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Forces Stabilizing Proteins

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Abstract

The goal of this article is to summarize what has been learned about the major forces stabilizing proteins since the late 1980s when site-directed mutagenesis became possible. The following conclusions are derived from experimental studies of hydrophobic and hydrogen bonding variants. 1. Based on studies of 138 hydrophobic interaction variants in 11 proteins, burying a $–CH_2–$ group on folding contributes 1.1 ± 0.5 kcal/mol to protein stability. 2. The burial of nonpolar side chains contributes to protein stability in two ways: first, a term that depends on the removal of the side chains from water and, more importantly, the enhanced London dispersion forces that result from the tight packing in the protein interior. 3. Based on studies of 151 hydrogen bonding variants in 15 proteins, forming a hydrogen bond on folding contributes 1.1 ± 0.8 kcal/mol to protein stability. 4. The contribution of hydrogen bonds to protein stability is strongly context dependent. 5. Hydrogen bonds by side chains and peptide groups make similar contributions to protein stability. 6. Polar group burial can make a favorable contribution to protein stability even if the polar group is not hydrogen bonded. 7. Hydrophobic interactions and hydrogen bonds both make large contributions to protein stability.

Introduction and Historical Perspective

By the mid-1930s, the structure of proteins was emerging and a discussion of the forces that might stabilize the structures had begun. In 1936, Pauling and Mirsky [1] suggested "⋯ this chain is folded into a uniquely defined configuration, in which it is held by hydrogen bonds between the peptide nitrogen and oxygen atoms ⋯ The importance of the hydrogen bond in protein structure can hardly be overemphasized."; and they suggested that each hydrogen bond would contribute 5 kcal/mol to the stability of the uniquely defined configuration. Three years later, Bernal [2] impressively guessed: "Ionic bonds are clearly out of the question ⋯ the hydrophobe groups of the protein must hold it together ⋯ the protein molecule in solution must have its hydrophobe groups out of contact with water, that is, in

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contact with each other, ⋯ In this way a force of association is provided which is not so much that of attraction between hydrophobe groups, which is always weak, but that of repulsion of the groups out of the water medium." The purpose of this review is to summarize the major contributions to our understanding of the forces stabilizing proteins over the past 75 years and to suggest where we stand at present. In line with these good ideas from the 1930s, this review will focus on the contribution of hydrogen bonds and hydrophobic interactions to protein stability.

The next major advance occurred in 1951 when Pauling's group used constraints derived from studies of model compounds and their ideas about hydrogen bonds to discover the most important structural elements in globular proteins: the alpha helix [3] and the beta sheet [4]. In their paper describing the alpha helix [3], they suggested that hydrogen bonds would contribute about 8 kcal/mol to the stability. But, in their next paper describing the beta sheet [4], they had reached a better understanding and suggested that "With proteins in an aqueous environment the effective energy of hydrogen bonds in not so great, inasmuch as the difference between the energy of the system with N-H ⋯ O hydrogen bonds surrounded by water and a system with the N-H group and the O atom forming hydrogen bonds with water molecules may be no more than about 2 kcal/mol." This is in line with most current thought.

Eight years later, Kauzmann [5] published his groundbreaking review with a focus on hydrophobic bonds. He presented convincing evidence that "⋯ the hydrophobic bond is probably one of the more important factors involved in stabilizing the folded configuration in many native proteins." This was supported by the first high resolution structure of a protein [6], myoglobin, by Kendrew's group and he suggested [7] : "⋯ it is clear that by far the most important contribution comes from the van der Waals forces between nonpolar residues which make up the bulk of the interior of the molecule."

Soon after Kauzmann's review was published, Tanford [8] used the limited model compound data available to make an even more convincing case for the importance of the contribution of hydrophobic bonds to protein stability. He concluded that "⋯ the stability of the native conformation can be explained ⋯ entirely on the basis of the hydrophobic interactions of the nonpolar parts of the molecule." To gain better insight, Tanford and Nozaki [9] began experimental studies using model compounds that led to the first hydrophobicity scale. Later, Tanford wrote an interesting review of the history of the hydrophobic effect [10].

In 1964, Brandts [11] published an experimental study and analysis of the thermodynamics of folding of chymotrypsinogen. This work was the first to show the strong temperature dependence of protein folding that results because the unfolded (denatured) state has a much higher heat capacity than the folded (native) state, denoted ΔC_p. Because of this, proteins are most stable between about −40 and 40°C and can be unfolded by either raising or lowering the temperature. Cold denaturation was first clearly shown by Pace and Tanford [12] in 1968. This results because most of the nonpolar side chains are exposed to water in the unfolded protein but buried in the folded protein and this shows the important contribution that hydrophobic bonds make to protein stability [13]. However, when Brandts analyzed his

data, he concluded that hydrophobic bonds and hydrogen bonds both make important contributions to protein stability. More extensive studies were underway by the Privalov group who studied the thermodynamics of unfolding several proteins and reached similar conclusions [14].

The hydrogen bonding reaction of most interest is:

$$(-\mathrm{NH}\cdots\mathrm{O}{=}\mathrm{C}-)_{\mathrm{protein}}+3\ \mathrm{H_2O} \leftrightarrow \mathrm{H_2O}\cdots\mathrm{HN}- + -\mathrm{C}{=}\mathrm{O}\cdots(\mathrm{H_2O})_2$$

Based on model compound data, it was not clear whether the free energy change for this reaction was favorable or unfavorable. For example, Klotz [15] suggested − 750 cal/mol and Schellman [16] suggested + 400 cal/mol as the free energy change for this reaction. The analysis of the data is difficult because the contribution of entropy is difficult to estimate. These results are discussed further in two interesting reviews one by Dill[17] and one by Rose and Wolfenden [18] that consider both hydrogen bonds and hydrophobic interactions from different perspectives.

So, until about 1990, protein chemists thought that hydrophobic bonds made the major contribution to protein stability and that hydrogen bonds were necessary for maintaining the structure of proteins but it was not clear if they also made a large contribution to protein stability. In this review, we will summarize what has been learned about the forces stabilizing proteins by studying the stability of variants in which hydrophobic groups or hydrogen bonding groups have been removed.

Discussion

On average, proteins bury 85% of their nonpolar side chains [19] and form 1.1 hydrogen bonds per residue when the protein folds [20]. In the following sections, we will consider how much stability proteins gain from the burial of nonpolar groups (hydrophobic interactions) and from the burial and formation of intramolecular hydrogen bonds by polar groups. We will then consider other forces that contribute to protein stability and the forces that contribute to protein instability.

Protein Interiors are tightly packed

The tight packing of protein interiors is important to protein stability for the burial of both polar and nonpolar groups. The tight packing of protein interiors was first pointed out by the Richards' group [21,22] and by Klapper [23] who wrote "We may conclude that the protein interior contains little space, and is closer to a solid than a liquid." The tight packing is illustrated in Table 1. The protein interior is more tightly packed than close-packed spheres and twice as tightly packed as water. The importance of this can be seen in Table 2. The Karplus group calculated the van der Waals interactions of a $–CH_2–$ group with three different environments [24]. It is clear that the energy of interaction of the $–CH_2–$ group is much greater in the interior of a protein than with water or cyclohexane.

Hydrophobic interactions makes the major contribution to protein stability

To study the contribution of hydrophobic interactions to protein stability, a variant is made in which a buried hydrophobic group is removed and then the stabilities of the wild type and variant protein are measured to find the change in stability which is denoted a Δ(ΔG) value. The Δ(ΔG) values can be determined quite accurately from urea or thermal denaturation experiments. We have previously discussed this approach in detail [25].

Table 3 summarizes studies of 138 variants from 11 different proteins [13]. For each of the hydrophobic variants, a larger side chain is replaced by a smaller. The best variant to study is Ile to Val where a single $–CH_2–$ group is removed from the wild type protein. However, it can be seen that the average Δ(ΔG) values per $–CH_2–$ is about the same for all of the four aliphatic variants. These results show that proteins gain 1.1 ± 0.5 kcal/mol for every $–CH_2–$ group that is buried on folding. They also show that the experimental Δ(ΔG) values are in good agreement with ΔG_{tr} values measured for the transfer of the groups removed from water to cyclohexane. Based on the results in Tables 1 and 2, this is fortuitous. The agreement probably results because the packing is tighter in the protein than in cyclohexane but the polarizability is greater in cyclohexane than in the protein. The only forces involved here are London dispersion forces [26].

The results in Table 3 show that the errors are large. This suggests that the environment surrounding the side chain in the protein is important. This is illustrated in Table 4. The ΔG_{tr} from water to vapor is favorable because of the classic hydrophobic effect that was discussed in an earlier review in this journal by Baldwin [27]. The ΔG_{tr} values into octanol and cyclohexane are more favorable because the $–CH_2–$ groups can have favorable London dispersion interactions. Note that the ΔG_{tr} to cyclohexane in Table 4 is close to the average Δ(ΔG) value determined by the experimental studies. Again, this is probably fortuitous but it suggests that we can get a rough estimate of the contribution of hydrophobic interactions to protein stability using the ΔG_{tr} values for the side chains into cyclohexane [13]. (Footnote: In a recent article in this journal, Baldwin wrote [27]: “In the new view, hydrophobic free energy is measured by the work of solute transfer of hydrocarbon gases from vapor to aqueous solution.” By hydrophobic free energy, he means just the ΔG_{tr} from water to vapor in Table 4. He implies that the goal of experimental results such as those in Table 3 was to measure this “hydrophobic free energy,” but that was not the case. The goal was to find how much the burial of a hydrophobic side chain in protein folding contributes to protein stability. It includes both the “hydrophobic free energy” and the difference in van der Waals interactions of the hydrophobic side chains in the folded and unfolded protein. For this reason, we use the term hydrophobic interactions to characterize this process in this paper.)

The Matthews lab was the first to study hydrophobic variants [28,29]. They determined structures for the variants and showed that the larger the size of the cavity left in the variant, the larger the Δ(ΔG) value. Their explanation was that the larger the cavity, the more van der Waals interactions lost and the larger the Δ(ΔG) value. This suggests that van der Waals interactions make an important contribution to the stability gained by proteins when nonpolar groups are buried. Several other lines of reasoning have led to the same conclusion [30–32].

Studies of hydrophobic variants of a membrane protein showed a similar contribution from van der Waals interactions, but the contribution of the classic hydrophobic effect was smaller [33]. This probably results because SDS was used to unfold the proteins so that the nonpolar side chains were in a more nonpolar environment after unfolding than for a soluble protein where they would be in contact with water and this reduces the contribution of the classic hydrophobic effect.

So, the Δ(ΔG) values for hydrophobic variants are determined mainly by two factors: first, a constant term that depends on the difference in hydrophobicity between the WT and variant side chains, and, second, a variable term that depends on the difference in the van der Waals interactions of the side chains.

Hydrogen bonds make a large contribution to protein stability

When a proteins folds, 70% of the peptide groups and 65% of the polar side chains are buried in the interior of the protein out of contact with water [19]. The burial of polar groups is more complicated than the burial of nonpolar groups because now hydrogen bonds and longer range Coulombic interactions also contribute. The experimental results are more difficult to interpret and some of the theoretical results are not in agreement with the experimental results. Whether the burial of polar groups makes a favorable contribution to protein stability is still contentious.

In a recent review, Bowie analyzed two different approaches for estimating the contribution of individual hydrogen bonds to protein stability [34]. In the first approach, double mutant cycles are used to isolate the contribution of a hydrogen bond to stability. In the paper where this approach was used, the contribution estimated using double mutant cycles was the same, within experimental error, as the estimate obtained by just removing the side chain involved in the hydrogen bond, as was done in most of the earlier studies [35]. In the second approach, the estimate is based on comparing the Δ(ΔG) values for the same mutation, e.g., Ser to Ala, when the Ser −OH is or is not hydrogen bonded [34]. This is the approach we use below. The assumptions used in both of these approaches are discussed in the Bowie review [34].

When a mutation is made that replaces a Tyr residue with a Phe residue, an increase in stability of about 1.6 kcal/mol is expected because of the differences in hydrophobicity and conformational entropy between the two residues [36,37]. But, as shown in Table 5, we observe a decrease in stability of 0.2 kcal/mol when the Tyr −OH group is not hydrogen bonded and 1.4 kcal/mol when it is [37]. This is concrete evidence that hydrogen bonds make a favorable contribution to protein stability and that the −OH group can make a favorable contribution to protein stability even if it is not hydrogen bonded.

Table 5 shows results from many proteins for three types of variants of polar side chains. For each mutation, an −OH group is removed that is either hydrogen bonded or not. In each case, the stability decrease is larger when the groups are hydrogen bonded and this is convincing evidence that hydrogen bonds make a favorable contribution to protein stability. Note that the −OH groups of Tyr residues that are not hydrogen bonded also make a small favorable contribution to protein stability, but that the −OH group of Ser residues that are

not hydrogen bonded make a small unfavorable contribution. For a large sample of proteins, the −OH groups are buried to a greater extent for Tyr residues (67%) than for Ser residues (61%) [19]. Consequently, the −OH groups of Tyr residues will generally have more favorable van der Waals interactions than the −OH groups of Ser residues and this may account in part for this difference. Note also, as discussed previously (89), that the −OH group of Thr residues that are not hydrogen bonded make a contribution to the stability that is as large as that of the methyl groups that replace them in the Thr to Val variants.

It is interesting to compare the results for the ΔG_{tr} of a $–CH_2–$ group from Table 4 with those of an amide group in Table 6. The transfer from water to vapor is very unfavorable because dehydration of the peptide group results in the loss of all of the hydrogen bonds to water. However, transfer to cyclohexane is much less unfavorable because of favorable van der Waals interactions between the amide group and cyclohexane. The transfer to octanol is even less unfavorable because the amide group can now form hydrogen bonds to octanol and the 3.5 M water present in wet octanol. The fact that transfer to wet octanol is still unfavorable suggests that the van der Waals and longer range electrostatic interactions in the protein are more favorable than those in wet octanol.

These results show that hydrogen bonds make a favorable contribution to protein stability. They also show that the hydrogen bonding and other interactions of −OH groups in folded proteins can be more favorable than interactions with water in the unfolded protein. We have shown previously that hydrogen bonding increases the packing density in the interior of proteins [38]. In addition, the results show that buried polar groups that are not hydrogen bonded can make a favorable contribution to protein stability. It is surprising that the van der Waals and longer range electrostatic interactions of a buried polar group can sometimes be more favorable than the hydrogen bonding interactions of the polar group with water molecules in the unfolded protein.

Hydrogen bonds by side chains and peptide groups make similar contributions to protein stability

In Table 7, we compare the results from Table 5 with previous studies of Asn to Ala mutations in which the Asn side chains were hydrogen bonded to peptide groups [39,40] and to studies by the Kelly group in which the peptide group is converted to an ester to estimate the contribution of hydrogen bonds by peptide groups to protein stability [41,42]. The hydrogen bonds by peptide groups make a contribution to protein stability that is similar to those of the −OH groups of Tyr, Ser, and Thr residues.

These results are supported by studies using a completely different approach, equilibrium hydrogen/deuterium fractionation factors, which have been shown to correlate with hydrogen bond strengths. This approach was first used to look at hydrogen bond strengths by the Sosnick group [43] and more recently was improved to look at the contribution of hydrogen bonds to protein stability by Cao and Bowie [44]. They showed the hydrogen bonds formed by backbone amide groups contributed, relative to the weakest hydrogen bond, −2.2 ± 1.2 kcal/mol for staphylococcal nuclease, −1.9 ± 0.7 for His containing protein, −1.0 ± 0.2 for human ubiquitin, and −1.1 ± 0.3 for protein G. They also showed that

hydrogen bonds in α-helices are marginally stronger by ≈ 0.2 kcal/mol than those in β-sheets, and that charge stabilized hydrogen bonds are stronger than neutral hydrogen bonds by ≈ 2 kcal/mol on average.

The contribution of the hydrogen bonds by peptide groups is especially important because the average number of hydrogen bonds formed in a folded protein is 1.1 per residue and 65% of these are between peptide groups, 23% are between peptide groups and side chains, and just 12% between side chains [20]. (Several informative reviews of hydrogen bonding in folded [20,45–47] and unfolded [48] proteins are available.) Thus, the hydrogen bonds formed by peptide groups make a much larger contribution to protein stability than side chain hydrogen bonds.

The contribution of hydrogen bonds to protein stability is context dependent

The errors are large for the ΔG_{HB} estimates in Table 7. This reflects in part the fact that the contributions of individual hydrogen bonds depend on their distance and geometry [49,50]. Equally important, we think, is the environment of the individual hydrogen bonds. Studies by Kelly's group have shown that hydrogen bonds can be more than 1 kcal/mol stronger in a hydrophobic environment [51,52]. This is expected because hydrogen bonds are mainly electrostatic interactions and they will be stronger in more nonpolar environments with a lower dielectric constant [53]. The importance of neighboring group interactions was also shown by Schreiber's group [54]. A double-mutant cycle analysis of hydrogen bonds in the native protein environment showed a stabilization of 1 to 1.5 kcal/mol, but when the neighboring groups were removed, the stabilization was only ≈ 0.3 kcal/mol.

Worth and Blundell [55] have analyzed the hydrogen bonding of the polar amino acids in a set of structurally aligned protein families. They find that the polar side chains that are hydrogen-bonded are more conserved than those that are not, particularly for buried residues. These buried, hydrogen-bonded polar residues are substantially more conserved than buried hydrophobic residues. This interesting finding may have more to do with the specificity that hydrogen bonds contribute to protein structure than to the stability.

Eisenberg's group determined the crystal structure of the peptide GNNQQNY, and, in collaboration with Baker's group, studied the energetics of amyloid fiber formation using classical and quantum methods [56]. The remarkable structure is so tightly stabilized by 22 hydrogen bonds and van der Waals interactions that it is anhydrous and almost completely insoluble. This shows that the interactions in the crystal are much stronger than the interactions that the peptide would have with water in solution. When two of the peptides associate, each hydrogen bond contributes 7.8 kcal/mol to the stability, but when a peptide is added to the crystal, the contribution is 9.1 kcal/mol. (For comparison, in ice, the contribution is 6.7 kcal/mol per hydrogen bond.) This suggests that hydrogen bond formation in amyloid is cooperative, and that hydrogen bond strength can be increased even by a polar environment.

Other forces contributing to protein stability

Disulfide bonds

Disulfide bonds can make a substantial contribution to protein stability mainly by reducing the conformational entropy of the denatured state. Based on experimental evidence, the contribution of a disulfide bond to stability can often be estimated reasonably well with this equation [57]:

$$\Delta S = -2.1 - (3/2)\,R\,\ln(n)$$

Where n is the number of residues in the loop formed by the crosslink and R is the gas constant. The equation predicts that the stability will be increased by 3, 4, and 5 kcal/mol by loops of 15, 45, and 135 residues, respectively. For some proteins, such as RNase A and trypsin inhibitor, disulfide bonds are essential to the stability and the proteins unfold if the bonds are broken. Proteins can also be stabilized by adding disulfide bonds. For example, the melting temperature of arc repressor was raised ≈ 30°C by adding one disulfide bond [58], and the melting temperature of T4 lysozyme was increased by 23.4°C by adding 3 disulfide bonds [59]. A comprehensive review of this subject was recently published in this journal [60].

Charge-charge interactions

The charges on the surface of a protein are generally arranged so that there are more attractive than repulsive interactions near neutral pH [61–64]. Consequently, these electrostatic interactions will generally contribute favorably to a protein's stability. Nevertheless, several recent studies have shown that it is possible to stabilize proteins by making charge reversal mutations on the surface that improve the electrostatic interactions even further [64–69]. These stability increases are always less than predicted using Coulomb's law with a dielectric constant of 80 to sum up the electrostatic interactions in the native state. This led us to conclude that charge – charge interactions in the denatured state ensemble are also favorable so that the increase in stability is less than expected [70]. Thus, it is unlikely that charge – charge interactions will make contributions to protein stability greater than 10 kcal/mol at 25°C [71]. At higher temperatures, the contribution might be considerably greater, and it appears that proteins from thermophilic organisms often use this strategy to increase their stability [72,73].

Hydrogen bonds to buried charged side chains

In RNase T1, Asp 76 is buried forms three good hydrogen bonds and has a pK ≈ 0.6 [74,75]. In RNase Sa, Asp 33 is buried forms three good hydrogen bonds and has a pK ≈ 2.4 [75,76]. The hydrogen bonds to these carboxyl groups lower their pKs from the unperturbed value of 3.6 and make a large contribution to the stability of the protein. In contrast, buried charged side chains that are not hydrogen bonded can make large unfavorable contributions to the stability [76,77]. These and more recent studies [44,78] show that hydrogen bonds to charged residues can make substantially larger contributions to protein stability than hydrogen bonds between uncharged groups.

Salt bridges

When oppositely charged groups in proteins are within 5Å, they are generally referred to as ion pairs or salt bridges. Ion pairs on the surface generally contribute less than 1 kcal/mol to the stability [64]. However, a buried salt bridge can contribute more than 4 kcal/mol to the stability [79], but the number of buried salt bridges in proteins is small so they do not make a large contribution to stability.

Contribution of n→π* interactions to protein stability

The Raines and Woolfson groups have recently considered the possibility that n→π* interactions make an important contribution to protein stability [80]. They state [81]: "⋯ the hydrogen bond is distinct in having its origins in electron delocalization. Recently, another type of electron delocalization, the n→π* interaction between carbonyl groups, has been shown to play a role in stabilizing protein structure. ⋯ We found that an n→π* interaction is worth ≈5–25% of a hydrogen bond ⋯ Thus, these two interactions conspire to stabilize local backbone-side chain contacts, which argues for the inclusion of n→π* interactions in the inventory of non-covalent forces that contribute to protein stability and thus to the force fields for biomolecular modeling."

Gain in configurational entropy of water on protein folding

When a protein folds, much of the water hydrogen bonded to the protein will be released. Kinoshita's group has suggested that the gain in the configurational entropy of the water when this occurs may contribute to protein stability. This interesting possibility is discussed in several recent papers. See, for example [82].

Forces contributing to the instability of proteins

The major force destabilizing proteins is conformational entropy. Rotation around the many bonds in a protein is much freer in the denatured state than in the native state and provides a strong entropic driving force for protein unfolding. Two different approaches suggested that the energetic cost of folding a protein is about 1.7 kcal/mol per residue; [83,84]. We have used this estimate in previous papers [85,86]; however, this estimate may be too low. Brady and Sharp [87] have reviewed this subject and suggested that the conformational entropy cost of protein folding may be between 2.4 and 3.7 kcal/mol per residue. This is in line with an estimate of 2.4 kcal/mol per residue that we obtained in a previous study of the forces contributing to the stability of 22 proteins with the number of amino acids varying from 36 to 548 [13].

During the period when hydrogen bonds were thought to make at most a small contribution to protein stability, it was assumed that buried polar groups that were not hydrogen bonded would make a large unfavorable contribution. For example, Finney's group showed that about 90% of the polar groups formed intramolecular hydrogen bonds or hydrogen bonds to water molecules, but that still left a number of "lost hydrogen bonds" [88]. Since then Fleming and Rose have analyzed the hydrogen bonding of the backbone peptide groups and concluded, "Unsatisfied backbone polar groups are energetically expensive to the degree that they almost never occur." [47] The results in Table 5 show that the polar −OH groups of

Tyr and Thr can make a small favorable contribution to protein stability and that the −OH groups of the non-hydrogen bonded Ser residues make only a small contribution to protein instability. Even for 40 Val to Thr mutations, $\Delta(\Delta G) = 1.8 \pm 1.1$ kcal/mol showing that when a polar −OH group is placed at a site designed for a $-CH_3$ group, the penalty is small [89]. Consequently, we doubt that non-hydrogen bonded, side-chain polar groups will make a large contribution to protein instability, but it is not clear if this is the case for buried peptide groups.

Another interesting suggestion was made by Kajander et al. [90]. They analyzed the structures of a large sample of proteins of varying molecular weight and showed that 65% more charged groups are buried in proteins containing 700 amino acids than in proteins containing 100 amino acids. From this they concluded, “Nature may use charge burial to reduce protein stability; not all buried charges are fully stabilized by a prearranged protein environment.” [90]. Our results supported this idea [13]. If so, removing buried charges might be a way of increasing protein stability. We observed that removing a buried charge in RNase Sa increased the stability by over 3 kcal/mol [76]. The Garcia Moreno group has shown that burying a charge in a nonpolar environment in staphyloccocal nuclease can decrease the stability by over 5 kcal/mol [77]. However, if buried charged groups are hydrogen bonded they can make a large favorable contribution to protein stability [75].

Concluding Remarks

Until about 1990, the prevailing view was that intramolecular hydrogen bonds were necessary for maintaining the structure of proteins, but made, at most, a small contribution to the stability. The Fersht group was the first to show that the contribution of hydrogen bonds to binding energies is large [91], and our group was the first to show that the same was true for the hydrogen bonds stabilizing proteins [92]. The experimental results from many groups over the past few decades have confirmed that hydrophobic interactions do make the major contribution to protein stability but that hydrogen bonds also make a large contribution. In addition, global analyses based of the experimental results support this and show that hydrogen bonds make a favorable contribution to protein stability [93,94]. Applying the results presented in this review to 22 proteins containing from 36 to 548 amino acids, we found, on average, that hydrophobic interactions contribute 60 ± 4% and hydrogen bonds 40 ± 4% to protein stability (13).

In contrast, theoretical studies have been less successful. In an assessment of results from CAPRI (Critical Assessment of Predicted Interactions) the conclusion was that [95]: “We have generated a number of designed protein-protein interfaces with very favorable computed binding energies but which do not appear to be formed in experiments, suggesting that there may be important physical chemistry missing in the energy calculations. ⋯ the designed complexes typically stand out as having, on average, less optimal values than a majority of the natural complexes in terms of their van der Waals contacts, solvation self-energy, and electrostatic complementarity.” This was analyzed in more detail by Stranges and Kuhlman [96] who concluded: “These results suggest that Rosetta may not be accurately balancing hydrogen bonding and electrostatic energies against desolvation penalties and that design processes may not include sufficient sampling to identify side chains in preordered

conformations that can fully satisfy the hydrogen bonding potential of the interface." But there has been some success. Baker's group designed hydrogen bonds that contribute ≈ 2 kcal/mol to the energy of binding a ligand to a protein [97], in line with experimental studies of the contribution of hydrogen bonds to the stability of protein – peptide complexes [98].

Theoretical studies are clearly needed to reach a better understanding of the experimental results. With regard to nonpolar group burial, theoretical studies are needed to determine the relative contribution of the classic hydrophobic effect and van der Waals forces to the contribution of hydrophobic interactions to protein stability. With regard to polar group burial, theoretical studies are needed to determine the relative contributions of desolvation, hydrogen bonds, longer-range electrostatic interactions, and van der Waals forces to protein stability. The improved molecular dynamics simulations by Shaw's group look especially promising for improving our understanding of forces contributing to protein stability [99].

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Table 1

Protein interiors are tightly packed.[a]

Compound	Space Occupied By Atoms (%)
Water	36
Cyclohexane	44
Ethanol	47
Close-Packed Spheres	71
Protein Interior	75

[a] From [23].

Table 2

Calculated van der Waals interactions of a – CH_2– group with three environments.[a]

Surrounding Environment	Energy of interaction (kcal mol^{-1})
Water	−1.8
Cyclohexane	−1.9
Protein Interior	−3.1

[a] from [24].

Table 3

Δ(ΔG) values for 138 hydrophobic variants from 11 proteins.[a]

Variant[a]	Volume[b] (Å^3)	ΔG_{tr} to Octanol[c]	ΔG_{tr} to Cylohexane[d]	Range	Δ(ΔG)[e] Average	Per –CH_2–
Ile → Val (33)	25.8	−0.80	−0.88	−2.3 to 0.1	−1.1 ± 0.6	−1.1 ± 0.6
Val → Ala (39)	49.0	−1.24	−2.23	−4.9 to 0	−2.4 ± 1.1	−1.2 ± 0.5
Ile → Ala (26)	74.8	−2.04	−3.11	−5.4 to 0.7	−3.1 ± 1.2	−1.0 ± 0.4
Leu → Ala (40)	74.5	−1.90	−3.11	−6.2 to 0.2	−3.2 ± 1.2	−1.1 ± 0.4

[a] From [13].

[b] From [100].

[c] In kcal mol^{-1} [101].

[d] In kcal mol^{-1} [102].

[e] In kcal mol^{-1}. The negative values indicate a decrease in stability.

Table 4

ΔG_{tr} of a $-CH_2-$ group from water to three environments.[a]

Solvent	ΔG_{tr} (kcal mol)$^{-1}$)
Vapor	−0.2[b]
Octanol	−0.6[c]
Cyclohexane	−1.0[d]

[a] Experimental results show that each $-CH_2-$ group buried on folding contributes -1.1 ± 0.5 kcal mol^{-1} to protein stability (see Table 3).

[b] From [103].

[c] From [101].

[d] From [102].

Table 5

Δ(ΔG) values for 151 hydrogen bonding variants from 15 proteins for Tyr → Phe, Thr → Val, and Ser → Ala variants.

	Hydrogen-bonded		Not hydrogen-bonded	
Variant	**number**	**Δ(ΔG)[d] (kcal mol^{-1})**	**number**	**Δ(ΔG)[d] (kcal mol^{-1})**
Tyr → Phe [a]	35	−1.4 ± 0.9 (−3.6 to 1.2)	17	−0.2 ± 0.4 (−1.2 to 0.5)
Thr → Val [b]	25	−1.0 ± 1.0 (−3.5 to 1.9)	15	0.0 ± 0.5 (−1.7 to 1.0)
Ser → Ala [c]	44	−0.8 ± 0.9 (−3.8 to 1.3)	15	0.1 ± 0.4 (−0.8 to 0.5)

[a] Fifty-two Tyr → Phe variants from [37].

[b] Forty Thr → Val variants from [89].

[c] Fifty-nine Ser → Ala variants from [104].

[d] The negative values indicate a decrease in stability.

Table 6

ΔG_{tr} of an amide group from water to three environments.

Solvent	ΔG_{tr} (kcal mol^{-1})
Vapor	11.2 [a]
Cyclohexane	7.6 [b]
Octanol	1.4 [c]

[a] From [103].

[b] From [102].

[c] From [101].

Table 7

Contribution of hydrogen bonds to protein stability.

Variant	ΔG_{HB} (kcal mol^{-1})[a]
Ser → Ala[b]	−0.9 ± 0.9
Thr → Val[c]	−1.0 ± 0.5
Tyr → Phe[d]	−1.2 ± 0.6
Asn → Ala[e]	−1.1 ± 0.6
Peptide → Ester[f]	−1.1 ± 1.6

[a] ΔG_{HB} = Δ(ΔG)(hydrogen bonded) – Δ (ΔG)(not hydrogen bonded). See [34].

[b] From [104].

[c] From [89].

[d] From [37].

[e] From [39,40].

[f] From [51] and a personal communication from Evan Powers and Jeff Kelly.