c* above. [g] The observed Δ(Δ*G*) values have been divided by the Δ*V* values to normalize them to give the Δ(Δ*G*) per 1 Å^3 of buried surface.

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* To whom correspondence should be addressed. Phone: (979) 845-1788. Fax: (979) 847-9481. E-mail: nickpace@tamu.edu.

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interactions of these amide groups in the folded protein are more favorable than the interactions with water in the unfolded protein, and consequently, the burial of amide groups in a folded protein makes a large, favorable contribution to their stability.

Contribution of Buried −CH_2− Groups to Protein Stability. For a comparison of the energetics of −CH_2− group burial with amide group burial, we consider Ile to Val mutants where a single −CH_2− group is removed and Leu to Ala mutants where >CH−, −CH_2−, and CH_3− groups are removed. The average results for eight mutants of each type are given in Table 1. Since the size of the groups removed differ, we normalize the results in the last column of Table 1 to compare the stability gains. Note that the results per −CH_2− group are very similar for the two hydrophobic mutations and show that the burial of −CH_2− groups makes a favorable contribution to protein stability. The stability gain from burying an amide group, 78 cal/(mol Å^3), is about 60% greater than the stability gain from burying the equivalent volume of −CH_2− groups, 49 cal/(mol Å^3). An analysis of 40 Tyr to Phe mutations (unpublished observations) suggests that the burial and hydrogen bonding of the polar −OH groups of Tyr residues are also more favorable than the burial of the equivalent volume of −CH_2− groups, but the difference is smaller than in the case of the amide groups.

Is the Amide Group of Asn a Reasonable Model for a Peptide Group? The volume of the Asn amide groups buried in proteins is 37.4 Å^3, and the comparable volume for buried peptide groups is 39.2 Å (*8*). Amide groups can form four hydrogen bonds but peptide groups only three. Thus, it is not surprising that Asn amide groups form 2.65 and peptide groups 2.10 hydrogen bonds on average in folded proteins (*10*). If we reduce the 78 cal/(mol Å^3) in Table 1 in proportion to the difference in the number of hydrogen bonds formed, it becomes 62 cal/(mol Å^3), still larger than the value for −CH_2− group burial. This is likely to be a lower limit for the stability gain from burying a peptide group because the Asn amide groups will be more accessible to solvent and form more hydrogen bonds than peptide groups in the unfolded states of a protein. Thus, the stability gain from burying a peptide group may be smaller than the stability gain from burying an amide group, but probably not by more than 10%. This will not change the conclusion we reach in the next section.

Comparison of the Contribution of Peptide Group Burial and Nonpolar Side Chain Burial to Protein Stability. Harpaz et al. (*8*) have analyzed the completely buried residues in 108 folded proteins. The total volume of the buried residues is 298 100 Å^3. Of this, 118 200 Å^3 comes from the nonpolar side chains, and 92 000 Å^3 comes from the peptide groups. Thus, the burial of the nonpolar side chains contributes 5800 kcal/mol [118 200 Å^3 × 49 cal/(mol Å^3)] to the stability, and the burial of the peptide groups contributes 7200 kcal/mol [92 000 Å^3 × 78 cal/(mol Å^3)] to the stability. This analysis suggests that the burial of peptide groups makes a 25% larger contribution to protein stability than the burial of the nonpolar side chains. [A similar conclusion was reached when the polar group contribution was analyzed in terms of hydrogen bonding (*11*).] Note that this analysis does not take into account the contribution of the burial of the C^α atoms of the main chain or the burial of the aliphatic carbons of the polar and charged side chains. Also, these volumes are based only on the residues that are completely buried in the folded protein (*8*). Nevertheless, the conclusion is surprising and suggests that the burial and hydrogen bonding of peptide groups make a more favorable contribution to protein stability than generally thought. In the next section we consider why.

Table 2: ΔG_{tr} Values for −CH_2− and Amide Groups

	ΔG_{tr} (kcal/mol)		
group	H_2O to vapor[a]	H_2O to Chex[b]	H_2O to Oct[c]
−CH_2−	−0.2	−1.0	−0.6
amide	11.2	7.6	1.4

[a] The ΔG_{tr} values are from Privalov and Makhatadze (*32*). [b] The ΔG_{tr} values for transfer to cyclohexane (Chex) are from Radzika and Wolfenden (*33*). [c] The ΔG_{tr} values for transfer to 1-octanol (Oct) are from Fauchere and Pliska (*34*).

The Desolvation Penalty for Burying Peptide Groups Is Small Because of the Tight Packing of Groups in the Protein Interior. There is widespread belief that burying polar groups in protein folding incurs a large desolvation penalty. For example, Honig and Cohen (*12*) write: "... a crucial property of the polypeptide backbone is that it contains polar NH and CO groups whose removal from water involves a significant energetic penalty". We think that there may be little if any net desolvation penalty for burying peptide groups in protein folding (*5*). We agree that the contribution of electrostatic interactions to polar group burial may be unfavorable, as calculations by programs such as DelPhi (*13*) indicate but that the contribution of van der Waals interactions to polar group burial is favorable enough to overcome this and make the overall process favorable.

van der Waals interactions are also known as London dispersion forces (*14*), and they are the basis for the attractive term in the Lennard-Jones 6-12 potential used in many force field programs such as CHARMM, AMBER, and GROMOS (*15*). The strength of the interaction is directly proportional to the polarizabilities of the atoms and inversely proportional to the sixth power of the distance between them (*15*). Thus, van der Waals interactions are very short range. The importance of van der Waals interactions is illustrated by the data shown in Table 2. The transfer of an amide group from H_2O to the vapor phase is unfavorable because of the dehydration of the amide group. However, the transfer to cyclohexane is less unfavorable mainly because of favorable van der Waals interactions between the amide group and the cyclohexane molecules. [There will also be an energetic cost for creating a cavity in the cyclohexane (*16*).] The transfer of the amide group to octanol is even more favorable because the amide group can hydrogen bond to the −OH groups of octanol and to the 3.5 M H_2O that the wet octanol contains. Note that the transfer of the amide group from H_2O to octanol is still unfavorable. However, we expect the transfer of an amide group from H_2O to the interior of a protein to be favorable for reasons explained below.

For comparison, ΔG_{tr} values for a −CH_2− group are also shown. In this case, all of the ΔG_{tr} values are favorable, reflecting both the aversion of nonpolar groups for water and the favorable van der Waals interactions that are possible for transfer to cyclohexane or octanol. Ratnaparkhi and Varadarajan recently suggested "... that the loss of packing interactions rather than the hydrophobic effect dominates the

observed energetics ...” of cavity creating hydrophobic mutations in proteins (*17*).

Protein interiors are tightly packed. For example, the fraction of space occupied by atoms is 0.36 in water, 0.44 in cyclohexane, 0.71 for close packed spheres, and 0.75 in protein interiors (*18*, *19*). Thus, protein interiors are much more tightly packed than water and cyclohexane. As Klapper (*18*) noted in 1971: “... we may conclude that the protein interior contains little space and is closer to a solid than a liquid”. Four recent studies have used more sophisticated analyses but reached similar conclusions (*20*−*23*). In addition, the regions that contain hydrogen-bonded polar groups may be more tightly packed than regions that contain nonpolar side chains (*20*, *24*). Consequently, van der Waals interactions among groups in the interior of a folded protein will be more favorable than the interactions of the same groups with water in the unfolded protein. This is supported by the observation that the transfer of peptide groups in cyclic peptides from H_2O to the crystal is favorable (*25*). In these crystals, the atoms are slightly more tightly packed than they are in protein interiors (*25*). Further support comes from the calculations of Lazaridis et al. (*26*), who show that the binding energy of a $-CH_2-$ group is significantly smaller in magnitude in liquid alkanes (≈ -1.8 kcal/mol) than it is in alkane crystals (≈ -3.5 kcal/mol) or the interior of a protein (≈ -3.1 kcal/mol). In another interesting example, Lazaridis et al. (*26*) calculate the contribution of van der Waals interactions to the enthalpy of folding of four proteins in a vacuum. For RNase A, they find that ΔH(van der Waals) $= -140$ kcal/mol for polar−polar group interactions, −148 kcal/mol for nonpolar−nonpolar group interactions, and −366 kcal/mol for polar−nonpolar group interactions. These results emphasize two important points. First, as noted above, many polar groups are buried on protein folding. Second, some polar groups such as the carbonyl oxygen are more polarizable than $-CH_2-$ groups and will accordingly have more favorable van der Waals interactions with the other groups in the protein.

On the basis of the thermodynamics of melting of alkanes, Nichols et al. (*27*) showed that per $-CH_2-$ group melted at 25 °C: $\Delta H \approx 0.59$ kcal/mol, $-T\Delta S \approx 0.45$ kcal/mol, and $\Delta G \approx 0.14$ kcal/mol. They concluded: “... close packing of the protein interior makes only a small free energy contribution to folding because the enthalpic gain resulting from increased dispersion interactions (relative to the liquid) is countered by the freezing of side chain motion”. This is true, but the large loss in conformational entropy that accompanies protein folding is compensated by all of the stabilizing forces and is generally treated separately from the individual stabilizing forces. Taking this view, these results also suggest that improved van der Waals interactions resulting from the close packing of groups in the protein interior make a large favorable contribution to the enthalpy of protein folding. Programs such as DelPhi (*13*) do not take the close packing in protein interiors into account and, consequently, overestimate the net cost of polar group desolvation in protein folding.

Concluding Remarks. The results in Table 1 suggest that burying side chains containing amide groups will make a more favorable contribution to protein folding than burying aliphatic side chains. This is supported by the results in Table 2 when the tight packing of groups in the protein interior is taken into account. It is also supported by the studies of Maxwell and Davidson (*28*) showing that the stability of an SH3 domain could not be increased by replacing groups forming a buried hydrogen bond by different combinations of hydrophobic residues. It is further supported by studies of peptide group solubility from the Bolen laboratory (*29*). They concluded: “The high insolubility of glycine peptides suggests that water is a relatively ‘poor’ solvent for the peptide backbone. This possibility raises the intriguing question of whether solvophobicity of the backbone in water has been overlooked as a contributor to the collapse and folding of a protein in aqueous solution”. We think it has. In summary, perhaps we have been wrong for the past 40 years, and the burial of polar groups makes a larger contribution to protein stability than the burial of nonpolar groups.

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