Protein−Protein Interactions: Interface Structure, Binding Thermodynamics, and Mutational Analysis

Wesley E. Stites

Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, Arkansas 72701-1201

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I. Introduction

It is difficult to overstate the importance of protein−protein interactions in biological processes. Some of these interactions and their roles are well known, for instance, the formation of hormone−receptor, protease−inhibitor, or antibody−antigen complexes. Other instances may be less well-known, but no less important. For instance, in *Escherichia coli* regulation of the gene-regulatory catabolite activator protein's binding affinity has been linked to a dimer-monomer equilibrium.[1] Quite frequently in oligomeric proteins one finds binding sites, e.g., antibodies, or active sites, e.g., HIV protease, located at subunit interfaces. More subtle aspects of protein interactions are also important. Intersubunit interactions are vital to the function of allosteric proteins as exemplified by the relatively minor conformational changes at the hemoglobin protein−protein interface that are responsible for the cooperative nature of oxygen binding. Even when a direct functional role for quaternary structure is not obvious oligomeric forms of proteins may be less likely to unfold or be degraded than monomeric versions.

Wesley Stites was born in Ponca City, OK, in 1961 but from age 2 to 17 lived in Cañon City, CO. He received simultaneously his B.A. and M.A. in chemistry from the Johns Hopkins University in 1983. Working under the direction of Dr. Daniel Kemp at the Massachusetts Insititute of Technology in organic chemistry and peptide synthesis, he synthesized a conformationally locked medium ring β-turn template, earning his Ph.D. in biochemistry in 1988. Switching focus to molecular biology and biophysical chemistry, from 1988 to 1991 he worked as a postdoctoral fellow with Dr. David Shortle in the Department of Biological Chemistry at the Johns Hopkins University School of Medicine. In 1991 he took a faculty position in the Department of Chemistry and Biochemistry at the University of Arkansas. His research program revolves around exploring and understanding the underlying physical and chemical principles that determine the tertiary and quaternary structures of proteins.

Abnormal protein−protein interactions are also important in various disease processes. The conversion of the *neu* gene into an oncogene apparently occurs when mutations in the Neu receptor protein favor dimerization, which activates the tyrosine kinase domain of the protein.[2] The impact of an unwanted protein−protein interaction is illustrated in the textbook example of mutant hemoglobin oligomerizing in sickle cell anemia.[3] Less specific protein−protein interactions occur when proteins aggregate. Amyloid fibrils composed of aggregated protein form in the tissues in several diseases such as Alzheimer's, familial amyloidotic polyneuropathy, the spongiform encephalopathies (scrapie, Creutzfeldt−Jakob disease, kuru, etc.), familial Mediterranean fever, and some forms of rheumatoid arthritis.[4] Amyloid deposition can be a byproduct of some other underlying life-threatening condition such as renal

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failure, but for some diseases, possibly including Alzheimer's,[5] it appears that amyloid deposition is the causative agent of the illness. The loss of a protein–protein interaction can have unpleasant consequences as well. The oxidation of a specific methionine in α-1-proteinase inhibitor (also known as α-1-antitrypsin) obstructs its binding to various proteases, including elastase.[6] This oxidation occurs in the lungs of smokers.[7] The disruption of the protease–inhibitor interface leads to elastase activation which in turn appears to lead to the development of adult respiratory distress syndrome and emphysema.[8]

Aside from their considerable intrinsic importance, study of protein–protein interactions is also warranted because of the potential to bear on the fundamental problem of protein folding. The forces that are important in protein folding (hydrophobicity, hydrogen bonding, electrostatic interactions, van der Waals interactions, and so on) are precisely those that are responsible for protein–protein interaction. However, when approaching the problem of how two folded proteins, of relatively well-defined conformation at least on the level of the backbone, interact, we are relieved of the enormous conformational problem confronted in study of protein folding. The immense conformational flexibility of the denatured state and the lack of adequate models or even a basic understanding of the character of the denatured state has been a major barrier to theoretical calculation of protein stabilities. Quantification of the exact magnitude of the forces listed above is vital to the solution of the protein folding problem. Study of protein–protein interactions is an attractive route to the needed quantification because it removes most of the conformational uncertainty from issue.

Another aspect of the protein-folding problem is directly related to the problems of protein–protein interaction. Crystal structures of large proteins show that beyond a certain size proteins are invariably divided into fairly independent subunits known as domains, even though there may be just a single polypeptide chain. These domains can be seen to interact in ways that are analogous to the interactions among the subunits of oligomeric proteins and are believed to be relatively independent folding units.[9] Thus studies of interprotein interactions throw light on the intraprotein interactions of domains.

Protein–protein interactions have received less attention than the extensive study of the protein-folding problem. Nevertheless, a large amount of information is available and reviews of this area have appeared.[10−17] However much material has appeared recently, and no single review, including this one, can exhaustively cover all the possible topics involved in protein–protein interactions. In this article some of the common structural aspects of the interfaces of protein–protein complexes are first reviewed. The thermodynamics of protein–protein interactions have been thoroughly investigated in an increasing number of cases recently and this work is summarized. Lastly, mutational studies of protein–protein interactions are surveyed as well. Mutagenesis, of virtually every residue in some protein–protein interfaces, has revealed many interesting points about what factors are critical to complex formation.

II. Analysis of Protein–Protein Complex Crystal Structures

Many structures are now known of protein–protein complexes. Naturally, there are several distinct types of protein–protein complexes. An oligomeric protein composed of multiple identical subunits that do not have a stable monomeric folded state is clearly different in many regards from a heterodimer such as a protease–inhibitor or antibody–antigen complex where the subunits are stable in isolation. Even a dimeric protein such as triosephosphate isomerase[18] clearly differs from the somewhat special case of dimerization through leucine zippers (coiled-coils) observed for proteins such as Fos and Jun.[19] Nevertheless, there are some broad similarities in the structures of all these different types of protein–protein complexes. Several groups have analyzed large numbers of protein–protein complexes. This work has provided interesting insight into the structural similarities and differences between various types of protein–protein complexes. Some of these findings are summarized below.

A. Interface Size

What is considered as part of a protein–protein interface differs slightly from study to study. Definitions usually rely on proximity to the other protein or upon whether solvent accessibility on one protein is blocked by the other protein. However defined the surface area involved in protein–protein interactions can be easily measured in crystal structures. In dimeric proteins, the size of the interface area per subunit was found by Janin *et al.*[20] to range from 670 to 4890 Å^2. A more recent examination of 32 nonhomologous dimers by Jones and Thornton[21] found a slightly wider range of interface sizes, from 368 to 4761 Å^2. Argos[22] found in his examination of dimers that this represented slightly more than 12%, on average, of the accessible surface area of the individual monomers, but there is a wide range of 6.6–23.3%. Again, Jones and Thornton[21] found in their more recent survey a slightly wider range of 6.5–29.4% of the monomer surface area. The relatively small change despite the increase in the number of dimeric structures appearing in the seven years separating these surveys might imply that major excursions outside of these ranges are unlikely. The lower limit of observed interface area agrees well with theoretically based estimates of 1200 Å^2 of buried surface area required to allow stable association of a dimer, 600 Å^2 per monomer.[10, 23]

For oligomeric proteins with more subunits the interface areas tended to be at the high end of or above the range for dimers.[20] Similarly the percentage of monomer surface area involved in interactions climbs in higher oligomers. Tetramer monomers averaged 20.9% of their surface area buried upon oligomerization.[22] Crystal structures of protease–inhibitor complexes[24] and antibody–protein antigen complexes[24,25] show 600–1000 Å^2 buried per individual protein, for a total surface area buried in the

complexes of 1600 ± 350 Å^2. The percentage of the accessible surface area of the proteins that was buried ranged from 5 to 20%.

The amount of solvent-accessible surface area for any one of 23 oligomeric proteins examined in another study[26] was found to be related to the molecular weight of that protein by a simple power law. A later examination of a slightly larger group of proteins found a similar result.[27] The implication of this point is that given subunits of identical molecular weight, a greater proportion of the subunit's surface area is buried in subunit interfaces as the number of subunits in an oligomer increases. While this seems intuitive, the tight relationship observed between molecular weight and surface area is somewhat surprising. A similar relationship between surface area and molecular weight holds for monomeric proteins.[28] These two empirical relationships allow the rough prediction of amount of surface area involved in subunit interactions in an oligomeric protein if one knows the molecular weight of the subunits.

It is important to note that where large surface areas are buried by subunit association it appears that the tertiary structure of individual monomers is unstable presumably because of the large amounts of hydrophobic surface exposed in an isolated monomer.[9]

B. Number of Amino Acids Involved in Interfaces

The need to bury 600 Å^2 or more to form a stable protein–protein complex is at first a bit daunting. This would seem to require a square 25.5 Å on the side, much larger than most reasonably flat surfaces on a small protein. Of course, when protein surface area is measured using a probe the approximate size of a water molecule the surface is quite irregular with many side chains protruding out from the monomer surface.[22] This increases the surface area buried upon complex formation considerably. In an extended tripeptide model the solvent accessible surface area of the side chain of a phenylalanine is 135 Å^2, of a methionine, 115 Å^2, and tryptophan is 180 Å^2. Thus, the complete burial of one or two hydrophobic residues could, in theory, contribute the majority of the surface area buried in making a dimer. However, in practice, many more residues are involved in the average interface. One study defined any residue with an atom within 0.5 Å of an atom across the interface as a contact residue.[24] In this survey of 15 protease–inhibitor complexes and four antibody–antigen complexes the average number of interface residues was 34 ± 7 (in both proteins in the complex, 17 per monomer). This number is perhaps a bit more graspable measure of interface size than surface area buried.

C. Types of Amino Acids Involved in Interfaces

Examination of protein–protein complexes provides strong evidence for a principal role for hydrophobic residues in protein–protein association. Young *et al.*[29] examined 38 different proteins in complexes with other proteins, peptides, or peptide-like fragments for clusters of solvent accessible hydrophobic residues. The most hydrophobic cluster was found to compose more than one-third of the protein–protein interface in 25 out of 38 cases. The remaining cases all had one of the top six ranked hydrophobic clusters buried in the interface. Korn and Burnett[30] determined the hydropathy of the protein interior, interface surface, and noninterface surface of 40 multisubunit proteins and two antibody–protein complexes. They also found that interfaces were generally more hydrophobic than the remainder of the exterior. However there were some exceptions that were fairly hydrophilic. As one might expect, there was a complementarity of the interface hydropathy. Hydrophobic regions in one protein were matched across the interface with hydrophobic regions, and hydrophilic regions with hydrophilic.

Janin *et al.*[20] found that the amino acid composition of oligomeric protein interfaces, while intermediate between the composition of the interior and exterior, more closely resembled the protein's interior rather than the surface exposed to solvent. However, as we shall see this is in marked contrast to the results of others. They did note that compared to the protein interior there were a high number of charged residues found buried in interfaces. The number of charged residues found in the interior of proteins is quite low.[31] In particular, a disproportionate number of arginines were found at interfaces. Indeed, arginines comprised 9.9% of the surface area of the interfaces, second only to leucine with 10.5% and well ahead of the third most important contributor, valine, at 7.3%. Approximately 25% of the arginines were completely buried.

Argos[22] found the amino acid composition of oligomeric protein interfaces to be generally intermediate between the composition of the protein interior and exterior. The number of both charged and uncharged polar residues in the interface was intermediate between the numbers found for interior or exterior as was the number of nonaromatic hydrophobic residues. However, the aromatic hydrophobic residues were as well represented at interfaces as in the protein interior. He noted a preference for larger residues in form of the aromatics and also, interestingly, arginine. The intermediate nature of interfaces was also recently noted by Tsai *et al.*[32]

In their recent comprehensive analysis of dimeric proteins Jones and Thornton[21] describe the interface as more similar to the exterior of the protein than the interior. Their data do indicate that when dimer interface amino acid compositions differ from that of the exterior, they tend to differ in the direction of the composition found in the interior. Asparagine and, again, arginine were polar residues identified as having a particular affinity for the interface. Among nonpolar residue methionine and proline were slightly preferred in interfaces. Tyrosine was favored in interfaces, while phenylalanine and tryptophan were not.

When Janin and Chothia[24] examined interfaces in protease–inhibitor and antibody–protein complexes they also found little difference between interface surfaces and solvent exposed surfaces in the percentages of polar, nonpolar, and charged surfaces. In antibodies, they found that nonpolar residues were

disproportionately aromatic in character. Padlan[33] examined the complementarity-determining region (CDR) of seven crystallographically determined Fab structures. He also found that aromatic residues, in particular tyrosine, were more likely to be in the binding pocket than elsewhere in the molecule. (No distinction was made between the interior or exterior of the rest of the Fab.) However, histidine and asparagine were the most favored, by a factor of 8, to appear in the CDR relative to the rest of the Fab. Serine residues, although only slightly more likely to be found in the CDR than elsewhere in the Fab, were still the most common residue in the CDR, comprising 14.7% of residues. Next most common, at 13.13% of the residues in the CDR, was tyrosine. Asparagine was third at 8.32%. Although no explicit analysis of percent surface areas was made, it is clear from size and prevalence that tyrosines form the largest part of the surface of the CDRs. Mian *et al.*[34] also examined antibody CDRs, supplementing data from structures of six complexes and four free Fab fragments with the large database of known antibody sequences. They did not limit their survey to antibodies that bind proteins, but in reasonable agreement with Padlan, they found that the amino acids most likely to be in the binding site were tyrosine, tryptophan, serine, and asparagine.

There are clearly some differences in these results. However, there are more similarities. It appears that interfaces are more likely to incorporate nonpolar amino acids than the remainder of the protein exterior. There are some clear preferences for certain amino acids. Some of the differences and similarities can be rationalized. Janin *et al.*[20] speculated that ability of the guanidinium group to donate multiple H-bonds (see below) might be the reason for its statistical over representation. Aromatics, in particular tyrosine, seem to be the next most favored type of residue. There is considerably more variation here than in the case of arginine. Preference for aromatics appears to depend somewhat on the type of interface.[17] The preference is less clear, for example, in homodimers than in antibody–protein complexes.

One possible explanation for this is the fact that most homodimers spend most of their time as dimers while both the antibody and the protein it binds must be able to maintain a stable native fold as a monomer. It is not unreasonable to suppose that different amino acid side chains would thus be favored in these different types of interface. Consider, the case of a protein which requires stable monomeric state. Exposure of hydrophobic residues on the surface of proteins is often destabilizing.[9,35] While hydrophobic, for their sizes the aromatic residues have considerably lower hydrophobic transfer free energies than the aliphatic side chains.[36] It seems conceivable that the aromatics provide a way to cover relatively large amounts of normally solvent exposed surface, without paying too much of a price in destabilization of the protein native state.

A similar rationalization can be extended to methionine. Calmodulin, which binds to many different proteins, has eight methionines exposed in the binding site. It has been proposed by Gellman[37] that the flexibility of the methionine side chain[38] and the high polarizability of the sulfur (which increases the favorable enthalpy when interacting with nonpolar surfaces) allows the plastic interaction of the calmodulin binding site with different proteins. Janin *et al.*[20] did not identify methionine as an important interface residue, for it composes only 3.9% of oligomeric subunit interface surface areas. However, their data show that methionine is much more prevalent in interfaces than elsewhere in the proteins. Contrast the 3.9% contribution of methionine to interfacial surface area with its 2.9% contribution to other buried surface area and the even smaller 1.9% of exposed surface that it contributes. Given methionine's infrequency of occurrence one may question how much statistical significance this observation holds. But the idea that methionine is favored in protein–protein interfaces is buttressed by data from Padlan's analysis of Fab CDRs,[33] where methionine was the only small nonpolar (i.e., nonaromatic) residue that was more likely to be in the CDR than elsewhere in the molecule. Jones and Thornton[21] also report a slight preference for methionine in dimeric interfaces.

Just as for aromatics, methionine has a lower hydrophobic transfer free energy relative to its size than aliphatic side chains.[36] Further, there is now much evidence that methionine–aromatic interactions are particularly favorable because of the polarizability of the sulfur and the aromatic electrons.[39] Aromatic residues are quite certainly found in interfaces with high frequency. Thus it appears fairly safe to say that between theoretical considerations and empirical observations there is reason to believe methionine is indeed found more frequently in protein interfaces than chance alone would dictate. It is also clear that the interface amino acid composition depends in part upon the type of interface. More speculatively, aromatics and methionines may be favored over aliphatic side chains in interfaces that must spend considerable amounts of time exposed to solvent.

A related argument, based on a statistical analysis of the types of amino acids found in interfaces, has recently been made by Tsai *et al.*[32] They find that interfaces are more hydrophobic than protein exteriors in general, yet more polar than protein interiors. They conclude that this intermediate character is dictated by the need to promote association without destabilizing the unassociated monomer.

D. Structural Motifs and Secondary Structure at Interfaces

Closely related structural motifs for protein–protein interaction have been found in large numbers of proteins[40] with the leucine zipper and helix–loop–helix being two of the better known examples. This might seem to imply that there are limited number of ways for proteins to interact. Tsai *et al.*[41] recently published an exhaustive survey of the structures of protein–protein interfaces. Using a computer vision-based technique, they examined 1629 two-chain interfaces represented in the protein databank. These interfaces were clustered into 351 families. These families are based on the architecture of the interface

and were independent of the sequence and fold of the proteins participating in the interface. Indeed, several instances of similar interface interactions between proteins with dissimilar folds and dissimilar interface interactions between protein with similar folds are given. Clearly, a large number of different protein–protein interaction motifs are possible.

Is there then any strong tendency for certain types of secondary structure to dominate interface interactions? Argos[22] found that the secondary structural distribution of oligomeric protein interfaces more closely resembled the secondary structure found in exterior rather than interior residues of proteins, although there were not large differences between the distribution of secondary structural states in interior and exterior residues. Further he found that, for the most part, interfaces do not consist of long lengths of helix or sheet interacting. Leucine zippers are perhaps the exception to this rule. Instead in most protein–protein interfaces many distinct secondary structural elements contribute. Indeed, 70% of interfacial residues were the only contributing residue from a given secondary structural element. In an apparent contrast, Miller[42] found that sheet–sheet, helix–helix, and/or helix–sheet interactions were fairly common, appearing in virtually all interfaces of oligomer proteins examined. These seemingly contradictory observations can be reconciled by noting that while, as Miller shows, most interfaces have such interactions, Argos shows these interactions do not make up the majority of residues or the majority of the surface area of the interface.

Jones and Thornton[17,21] found that the amino acids that make up interfaces are discontinuous, with multiple segments separated by more than five residues in primary structure contributing to the interface. The number of different segments in dimeric interfaces ranged from two to 15. There was a weak correlation between the number of segments and the size of the interface. They found in agreement with Argos[22] that dimeric interfaces had levels of sheet, turn, and coil more similar to the protein interior than exterior. However, the amount of helix was much higher in interfaces than in the rest of the protein exterior.

The evidence for oligimerization of proteins by exchange of entire elements of secondary structure was recently reviewed.[43] The exchange, called 3-D domain swapping, has now been observed in a number of proteins and apparently is a reasonably common evolutionary mechanism for the generation of dimeric and higher oligomeric forms from ancestor monomers.

E. Hydrogen Bonding and Electrostatic Interactions

Janin *et al.*[20] found that the number of hydrogen bonds between subunits was found to be proportional to the area of the subunit interface. On average, one hydrogen bond is found for each 200 Å^2 of interface surface. However there is a fair amount of variability and several interfaces have few or no hydrogen bonds present. A side chain was involved as donor and/or acceptor in 78% of polar interactions (hydrogen bonds or salt bridges). Most polar interactions found by Janin *et al.* involved charges. Salt bridges, close interactions between oppositely charged groups, make up 22% and another 35% of these interactions have one charged and one neutral group. Arginine was the major side chain donor of hydrogen bonds, involved in 33% of all hydrogen-bond interactions and 42% of all polar interactions involving side chains. Jones and Thornton[17] also found that the number of hydrogen bonds in dimeric interfaces was roughly proportional to interface size. Four of the 32 proteins surveyed had no hydrogen-bonding interactions at all between subunits, and at the high end of the range 46 hydrogen bonds were found between subunits. Side chains were involved in 76.4% of the hydrogen bonds. In contrast to Janin *et al.*,[20] salt bridges were much less common. Eighteen proteins (56%) had no salt bridges between subunits and the highest number found was only five. There was no correlation between interface size and number of salt bridges.

In the 15 protease–inhibitor complexes and four antibody–antigen complexes examined by Janin and Chothia[24] there were an average of 10 hydrogen bonds across the recognition interface, more than in the oligomer interfaces. Both the protease–inhibitor and antibody–antigen complexes had comparable numbers of hydrogen bonds, however these hydrogen bonds were different in the nature of the groups participating. Antibody–protein antigen complexes were similar to oligomeric protein interfaces in that most hydrogen bonds involved side chains, but in protease–inhibitor complexes two-thirds of the hydrogen bonds involved main-chain atoms. While many charged groups were involved in hydrogen bonds, salt bridges were rare.

F. Packing, Cavities, and Shape Complementarity

The packing of the interfaces of the protease–inhibitor complexes and antibody–antigen complexes examined by Janin and Chothia[24] was as dense as the packing of crystalline amino acids with few cavities apparent. Walls and Sternberg[44] also found that three antibody–lysozyme structures have interface packing densities essentially identical to the global packing density. However tight packing does not mean that cavities are unknown, as an examination of the interfaces between the antibody constant domains CH1 and CL found a large cavity.[45] Because there are five different CH1 chains, α, γ, δ, ϵ, and μ, that each must be capable of interacting with two different CL chains, λ and κ, it was proposed the function of this cavity was to allow room for reorientation of the central interface residues without reorientation of the whole interface. In different crystal structures this cavity varies in size from 146 to 49 Å^3, at its largest more than sufficient to accommodate an aromatic side chain.

Again clear differences exist between types of protein–protein complexes. Lawrence and Colman[46] defined a shape complementarity statistic and found that dimeric proteins have the most complementary shapes, protease–inhibitor complexes less so, and antibody–protein complexes the worst. Jones and Thornton[17,21] define a gap volume index and confirm this general ranking of complementarity.

In perhaps the most thorough investigation of cavities at interfaces to date, Hubbard and Argos[47]

examined oligomeric complexes as well as domain interfaces. They found that 77% of oligomers have at least one cavity in their interfaces and that these cavities make up 30% of all cavity volume in the entire structure. Seven of the 52 oligomeric surfaces had cavities comprising more than 20% of the interface surface area. Most of these cavities were solvent filled, frequently with hydrogen bonds forming between the protein and the waters in these cavities. Interdomain surfaces were generally more tightly packed with fewer cavities. While some of these cavities may be merely tolerated, Hubbard and Argos point out that interface cavities may often be important to protein function, such as providing flexibility for subunit movement in allosteric enzymes, or channels for substrate movement.

An interesting study by Pawowski *et al.*[48] examined the source of quaternary structural diversity and specificity in the EF-hand calcium-binding protein family. They conclude that the interactions are controlled by the location of a hydrophobic interface on the surface of the protein and by blocking other potential interaction sites with additional sequence fragments. In other words, complementarity as dictated by interface topology appears to contribute to interface specificity.

G. Backbone and Side-Chain Rearrangements

Since many uncomplexed oligomeric proteins are unfolded in the monomeric state, the structures of the monomer are difficult to obtain and compare to the complexed form. However, as these proteins are folded in the complex, it is evident large changes in backbone and side chain conformations can occur upon complex formation. Comparison of the components of associated and unassociated forms of heterogeneous complexes has been possible. Padlan[33] found little evidence in Fab CDRs of conformational change, particularly in the backbone, upon binding. This was also implied by Argos's analysis of secondary structure distributions.[22] In their study of protease–inhibitor complexes and antibody–antigen complexes Janin and Chothia[24] found that when the crystal structure of one of the unassociated proteins was known, comparison to the associated form showed only minor rearrangements in the backbone. Side-chain conformations changed in some instances also. These appeared to be low-energy conformational changes to enable H-bond formation and packing of residues.

However, since these earlier surveys several instances of large conformational rearrangements have been reported. An antibody against foot and mouth virus showed significant changes in the main-chain conformation of a CDR loop upon complex formation with the antigenic peptide.[49] These changes may be low in energy as there is some evidence that the conformation found in the bound form may be present in low amounts in the unbound form. An antibody against a HIV peptide showed movement of the main chain of one CDR loop by several angstroms and movement of another CDR loop as a rigid body by over one angstrom.[50] A recent study of antibody bound to protein antigen also shows rearrangements of the main chain.[51] The antibody D1.3 was raised against hen egg white lysozyme but also binds, albeit less tightly, to the homologous turkey egg white lysozyme. The structure of D1.3 bound to the hen protein differs from D1.3 bound to the turkey protein by peptide flip in one of the CDR loops. This apparently facilitates hydrogen bonding with a contact residue that is different in the two antigens. This conformational change could be largely responsible for the reduced affinity of the antibody for the turkey protein. While structural changes clearly can occur whether they are "major" depends in good part on the scale used to define them. At the largest scale, that of gross topography, even the changes cited above are small, never resulting in a change of the overall interface shape from a cavity to a planar surface or *vice versa*.[52] Instead of using the distances or number of residues moved perhaps major conformational changes are better defined as those that come at an energetically significant price. While crystal structures are silent on energetics, the energetics of protein–protein interactions have been extensively investigated through other techniques and may be coupled with structural studies to investigate points such as these.

H. Summary

Interfaces make up an appreciable fraction of the surface of the monomers involved in a protein–protein complex, with nearly 20 residues per monomer participating in the interface on average. The amino acid composition of interfaces can be loosely described as intermediate between that of protein interiors and protein exteriors. There appears to be a high degree of variability in amino acid composition depending on type of protein–protein complex; however, arginines and aromatic residues seem to be particularly favored in interfaces. There does not appear to be a strong preference for any particular type of secondary structure or structural motif in protein–protein interfaces, although many disparate secondary structural elements typically contribute to a given interface. Hydrogen bonding and electrostatic interactions are also quite variable, although on average the number of such interactions are roughly proportional to interface size. The packing and shape complementarity of interfaces is generally fairly good. However cavities are still quite frequently found. Again, the type of protein–protein complex is reflected in the goodness of fit between the proteins, with dimeric proteins having the best matching of shape. In cases where the monomers are stable outside of the protein–protein complex, this complementation of shape appears to be achieved without major conformational rearrangement of the backbone and side chains away from the low-energy conformations found in the monomeric form.

III. The Thermodynamics of Protein–Protein Interactions

The value of the association constant is one of the first things determined in the detailed study of any protein–protein complex. New instrumentation such as Pharmicia's BIAcore has improved the speed, precision, and accuracy with which binding constants

can be determined and also allows ready determination of on and off rates. Association constants are of great practical utility for such purposes as determining at what protein concentrations a complex might be formed. However, they are also of great interest in the more theoretical comparison of the energetics and rates of complex formation to the structure of the complex. An association constant is of course determined by the free energy difference between the associated and unassociated states of the proteins. However, the free energy change of complex formation is only one part of the thermodynamics. The change in enthalpy, entropy, and heat capacity all provide useful information about the importance of various factors involved in the association. In combination ΔG and ΔH allow calculation of ΔS. If binding enthalpies are determined at a number of different temperatures then ΔC_p, which is the first derivative of ΔH with respect to temperature, can be determined as well. For many years it was impractical outside of a few specialized laboratories to directly determine ΔH for an association reaction. The variation of an association constant with temperature could and frequently has been used in a van't Hoff analysis to infer values for ΔH. But, as recently pointed out, disagreement between van't Hoff and calorimetric enthalpies is quite frequent,[53] perhaps because of inappropriate application of the simplified van't Hoff equation[54,55] or because the assumption that there is no thermodynamically significant conformational change in the unfolded state of a protein over an extended temperature range is incorrect.[56] Fortunately calorimetric instrumentation that can be used to directly determine binding enthalpies has recently become increasingly available from Microcal and CSC.[57] In the last review of protein–protein association thermodynamics 11 years ago only 10 cases of calorimetric study were listed.[58] This area has now grown dramatically with much new work appearing in just the last few years.

A. Calorimetrically Determined Thermodynamics of Association

Listed in Table 1 are the thermodynamics of association for 43 protein–protein (Table 1A) and 26 protein–peptide (Table 1B) systems.[56,59–101] The enthalpies were all calorimetrically determined at a constant temperature. Most of these values were determined with isothermal titration calorimetry, although some, usually earlier, determinations used other calorimetric techniques such as flow or batch calorimetry. The free energies were often derived calorimetrically, but the ΔG of formation for more stable complexes is difficult to determine with isothermal titration calorimetry so some were determined by noncalorimetric techniques such as surface plasmon resonance. Some of the values shown in Table 1 are calculated from data in the reference and not directly given in those references. Every effort has been made to make Table 1 as inclusive as possible in terms of the number of different protein–protein systems studied. If values of ΔG and calorimetric ΔH at constant temperature were available for an associating protein system of well-defined stoichiometry it was included. On the other hand, the same complex is often studied under different conditions of pH, ionic strength, type of buffer, and/or temperature. In addition, thermodynamics have been determined for complexes that are very closely related, such as studies of a series of single site mutants of one protein binding to another protein. In such instances, one representative case is typically shown in Table 1. The effects of some of these variations are discussed in more detail below in this and the subsequent section.

In the recent past it has been asserted that protein–protein interactions are enthalpically driven[102] and, contradictorily, that they are usually entropy driven.[54] Of course, if the value of ΔC_p is large, the values of ΔS and ΔH will be temperature dependent and a temperature at which either entropy or enthalpy drives the reaction may be found. The values in Table 1, most determined near 25 °C, support neither entropy or enthalpy as the primary driving force at this temperature. In 31 cases the enthalpy is favorable but the entropy of association is unfavorable. There are 18 cases where association is driven by entropy and enthalpically opposed. In the remaining 20 cases both enthalpy and entropy favor association. In 74% of the cases, enthalpy favors association while entropy favors complex formation only in 55%. However, no broad generalization is possible beyond this as a wide range of values are found. There are no obvious correlations between values of ΔH or ΔS with ΔG. Neither do ΔG, ΔH, or ΔS correlate with ΔC_p.

Although ranges and averages are not particularly meaningful in any fundamental thermodynamic sense they do allow us to compare the two classes of interactions, protein–protein and protein–peptide, that Table 1 is divided between. The range of ΔG is −7.0 to −17.2 kcal/mol for protein–protein interactions. For protein–peptide (or, in two instances, peptide-peptide) interactions the range is −5.3 to −11.7 kcal/mol. The range of ΔH and ΔS is +12.6 to −66.7 kcal/mol and +78.6 to −188.4 cal/(mol K) respectively for protein–protein interactions and +19.9 to −41.9 kcal/mol and +95.7 to −109 cal/(mol K) respectively for protein–peptide interactions. The values of ΔC_p for protein–protein interactions range from 2 to −767 cal/(mol K) and −100 to −1200 cal/(mol K) for protein–peptide interactions. The average protein–protein ΔG is −10.40 kcal/mol with a standard deviation of 2.49 kcal/mol and the average protein–peptide ΔG is −8.5 ± 1.88 kcal/mol. The average ΔH of protein–protein interactions is −8.60 ± 13.63 kcal/mol and that of protein–peptide interactions is −8.90 ± 11.23 kcal/mol. Protein–protein interactions have an average ΔS of 6.12 ± 43.68 cal/(mol K) and protein–peptide interactions average −1.13 ± 37.86 cal/(mol K). The average values of ΔC_p for protein–protein and protein–peptide interactions are −333 ± 202 and −447 ± 309 cal/(mol K) respectively.

B. Entropy and Conformational Restriction: Protein–Protein vs Protein–Peptide Interactions

It is striking and initially surprising that there is very little difference in the average thermodynamics of protein–protein and protein–peptide interactions.

Table 1[a]

complex	ΔG, kcal/mol	ΔH, kcal/mol	ΔS, cal/(mol K)	ΔC_p, cal/(mol K)	T, °C	buffer	ref
A. Protein−Protein Systems							
trypsin−soybean inhibitor	−12.3[a]	8.6	69.8	−442	25	200 mM KCl, 50 mM $CaCl_2$, pH 5.0	59
trypsin−cleaved soybean inhibitor	−10.8	12.6	78.6	−387	25	200 mM KCl, 50 mM $CaCl_2$, pH 5.0	59
trypsin−ovomucoid	−10.2	5.6	53.1	−270	25	200 mM KCl, 50 mM $CaCl_2$, pH 5.0	59
trypsin−lima bean inhibitor	−12.7	2.1	49.7	−430	25	200 mM KCl, 50 mM $CaCl_2$, pH 5.0	59
trypsin−bovine pancreatic trypsin inhibitor	−10.7	2.5	44.7		22	50 mM Tris−HCl, 20 mM $CaCl_2$, 0.005% Triton X-100, pH 8.2	60
subtilisin inhibitor−chymotrypsin	−7.1	4.5	38.2	−260	25	25 mM potassium phosphate, adjusted to 100 mM ionic strength with KCl, pH 7.0	61
subtilisin inhibitor−subtilisin	−13.8	−4.7	31.1	−240	25	25 mM potassium phosphate, adjusted to 100 mM ionic strength with KCl, pH 7.0	62
calmodulin Ca^{2+}−myosin light chain kinase	−11.5	−20.3	−29.2		25	50 mM Pipes/NaOH, 150 mM NaCl, 0.5 mM $CaCl_2$, pH 7.5	63
calmodulin Ca^{2+}−seminal plasmin	−12.0	−12.0	0.0	0	25	50 mM Pipes/NaOH, 150 mM NaCl, 0.5 mM $CaCl_2$, pH 7.5	63
calmodulin−myosin light chain kinase	−7.2	0.0	24.1		25	50 mM Pipes/NaOH, 150 mM NaCl, pH 7.5	63
calmodulin−seminal plasmin	−8.1	0.0	27.2		25	50 mM Pipes/NaOH, 150 mM NaCl, pH 7.5	63
ch4D5 Fab−p185HER2-ECD	−13.5	−17.2	−12.0	−400	25	20 mM sodium phosphate, 100 mM NaCl, pH 7.5	64
HyHEL-5−hen egg lysozyme	−14.5	−22.6	−27.2	−340	25	10 mM sodium phosphate, pH 8.0	65
HyHEL-10−hen egg lysozyme	−12.0	−21.9	−32.6	−335	30	50 mM phosphate, 200 mM NaCl, pH 7.2	66
D1.3−hen egg lysozyme	−11.5	−21.7	−34.4	−380	24.2	10 mM sodium phosphate, 150 mM NaCl, pH 7.1	67
F9.13.7−hen egg lysozyme	−12.0	−11.1	3.3	−650	23.9	10 mM sodium phosphate, 150 mM NaCl, pH 7.1	68
D44.1−hen egg lysozyme	−9.7	−10.3	−2.3	−280	24.2	10 mM sodium phosphate, 150 mM NaCl, pH 7.1	68
D11.15−hen egg lysozyme	−12.1	−19.0	−23.0	−240	24.9	10 mM sodium phosphate, 150 mM NaCl, pH 7.1	68
D1.3−E5.2	−10.4	−66.7	−188.4		25	10 mM sodium phosphate, 150 mM NaCl, pH 7.1	69
D1.3−E225	−7.3	1.8	30.4	0	28.3	10 mM sodium phosphate, 150 mM NaCl, pH 7.1	69
ferredoxin−ferredoxin:$NADP^{+}$ reductase	−9.3	−0.3	30.1	−160	27	50 mM Tris-HCl, pH 7.5	70
colicin N−OmpF	−7.7	−12.3	−15.3		25	20 mM Tris, 300 mM NaCl, 1% octyl-polyoxyethylene, pH 7.4	71
colicin N−OmpC	−7.1	−3.7	11.2		25	20 mM Tris, 300mM NaCl, 1% octyl-Polyoxyethylene, pH 7.4	71
colicin N−PhoE	−7.4	−6.0	4.8		25	20 mM Tris, 300mM NaCl, 1% octyl-Polyoxyethylene, pH 7.4	71
barstar−barnase	−17.2	−13.9	12.2	−190	25	50 mM Pipes, pH 7.0	72
human tissue factor−coagulation factor VII	−11.2	−32.0	−70.0	−730	25	20 mM Tris-HCl, 100 mM NaCl, 5 mM $CaCl_2$, pH 7.5	73
cytochrome c peroxidase−cytochrome c	−7.0	2.3	31.0	2	26	50 mM ionic strength, phosphate/KNO_3, pH 6.0	74
cytochrome b5−cytochrome c	−9.1	1.0	33.9		25	2 mM Tris-HCl, pH 7.4	75
Ab E3−cytochrome c	−9.7	−7.3	8.8	−350	25	20 mM sodium/potassium phosphate, 0.8% NaCl, 0.02% KCl, pH 7.2	76
Ab E8−cytochrome c	−9.5	−9.5	0.3	−165	25	20 mM sodium/potassium phosphate, 0.8% NaCl, 0.02% KCl, pH 7.2	76
Ab 2B5−cytochrome c	−12.6	−21.0	−28.2	−580	25	100 mM sodium phosphate, pH 7.0	77
Ab 5F8−cytochrome c	−13.9	−21.7	−26.3	−172	25	100 mM sodium phosphate, pH 7.0	77
CheY−$CheA_{1-233}$	−8.1	−12.3	−14.4	−230	28	20 mM sodium phosphate, 20 mM NaCl, 1 mM EDTA, 1 mM PMSF, 10% v/v glycerol, pH 7.4	78
Che B−$CheA_{1-233}$	−7.5	−10.1	−8.7	−450	28	20 mM sodium phosphate, 20 mM NaCl, 1 mM EDTA, 1 mM PMSF, 10% v/v glycerol, pH 7.4	78
Interleukin 5−IL5 receptor α subunit	−11.6	−11.4	0.7	−650	25	20 mM potassium phosphate, 150 mM NaCl, pH 7.4	79
erythropoietin−EPO receptor site 1	−11.7	−1.5	34.2		25	Dulbecco's phosphate buffered saline, pH 7.0	80
erythropoietin−EPO receptor site 2	−8.4	−3.4	16.8		25	Dulbecco's phosphate buffered saline, pH 7.0	80
human growth hormone G120R−hGHbp	−11.7	−9.4	7.7	−767	26.2	10 mM sodium phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.2	81
phosphocarrier protein−enzyme I N-domain	−7.0	8.8	53.0		25	10 mM potassium phosphate, 100 mM KCl, 1 mM EDTA, pH 7.5	82
stem cell factor−Kit extracellular domain	−9.0	−13.0	−13.4		25	phosphate buffered saline	83
Hck SH3 domain−HIV-1 Nef	−9.2	−12.8	−12		25	50 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 2 mM DTT, pH 7.4	84
Fyn SH3 domain−PI3-Kinase p85 subunit	−7.6	10.6	60.2		30	10 mM phosphate, pH 6.0	85
elastase−ovomucoid third domain	−14.5	−1.0	45.3	−222	25	50 mM imidazole chloride, pH 7.0	86

B. Protein−Peptide Systems							
Ab 131−angiotensin II	−11.0	−8.9	6.9	−240	30	20 mM buffer, 50 mM NaCl, pH 7.3	87
endothiapepsin−pepstatin A	−9.1	−2.5	22.8		16.1	20 mM phosphate, pH 7.0	88
HK 565 peptide−PK 262 peptide	−5.9	0.4	21.1		30	30 mM phosphate, pH 7.0	89
human Grb2 (SH3) −human Sos peptide	−6.4	−6.2	0.7		25	50 mM potassium phosphate, 1 mM DTT, pH 8.0	90
Fyn SH3 domain−P2L peptide	−6.6	−12.3	−18.6		30	10 mM phosphate, pH 6.0	85
Lck SH2 domain−Lck phosphopeptide	−7.3	−8.4	−3.5		25	50 mM Mops, 100 nM NaCl, 1 mM DTT, pH 6.8	91
p85 SH2 domain−PDGFR phosphopeptide	−8.7	−9.4	−2.4		25	50 mM Mops, 100 nM NaCl, 1 mM DTT, pH 6.8	91
Src SH2 domain−pYHmT phosphopeptide	−8.6	−8.4	0.4		25	50 mM Mops, 100 nM NaCl, 1 mM DTT, pH 6.8	91
Fyn SH2 domain−pYHmT phosphopeptide	−6.3	−4.3	6.6	−100	25	10 mM potassium phosphate, 30 mM NaCl, 5 mM DTT, pH 6.0	92
Fyn SH2 domain−pY531 phosphopeptide	−8.4	−8.7	−1.3	−270	25	10 mM potassium phosphate, 30 mM NaCl, 5 mM DTT, pH 6.0	92
SHC N-term. domain−EGFR1148 peptide	−10.3	−5.46	16.2	−185	25	100 mM Hepes, 100 mM NaCl, 1 mM DTT, pH 7.5	93
SHC N-term. domain−Trk490 peptide	−10.1	2.4	41.7	−207	25	100 mM Hepes, 100 mM NaCl, 1 mM DTT, pH 7.5	93
Ab 13AD−peptide LZ	−10.5	−12.6	−7.1	−251	27	1.46 mM KH_2PO_4, 6.46 mM Na_2HPO_4, 0.14 M NaCl, 0.27 mM KCl, pH 7.2	94
Ab 13AD−peptide LZ(7P14P)	−9.5	−17.4	−26.0	−366	27	1.46 mM KH_2PO_4, 6.46 mM Na_2HPO_4, 0.14 M NaCl, 0.27 mM KCl, pH 7.2	94
Ab 29AB−peptide LZ	−11.0	−13.8	−9.5	−335	27	1.46 mM KH_2PO_4, 6.46 mM Na_2HPO_4, 0.14 M NaCl, 0.27 mM KCl, pH 7.2	94
Ab 29AB−peptide LZ(7P14P)	−9.5	−17.1	−25.8	−392	27	1.46 mM KH_2PO_4, 6.46 mM Na_2HPO_4, 0.14 M NaCl, 0.27 mM KCl, pH 7.2	94
Ab 42PF−peptide LZ(7P14P)	−10.4	−13.4	−10.0	−691	27	1.46 mM KH_2PO_4, 6.46 mM Na_2HPO_4, 0.14 M NaCl, 0.27 mM KCl, pH 7.2	94
calmodulinCa^{2+}−melittin	−11.7	7.2	63.3		25	50 mM Pipes/NaOH, 150 mM NaCl, 0.5 mM $CaCl_2$, pH 7.0	95
calmodulin−melittin	−8.1	4.8	43.3		25	50 mM Pipes/NaOH, 150 mM NaCl, pH 7.0	95
ribonuclease S−truncated S peptide	−9.4	−41.9	−109.0	−1200	25	50 mM sodium acetate, 100 mM NaCl, pH 6.0	96
streptavidin−FSHPQNT peptide	−5.3	−19.3	−47.0		25	50 mM potassium phosphate, pH 7.62	97
streptavidin−pStrep-tag	−6.1	−12.6	−21.8		25	50 mM potassium phosphate, pH 7.62	98
CheR−receptor pentapeptide	−7.9	−13.6	−18.9		28	20 mM potassium phosphate, 20 mM NaCl, 1 mM EDTA, 1 mM PMSF, pH 7.0	99
profilin−Pro_{11}	−5.4	−5.1	1.1		28	10 mM Tris, 75 mM KCl, 3.1 mM NaN_3, pH 7.5	100
A−B heterodimeric coiled coil	−10.6	−24.7	−48.3	−720	20	10 mM sodium phosphate, ionic strength 22 mM, pH 7.2	56
GroEL−unfolded subtilisin BPN′ mutant	−7.6	19.9	95.7	−850	14.3	50 mM Tris, 100 mM KCl, 1 mM DTT, 0.1 mM EDTA, pH 7.8	101

[a] All figures are given to the same number of digits, 0.1 kcal/mol in the case of ΔG and ΔH. This is not meant to imply the actual significant digits or error in the values, which is frequently much higher. Errors reported in various publications are often not directly comparable, being based in some cases on returns from fitting programs, in others on the averages of multiple experimental determinations. For this reason and for considerations of space readers are referred to the original publications for further detail on errors of individual measurements. However as a rough estimate, a "typical" error in ΔG and ΔH is 0.3 to 1.0 kcal/mol, with ΔG generally being less reliable for tight binding complexes and ΔH being least reliable when values are near zero. The "typical" error for ΔS is higher, since error in both ΔG and ΔH contribute, but 10 cal/(mol K) is a reasonable estimate. An error of 20−100 cal/(mol K) is "typical" for ΔC_p.

Protein–protein interactions differ from protein–peptide interactions in two obvious ways. Perhaps most obvious is the size of one of the partners in the association reaction. However, it is not always clear how large the interaction domain is in a protein–protein interaction, so a protein–protein interaction might truly involve no more residues than a protein–peptide interaction. Indeed, in one case classified as a protein–peptide interaction, GroEL binding to a destabilized, unfolded subtilisin BPN′ mutant, both polypeptides are large. As this implies the major consideration in classification was whether or not both polypeptides had a well-defined tertiary structure before the formation of the complex. A peptide or unfolded protein binding to another protein should lose many degrees of conformational freedom. Again, there are some cases classified as protein–protein interactions where a surface loop might be quite mobile before binding, and some of the peptides have strong conformational preferences before binding (*vide infra*), but on the whole it seems likely that complex formation for the cases listed in Table 1B would involve the loss of more degrees of conformational freedom than those listed in Table 1A.

The naive expectation is that the change in entropy upon binding should be much less favorable for protein–peptide interactions than for protein–protein interactions. In fact there is some evidence to support this idea as the entropy of association for protein–peptide systems is favorable in 46.1% of the cases in Table 1B, while it is favorable in 60.5% of the cases in Table 1A. However, the averages and ranges cited above show little difference in either ΔS or ΔH between these two broad classifications of interactions. Other entropic factors common to both protein–protein and protein–peptide interactions, such as solvent reorganization, rotational, vibrational and the loss of side-chain conformational freedom[103] apparently so dominate the entropy of association that the signs of main chain conformational restriction are often obscured. The point that determinations of ΔH and ΔS at a single set of conditions are extremely difficult to interpret in terms of conformational and hydration changes of the associating system has previously been made[104] but is emphasized by the data in parts A and B of Table 1.

Nevertheless, an interesting experiment by Leder *et al.*[94] does seem to show a clear thermodynamic signal of major conformational rearrangement upon binding. Antibodies 13AD and 29AB were raised against the 29 amino acid peptide LZ, a peptide that forms a stable dimeric (and trimeric) coiled coil. These antibodies cross react with the homologous peptide LZ(7P14P). Differing by the substitution of two prolines, this monomeric peptide shows no helical CD signal in solution. Fluorescence and CD spectra indicate that LZ(7P14P) is forced into a coiled coil conformation when it binds to either 13AD or 29AB. As shown in Table 1B, the entropy of binding to the coiled coil LZ peptide is not favorable, but the entropy of binding to LZ(7P14P) is even more unfavorable. Presumably this reflects the lost conformational entropy of the transition from a random coil to a coiled coil as well as the lost degrees of translational and rotational freedom from the peptide dimerization required to form the coiled coil. While ΔS is quite different for the two peptides, ΔG for binding does not change by a great deal. The compensating change in ΔH that limits the change in ΔG likely reflects a favorable ΔH for the formation of the coiled coil. An antibody raised against the LZ(7P14P) peptide, 42PF, showed enthalpy and entropy of binding quite comparable to those of 13AD and 29AB against LZ. Although not shown in Table 1, data was presented in this work showing that binding 42PF to another coiled coil peptide similar to LZ caused that coiled coil to disassociate before binding to antibody. The enthalpy of antibody binding was correspondingly less favorable and the entropy actually became slightly favorable. Similar changes in enthalpy and entropy of binding were observed in the binding to Fyn SH3 domain of PI3-kinase p85 subunit and the P2L peptide derived from it[85] although this case is complicated by the fact that the intact protein apparently has other energetically significant binding interactions.[84,105]

Another protein–peptide system examined by Petrella *et al.*[100] was the binding of various polyproline peptides to profilin. In this case the peptide was disordered before binding. Substitution of a single glycine or alanine residue in place of a proline had relatively little effect on the enthalpy of binding, but a much larger effect on entropy of binding. These studies show that the entropic effects of conformational restriction upon binding can be discerned in favorable circumstances. Still, the overall range of data in Table 1, parts A and B, indicates that confidently stating that unfavorable entropies of binding are due to conformational restrictions upon binding is not possible in the absence of further information.

C. Relative Contributions of Hydrophobicity and Other Interactions to Association: The Meaning of ΔC_p

It is widely accepted that protein folding is driven to a large extent by hydrophobic interactions. This belief is based in part on calorimetrically derived thermodynamic evidence. What does Table 1 tell us about the contribution of hydrophobicity to protein–protein association? The transfer of a hydrophobic molecule from water to a nonpolar liquid at room temperature is characterized by an unfavorable enthalpy but a favorable entropy. Despite this fact simply examining the relative values of ΔH and ΔS of protein–protein interaction is not sufficient to determine the impact of the hydrophobic effect as many other processes can contribute to these quantities. A more distinctive thermodynamic signature for the burial of hydrophobic surface is the large negative ΔC_p commonly found for this process.[106] The burial of polar surface in a nonpolar environment has a positive ΔC_p,[107] but is of lower magnitude and it has been widely accepted that the hydrophobic effect dominates ΔC_p for protein folding and binding.[103,108] Determination of ΔC_p requires carrying out the binding experiment at different temperatures, and, as Table 1 shows, not all workers have done this. In those systems were it has been measured ΔC_p of association is usually large and negative. Although the magnitudes of ΔC_p in protein folding reactions

are generally larger, this is consistent with the larger amounts of hydrophobic surface buried in protein folding compared to protein association.[109] This seems to indicate that the hydrophobic effect is usually quite important in protein–protein interactions. Nonetheless, there are three prominent exceptions to this trend and, more generally, some reason to doubt whether conclusions about the relationship between ΔC_p and buried hydrophobic surface are reliable in the study of protein–protein interactions.

The Ca^{2+} saturated form of calmodulin binds to a variety of proteins, including seminalplasmin. Using data from "preliminary experiments" found in Milos *et al.*[63] the ΔC_p for this process appears to be approximately zero. The binding of monoclonal antibody D1.3 to the antiidiotopic antibody E225 also appears to have a very low value of ΔC_p.[69] The most thoroughly characterized case is the binding of cytochrome *c* peroxidase to cytochrome *c*.[74] The binding is entropy driven, at first glance consistent with a large hydrophobic component to binding. The more detailed analysis carried out in this work shows the danger of leaping to that conclusion. Although correction for buffer ionization (*vide infra*) lowers ΔC_p slightly from the uncorrected value of 2 cal/(mol K) in Table 1 to -28 ± 10 cal/(mol K) this is still remarkably close to zero. This seems to be strong evidence that this association reaction is driven by factors other than hydrophobicity. Adding to the plausibility of this idea is the fact that the binding of cytochrome *c* not only to cytochrome *c* peroxidase but to a number of other proteins in the electron transport system shows a very strong dependence of binding constant upon ionic strength.[110] Perhaps for this protein–protein interaction the principal driving forces are electrostatic or van der Waals interactions. Kresheck *et al.*[74] explored this possibility by examining the binding thermodynamics as a function of ionic strength. The ΔG of binding extrapolated to infinite ionic strength, where electrostatic interactions should be negligible, is essentially zero, indicating that electrostatic effects dominate this particular protein–protein interaction.

It is clear from other entries in Table 1 that this is not a property inherent to all protein–protein interactions involving cytochrome *c*. Four different antibodies binding to cytochrome *c* have been examined by two different groups.[76,77] In none of these four association reactions is the association enthalpy temperature independent in the manner of cytochrome *c*–cytochrome *c* peroxidase. This would seem to indicate that hydrophobic interactions are important in these protein–protein associations. Notwithstanding this, there are clear differences between the four binding reactions, with two lower magnitude values of ΔC_p and two higher magnitude values. This brings us back to the question implicit in the earlier discussion. Does this and similar variation in ΔC_p for other association reactions tell us something specific and quantitative about the relative contribution of hydrophobic interactions to the stability of different complexes? In protein-folding correlation between the value of ΔC_p and the amount of hydrophobic and polar surface area buried seems to be present, although the precise weights assigned to the effect upon ΔC_p of burying hydrophobic and polar surface area vary.[88,109,111] Finding a correlation for a protein–protein complex requires both a crystal structure and a value for ΔC_p. From those cases where it has been examined it is not completely clear if such a relationship exists for protein–protein association. Good correlations between observed values for ΔC_p and the relative amounts of hydrophobic and polar surface area buried were found for the endothiapepsin–pepstatin[88] interaction, the binding of HyHEL-5 with hen egg white lysozyme,[65] and angiotensin II–Fab complex formation.[87] On the other hand in the ribonuclease S system the ΔC_p values of S-peptides differing by a single hydrophobic residue varied substantially and without apparent correlation to the amount of nonpolar surface buried.[96] Mutations in the interface of the human growth hormone–hGH receptor resulted in values of ΔC_p *more* negative by as much as 200 cal/(mol K), very surprising given that some of these mutations removed large hydrophobic residues buried in the interface. As this implies, the actual changes in buried hydrophobic and polar surface buried as measured from crystal structures of the original and mutated complexes differed substantially from those calculated from changes in ΔC_p.[81] Similar disparities were found in mutants of an antibody against epidermal growth factor receptor, although in this case mutant crystal structures were not available to confirm changes in buried surface area.[64,112] Again, changes in ΔC_p upon removal of buried hydrophobic amino acids appeared to indicate the burial of more hydrophobic surface rather than less.

Bhat *et al.*[67] found that the observed ΔC_p for the binding of antibody D1.3 to hen egg white lysozyme agreed well with a rough value calculated from the surface area buried. However, they conclude that this agreement is accidental. They argue that the rest of their data indicates that the hydrophobic effect is not a major driving force for the association. Indeed, subsequent more detailed calculations from the same group[68] resulted in a calculated ΔC_p value much lower than the experimental value.

Why does ΔC_p often not correlate well with the amount of polar and nonpolar surface buried in a protein–protein interaction? Another recent result might answer this and other questions about the interpretation of ΔC_p in association reactions. Guinto and Di Cera[113] found that the binding of a single sodium ion to thrombin has a ΔC_p of -1100 cal/(mol K). This very large, negative value is greater in magnitude than most of the values listed in Table 1. It appears that sodium binding is not linked to large conformational changes in the protein which might bury a large amount of hydrophobic surface.

There are a large number of buried waters known from crystallographic evidence to be immobilized concomitant with sodium binding to thrombin. Guinto and Di Cera speculate that these buried waters have a lower heat capacity than bulk solvent and this burial is responsible for this large change in heat capacity for the association. Bhat *et al.*[67] previously noted that there are a large number of waters buried upon D1.3–lysozyme complex formation. The possibility must be considered that the burial of water

in protein−protein interfaces is responsible for some of the negative changes in heat capacity listed in Table 1. It is interesting to recall that water-filled cavities are frequently found in protein−protein interfaces.[47] Whether or not this explanation is correct and if it accounts for all discrepancies between the value of ΔC_p and the amount of buried hydrophobic surface remains to be seen. Clearly though, there are discrepancies to be explained.

In general on the basis of calorimetric evidence it appears that hydrophobic interactions do not as thoroughly dominate protein−protein association as they do protein folding. This echoes conclusions reached reached on the basis of interface amino acid composition.[32] However, this should not be taken to imply that the contributions of hydrophobic interactions are negligible, even in cases such as cytochrome *c* and cytochrome *c* peroxidase. Hydrophobic interactions still appear to be among the most important energetic contributors to the formation of protein−protein complexes. However, the strength of these conclusions are weakened by the difficulties of interpreting the values of ΔC_p for these reactions.

D. Caveat Calorimetrist

Given that many of the comments on this section have dwelt on the difficulties in interpreting these thermodynamic quantities it may seem redundant to close on a cautionary note. Nonetheless, several important points should be made. As Table 1 testifies, the availability of high-quality commercial instrumentation has opened the floodgates for thermodynamic study of heterogeneous protein−protein interactions. It seems likely that the numbers of systems studied will increase rapidly in the near future. However, many workers carrying out these studies are not trained specifically in calorimetry, a category into which this reviewer falls as well. This is not necessarily bad as one need not be a specialist to make good use of a technique and, in fairness, it must be noted that in many cases the determination of association thermodynamics is secondary to the main thrust of the paper. The ease with which one can mix two proteins in the calorimeter and determine ΔG, ΔH, and ΔS is rather seductive. Less seductive are the number of controls and experimental variations that need to be carried out to confidently interpret these results. Also, given the relative newness of the technique, it is often not clear to the nonspecialist exactly what controls and experimental variations should be done. So while there are numerous papers that use this new technique, there are relatively few that can be called complete and rigorous studies of association thermodynamics.

For example, more studies exploring ionic strength effects need to be performed in order to determine how important electrostatic interactions are in protein−protein interactions. Likewise, at the relatively high protein concentrations at which isothermal titrating calorimetric experiments are performed nonideality is possible. To rule this out a second determination at different protein concentrations is appropriate, but not always carried out.

Another common problem is also often ignored. Formation of a protein−protein complex often proceeds with the net release or uptake of protons. Any protons taken up or given up by the proteins will result in an equal number of protonation or deprotonation events in the buffer. The ionization enthalpy of the buffer thus contributes to the total enthalpy measured. Since ionization enthalpies of common buffers[114] can be comparable in magnitude to the enthalpy values reported in Table 1 failure to recognize ionization events can seriously skew interpretation of data. Buffer ionization also has its own distinct ΔC_p contributed to the overall ΔC_p. In order to determine if protonation or deprotonation linked to binding occurs ΔG and ΔH of binding should be measured with different buffers and at different pHs.[115] Unfortunately, this is not frequently done. Indeed, it is sometimes difficult to ascertian exactly what solvent conditions were used in the experiment as concentration data and buffer counterion are not always given.

One case in the literature further points up some of the difficulties in evaluating protein−protein binding thermodynamics. The binding of barnase and barstar was studied by Martínez *et al.*[72] as cited in Table 1 but has also been examined by Yakovlev *et al.*[116] The latter group reports substantially different numbers from those of Martínez *et al.* The work of Yakovlev *et al.* reports for the association of barnase and barstar at 25 °C in 10 mM PIPES, 50 mM NaCl, pH 8.0 a ΔG of −17.2, in good agreement with the −16.4 kcal/mol cited in Table 1, given the slightly different buffer conditions. However Yakovlev *et al.*'s ΔH value of −26.4 kcal/mol is in significant disagreement with the Martínez *et al.* value of −13.9 kcal/mol and the ΔS value of −33.9 cal/(mol K) also disagrees with the Martínez *et al.* value of +12.2 cal/(mol K). Both sets of values are plausible on their face. The values Yakovlev *et al.* reported for ΔH at 37 °C are apparently unreliable due to previously unrecognized thermal unfolding of the proteins. However, current work by these authors is reproducing the values reported for ΔH at 25 °C (A. A. Makarov, personal communication). Why such profound disagreement? There may be an error in one of these studies that remains unrecognized or this system may be extremely sensitive to pH, ionic strength, and protein concentration differences. Until detailed studies of the effects of varying these parameters and temperature are forthcoming we cannot really answer this critical question.

In his earlier review[58] Ross said, "It is this reviewer's opinion that one...thorough investigation which describes the thermodynamic behavior of a protein over a wide range of variables is of much greater value than a large number of fragmentary studies on many different proteins." This reviewer adds his vigorous agreement. Until such studies are the norm, interpretations of compilations of data such as that in Table 1 will remain difficult and tentative.

IV. Mutational Investigation of Protein−Protein Interactions

The number of studies that use mutagenesis to probe protein−protein interactions is overwhelming, so much so that only a few can be reviewed here in any detail. Of course, mutation is not the only way

to probe protein–protein interfaces experimentally. For example, the interface between nicotinic acetylcholine receptor and κ-bungarotoxin[117] and the dimeric interfaces of the tat protein from HIV[118] and of HIV-1 protease[119] have been studied using peptides duplicating a portion of the protein–protein interface. However, compared to mutagenesis, other methods are often less efficient, less reliable, and certainly less used.

The first efforts to study protein association through mutagenesis often were aimed at disruption of the interface, usually by introduction or removal of charged groups. The effects of introducing isolated charges[120] and ion pairs[121] into the interface of tryrosyl-tRNA synthetase were examined. Isolated charges increased the tendency to disassociate while complementary charges did not. Charges were introduced into the interface of triosephospate isomerase[18] and insulin[122] and increased the tendency to disassociate. A lysine to aspartate mutation in ribulose-1,5-bisphosphate carboxylase prevented hetereodimeric association.[123] A naturally occurring ion pair was removed from the interface of aspartate transcarbamoylase lowering stability.[124] However, even the relatively simple goal of disturbing an interaction can yield unexpected results. The interaction of hen egg white lysozyme with an antibody was unexpectedly strengthened when a glutamate and a lysine in lysozyme that were thought to make important electrostatic interactions with the antibody were replaced with neutral hydrophilic residues.[125]

A. Disruption of Protein–Protein Interfaces

Since these beginnings, mutational studies have become more comprehensive in the range of mutations made and in the study of the effects. One theme that has continued is the disruption of interfaces, but with the aim of creating stable proteins with reduced tendency to associate. Disruption of an interface itself is relatively easy; finding the mutation or set of mutations that result in a folded monomer of appreciable stability can be much more difficult. In cases where the interaction interface is separate from most of the rest of protein it can be removed. Alternatively, the interface interactions can be satisfied by adding a second copy of the interface domain to the monomeric polypeptide in such a fashion to allow it to interact with the original interface. The latter strategy was employed by Mossing and Sauer[126] when they connected, via a β turn, a partial copy of the β ribbon interface of λ cro to the end of an intact copy. This allowed the second copy to loop back and interact with remainder of the protein to form a stable monomer. The first strategy, the removal of much of a binding domain, allowed the creation of a stable monomeric form of triosephospate isomerase. In this instance a 15 residue loop that contained most of the interface residues was replaced with an eight residue loop.[127]

Removal of a binding domain is not always feasible, or desirable. Another strategy is to disrupt the interface by removal of side chains that stabilize association and/or by introduction of side chains that destabilize association. Tetrameric fructose-1,6-bisphosphate aldolase was converted into a largely dimeric form through substitution of an interface aspartate with a number of other residues.[128] This aspartate interacts with three main-chain amides from an adjacent subunit. The removal of these interactions was sufficient to convert the protein from a tetramer in which subunit interchange could not be detected, to a dimeric form which associated to form small amounts of tetramers at relatively high protein concentration. While quaternary structure was disrupted, tertiary structure was affected as well since the dimeric form had substantially reduced thermostability.

The association of insulin has received attention because of association's effects on insulin pharmacology. This work shows that oligomer destabilization need not require introduction of large side chains or charges to prevent oligomer formation. It appears that a range of neutral and charged substitutions at proline B28 or lysine B29 are extremely disruptive to oligomer formation.[129] In one case, this appears to be due to the new interface residue causing a conformational change in the interface, stabilized by new hydrophobic interactions, which blocks formation of the normal insulin protein–protein complex.[130]

The normally tetrameric lactate dehydrogenase was converted to a dimer by replacement of a surface loop. In contrast to triosephospate isomerase where a smaller loop was used,[127] in this work[131] a larger loop was added to cause steric interference. This larger surface loop was modeled after a loop in the structurally similar enzyme, malate dehydrogenase, which is normally dimeric. Malate dehydrogenase itself has been the target of mutagenic studies. While dimeric at neutral pH, the wild-type version of this enzyme disassociates at pH 5. Replacement of an interface histidine with a leucine resulted in a dimeric protein stable over a broader pH range.[132] Unfortunately no information about the folding or association stability of this mutant was obtained. In later work malate dehydrogenase was converted from a dimer to a stable monomeric form.[133] The strategy employed was to replace a conserved aspartate and serine that interact with each other across the interface. The aspartate interacts with a threonine and a lysine on the other monomer as well as the serine. Tyrosines were chosen as replacements for the aspartate and serine in order to remove electrostatic interactions and to provide steric conflict. The aspartate to tyrosine single mutant was monomeric, while the serine to tyrosine single mutant remained dimeric. Interestingly, when this aspartate is replaced with an asparagine it results in an aggregated, apparently denatured protein.[132]

Triosephospate isomerase again served as a model system for Mainfroid *et al.*[134] when an interface methionine and arginine were replaced with glutamine. Either mutation alone resulted in a protein with monomeric and dimeric forms present. The double mutant was a stable monomeric form. However, in addition to lowering the stability of the dimer these mutations result in proteins with marginal folding stabilities. In subsequent work,[135] this group showed that the monomer's folding stability could be improved by other mutations.

Related to the creation of a stable monomer from an oligomer is the stabilization of a single domain removed from a multidomain protein. Leistler and Perham[136] replaced seven hydrophobic residues in the excised INT domain of glutathione reductase. These interface residues interacted with two other domains of the intact protein. Hydrophilic substitutions at these sites created a soluble, stable protein.

B. Dissecting Interfaces: Contributions of Specific Side Chains to Complex Stability

Another important theme in the literature is the systematic mutation of an interface in order to determine the contribution of specific residues and interactions to the overall stability of the complex. The general strategy employed is to replace residues one at a time and observe the effects of this mutation. Alanine is usually chosen as a neutral replacement likely to preserve the main chain conformation but removing most or all side-chain interactions. This "alanine scanning" mutagenesis has been used to map important residues in a number of protein–protein interfaces for a number of years now and its use in this capacity was reviewed in 1991.[137] Since then several new studies have appeared. The binding of 21 different monoclonal antibodies to human growth hormone[138] showed results typical of these studies. Functional epitopes were defined as composed of those sites where alanine mutants caused a significant change in binding affinity. On average only about eight residues in human growth hormone contributed to the functional epitope of each of these 21 different interfaces, even using the loose criteria of a 2-fold effect on binding affinity for inclusion in the functional epitope. This is presumably many fewer residues than those in apparent contact across the interface. Only three residues were found on average to reduce the binding affinity by more than 20-fold in each of the 21 epitopes. Similarly, in the binding of antibody D1.3 to lysozyme a small group of residues centrally located in the interface proved to be critical to binding.[139] The binding of neuraminidase to antibody NC41 also showed that only 5 of 19 contact residues in neuraminidase significantly affect binding.[140] These results are quite intriguing in themselves but the determination of the detailed energetics using such mutational strategies is generally more recent and more revealing.

The association of human growth hormone (hGH) with its receptor has been the subject of intense investigation by workers at Genentech as reviewed in depth by Wells and de Vos.[141] There are two binding sites on hGH for the receptor. The work discussed here has focused on site 1. Mutation of residues in hGH[142] and hGH receptor[143,144] to alanine was carried out to identify the relative importance of their contributions to binding affinity. The crystal structure of the complex where receptor is bound to site 1 shows that there are 31 contact residues in hGH and 30 in the receptor. Remarkably few of the contact residues are energetically significant. Five residues in hGH lowered binding affinity by 1.6 to 2.4 kcal/mol each and three more reduced it by 1.0 to 1.5 kcal/mol. These eight residues account for over 85% of the binding energy. On the receptor side of the interface the situation is similar. Two tryptophan residues each reduce binding affinity by over 4.5 kcal/mol when replaced with alanine. A proline contributes 3.5 kcal/mol to binding and seven other sites have 1.0–2.0 kcal/mol reductions. The energetically important residues in the receptor interact in a "hot spot" with the energetically important residues of the hormone. Interestingly, just such a "hot spot" had previously been predicted by theoretical calculations[145] on antibody–antigen complexes. The "hot spot" core in hGH and hGH receptor is largely hydrophobic and is surrounded by less important, more hydrophilic residues. Electrostatic interactions seem to be of little energetic importance in this interaction.

A more detailed examination of the thermodynamics of hormone–receptor association of three alanine substitution mutants of hGH was recently published.[81] The three sites chosen for this study were F25, Y42, and Q46, each buried but making little difference in the binding affinity when mutated to alanine. Although ΔG of association changed little, for the F25A mutant the enthalpy and entropy of association did show large, but obviously compensating, changes. Mutation of Y42 and Q46 caused little change in the enthalpy or entropy of association. Despite this, as discussed above, mutation of Y42 and Q46 did cause significant change in ΔC_p, as did mutation of F25. And despite the wealth of detail, complete understanding of these effects is not yet at hand.

The binding of a humanized monoclonal antibody with epidermal growth factor receptor has also been investigated by Genentech.[64,146] Alanine-scanning mutagenesis showed that four residues, a histidine, arginine, tryptophan, and tyrosine, in the antibody had energetic effects greater than 3 kcal/mol.[112] Again several residues that have minimal effect on ΔG of binding cause large changes in binding ΔH and ΔC_p. The substitution of other amino acids as well as alanine indicate that the hydrophobic effect is the major element favoring binding.

The prolific Genentech group has also analyzed the binding of Factor VIIa to the extracellular domain of human Tissue Factor (TF) through alanine-scanning mutagenesis of TF.[73] They again found a limited number of residues make large contributions to the binding affinity. Five residues, K20, W45, D58, Y94, and F140, reduce ΔG of binding by 1.0 –2.5 kcal/mol. A much larger number, 17 residues, contribute 0.3–1.0 kcal/mol to binding. This is a rather larger number than in other interfaces and many of these residues are polar or charged. Without further mutagenic experiments, it is not clear if the change in binding energy when these polar side chains are removed is due to the loss of the hydrophilic group itself or the aliphatic portion of the side chain. Again, decreases in affinity result primarily from increased rates of disassociation.

Upholding the honor of academic science, Castro and Anderson[60] have ventured onto Genentech's territory by carrying out a similar analysis of the binding of bovine pancreatic trypsin inhibitor (BPTI) to β-trypsin and α-chymotrypsin. Fifteen residues in the reactive surface of BPTI were replaced with

alanine. Again most residues do not significantly affecting binding energy. For the trypsin/BPTI complex three residues G12, I18, and G36, drop affinity by slightly more than 4 kcal/mol. One other residue, K15, is the side chain that interacts with the primary specificity site on the protease and it reduces affinity by 10 kcal/mol. BPTI binding to chymotrypsin is significantly less favorable, but again the biggest change in binding affinity is for the K15A mutant.

One general feature running through these studies is that if the loss of binding energy occurs it seems to be largely due to increases in the disassociation rate, while the association rates remain largely unchanged.[112,139,141] However, exceptions are known, such as the binding of D1.3 to turkey lysozyme, a "mutant" of the chicken lysozyme this antibody was raised against.[51] In this case there is a significant conformational rearrangement of the antibody required for binding turkey lysozyme relative to the original, lower energy structure and the association rate with turkey lysozyme is much slower than that with chicken while disassociation is unchanged. Another example of major conformational rearrangement upon binding, the antibody–coiled coil/random coil system discussed in the previous section, shows significant increases in half-lives for complex formation relative to the related complex that apparently does not require rearrangement.[94] Unfortunately neither precise on rates or any information about off rates was given, and the free energies of association for the particular systems used for lifetime measurement were apparently not determined. However, ΔG for closely related systems was determined and the change in ΔG indicated by the changes in half-lifes is roughly comparable to those energetic changes, suggesting off rates are less affected than on rates. Given that crystal structures of complexes with "subtractive" mutations such as alanine substitutions seldom show such conformational rearrangement and these mutations seldom affect association rates, we propose that decreases in association rates may be a signal that binding to the mutant requires conformational rearrangements with significant energy barriers (or removes the necessity of rearrangement, if association rates increase). However, it must be kept in mind that other factors may reduce association rates as well. The association rate drop observed for a trypsin/BPTI K15A mutant complex is unlikely to be due to conformational change and was attributed instead to the loss of the key electrostatic interaction at the primary specificity site.[60]

An approach that might be called orthogonal to alanine scanning mutagenesis is to select a smaller number of sites in an interface and make many different substitutions. This saturation mutagenesis has also generated interesting results. Three contact residues and one partially buried residue in hen egg white lysozyme were selected from residues that make up the binding interface with antibody HyHEL-10.[147] A large number of mutations were made at these four sites and the binding affinities were determined. At two sites, one of which was the partially buried residue, substitutions had little effect on ΔG of association. The remaining two sites did show large changes, but in very different ways. The nine substitutions made for aspartate 101 show a high correlation between side chain volume and loss of binding affinity. Glycine at this site had an affinity essentially equal to the wild-type lysozyme. Alanine has lost 1.2 kcal/mol, which would identify this residue as marginally important if the alanine scanning mutagenesis criteria discussed above had been applied. As this implies, every other residue examined, even those smaller in size than aspartate, had reduced affinity. The plot of $\Delta\Delta G$ versus side chain volume has the nine substitutions on a fairly straight line with the wild-type aspartate as the prominent outlier. Glutamate and asparagine, as measured by one assay, may form more stable complexes than their volumes would indicate, but this is not clearly significant. Arginine significantly destabilizes the complex even more than its volume would suggest and serine may have a similar effect. On the other hand, the seven substitutions at arginine 21 had a virtually uniform effect, causing the loss of about 2.2 kcal/mol. The authors suggest that this is strong evidence that the cost of losing the two hydrogen bonds the guanidinium group makes to two tyrosine hydroxyls in the antibody is about 2.2 kcal/mol.

An interesting coincidence adds another layer to this story. Another group[66] independently examined the D101G mutant of HyHEL-10 but used isothermal titration calorimetry. They found that the binding affinity of this mutant was essentially identical to the wild-type lysozyme, confirming the result above. However, as found for the three affinity neutral mutants of hGH, there is a large change in binding enthalpy of this mutant compensated for by a change in binding entropy. In this case there is one other notable complication. Very surprisingly, ΔH is not a linear function of temperature, indicating that ΔC_p is a function of temperature. The significance of this unusual observation is unclear.

This same group has also examined the effects of alanine, leucine, phenylalanine, and tryptophan substitution at each of four tyrosine residues in the heavy chain of the HyHEL-10 antibody.[148] Two mutants could not be expressed and two bound so poorly that they could not be affinity purified. All but one of the remaining mutations reduce binding affinity by at least 0.7 kcal/mol and one alanine mutation costs 3.3 kcal/mol. The hydrophobic substitutions, phenylalanine, leucine, and tryptophan, are all bound less tightly than wild-type, but are generally equivalent to each other, except for one leucine. One alanine is significantly lower in binding affinity than the hydrophobes, and one is equivalent. As before, the changes in ΔH are much larger than the changes in ΔG and there is little correlation between the two values. The values of ΔC_p also varied widely, again with no obvious correlation to type of mutation or energetic effects.

The effects of mutation of aspartate 32 in the heavy of HyHEL-10 antibody to asparagine, glutamate, and alanine was also investigated.[149] This aspartate makes a salt bridge with lysine 97 of hen egg white lysozyme in the complex. The D32A mutant lost 0.9 kcal/mol of binding energy, while the D32E and D32N mutants lost 0.6 and 0.4 kcal/mol, respectively. Very interestingly, the binding of the mutants was

much more favorable enthalpically, by ~5 kcal/mol in each case. As this implies, the binding of the mutants was entropically less favorable than the binding of wild-type, again by ~5 kcal/mol. The ΔC_p of binding became more negative than wild-type binding by ~200 cal/(mol K). In other words, the disruption of this particular salt bridge had approximately equivalent effects in each case. The effect on binding enthalpy was nearly canceled out by the effect on entropy, for little overall change in binding affinity, at least at 30 °C. In this case, the large change in ΔC_p can be rationalized. The loss of a polar interaction should mean that binding overall is dominated more by hydrophobic interactions, which in turn should lead to a more negative ΔC_p, which is what occurs. Unfortunately since other changes in ΔC_p of binding resist such rationalization, not much weight should be placed on this simple interpretation.

C. Summary

The principal findings of the alanine-scanning experiments are quite clear. A limited number of residues in each binding interface are very important to the interaction. The critical residues in one protein interact with the critical residues across the interface in its binding partner. It appears on the whole that the critical interaction between the critical residues is hydrophobic, whether with a hydrophobic residue or the hydrophobic portion of a hydrophilic residue. Once detailed thermodynamics are examined, even residues of little significance to binding affinity show changes in enthalpy and entropy of association upon mutation. The significance of these changes is not clear. The results of extensive mutation at a single site offer no generalizations other than one similar to alanine scanning experiments. That generalization is that changes in ΔH, ΔS, and ΔC_p upon mutation are the rule, even if association binding energy is unaffected, and no rules that govern their changes are yet obvious.

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Note Added in Proof

A review of the statistical thermodynamics of binding affinities recently appeared (Gilson *et al.*, *Biophys. J.* **1997**, *72*, 1047). Further calorimetric studies on the interaction of cytochrome *c* with cytochrome *c* peroxidase using mutant proteins have been reported (Erman *et al.*, *Biochemistry* **1997**, *36*, 4054). The binding of calmodulin to smooth muscle myosin light chain kinase was also examined by calorimetry (Wintrode and Privalov, *J. Mol. Biol.* **1997**, *266*, 1050) and, in contrast to the binding of calmodulin to seminalplasmin discussed previously in this paper, ΔC_p is large and negative ($\Delta C_p = -645$ cal/(mol K), $\Delta G = -8.2$ kcal/mol, $\Delta H = -4.3$ kcal/mol, $\Delta S = 13.1$ cal/(mol K) at 25.4 °C). The thermodynamics of barnase–barstar binding have also been further investigated (Frisch *et al.*, *J. Mol. Biol.* **1997**, *267*, 696).

VI. References

(1) Brown, A. M.; Crothers, D. M. *Proc. Nat. Acad. Sci. U.S.A.* **1989**, *86*, 7387.
(2) Sternberg, M. J. E.; Gullick, W. J. *Nature* **1989**, *339*, 587.
(3) Ingram, D. V. *Nature* **1957**, *180*, 326. Watowich, S. J.; Gross, L. J.; Josephs, R. *J. Struct. Biol.* **1993**, *111*, 161.
(4) Cohen, A. S. *Curr. Opin. Rheumatol.* **1994**, *6*, 68. Cohen, A. S. *Curr. Opin. Rheumatol.* **1994**, *6*, 55. Sipe, J. D. *Annu. Rev. Biochem.* **1992**, *61*, 947. Benson, M. D.; Wallace, M. R. *The Metabolic Basis of Inherited Disease*, 6th ed.; Scriver, C. R., Beaudet, A. L., Sly, W. S., Vale, D., Eds.; McGraw-Hill: New York, 1989; p 2439. Pepys, M. B.; Hawkins, P. N.; Booth, D. R.; Vigushin, D. M.; Tennent, G. A.; Soutar, A. K.; Totty, N.; Nguyen, O.; Blake, C. C. F.; Terry, C. J.; Feest, T. G.; Zalin, A. M.; Hsuan, J. J. *Nature* **1993**, *362*, 553.
(5) Rosenburg, R. N. *Neurology* **1993**, *43*, 851.
(6) Johnson, D.; Travis, J. *J. Biol. Chem.* **1979**, *254*, 4022. Swain, M. W.; Pizzo, S. V. *J. Leukocyte Biol.* **1988**, *43*, 365.
(7) Gadek, J. E.; Fells, G. A.; Crystal, R. G. *Science* **1979**, *206*, 1315. Boudier, C.; Pelletier, A.; Pauli, G.; Bieth, J. G. *Clin. Chim. Acta.* **1983**, *132*, 309.
(8) Abrams, W. R.; Cohen, A. B.; Damiano, V. V.; Eliraz, A.; Kimbel, P.; Meranza, D. R.; Weinbaum, G. *J. Clin. Invest.* **1981**, *68*, 1132. Evans, M. D.; Pryor, W. A. *Am. J. Physiol.* **1994**, *266*, L593.
(9) Jaenicke, R. *Biochemistry* **1991**, *30*, 3147.
(10) Chothia, C.; Janin, J. *Nature* **1975**, *256*, 705.
(11) Weber, G. *Protein Interactions*; Chapman and Hall: New York, 1992.
(12) Zvelebil, M. J. J. M.; Thornton, J. M. *Q. Rev. Biophys.* **1993**, *26*, 333.
(13) Sheriff, S. *ImmunoMethods* **1993**, *3*, 222.
(14) Wilson, I. A.; Stanfield, R. L. *Curr. Opinion Struct. Biol.* **1993**, *3*, 113.
(15) Whisstock, J. C.; Lesk, A. M. *Synthetic Vaccines*; Oxford: Boston, 1994; p 116.
(16) Schenkman, J. B. *Handb. Exp. Pharmacol.* **1995**, *105*, 527.
(17) Jones, S.; Thornton, J. M. *Proc. Nat. Acad. Sci. U.S.A.* **1996**, *93*, 13.
(18) Casal, J. I.; Ahern, T. J.; Davenport, R. C.; Petsko, G. A.; Klibanov, A. M. *Biochemistry* **1987**, *26*, 1258.
(19) For example: Vieth, M.; Kolinski, A.; Skolnick, J. *Biochemistry* **1996**, *35*, 955. Lumb, K. J.; Carr, C. M.; Kim, P. S. *Biochemistry* **1994**, *33*, 7361. Harbury, P. B.; Zhang, T.; Kim, P. S.; Alber, T. *Science* **1993**, *262*, 1401. Schuermann, M.; Hunter, J. B.; Hennig, F.; Muller, R. *Nucleic Acids Res.* **1991**, *19*, 739. Engel, M.; Williams, R. W.; Erickson, B. W. *Biochemistry* **1991**, *30*, 3161. Hu, J. C.; O'Shea, E. K.; Kim, P. S.; Sauer, R. T. *Science* **1990**, *250*, 1400. Landshultz, W. H.; Johnson, P. F.; McKnight, S. L. *Science* **1988**, *240*, 1759.
(20) Janin, J.; Miller, S.; Chothia, C. *J. Mol. Biol.* **1988**, *204*, 155.
(21) Jones, S.; Thornton, J. M. *Prog. Biophys. Mol. Biol.* **1995**, *63*, 31.
(22) Argos, P. *Protein Eng.* **1988**, *2*, 101.
(23) Finkelstein, A. V.; Janin, J. *Protein Eng.* **1989**, *3*, 1. Janin, J.; Chothia, C. *Biochemistry* **1978**, *17*, 2943.
(24) Janin, J.; Chothia, C. *J. Biol. Chem.* **1990**, *265*, 16027.
(25) Davies, D. R.; Padlan, E. A.; Sheriff, S. *Annu. Rev. Biochem.* **1990**, *59*, 439.
(26) Miller, S.; Lesk, A. M.; Janin, J.; Chothia, C. *Nature* **1987**, *328*, 834.
(27) Miller, S.; Janin, J.; Chothia, C. *UCLA Symp. Mol. Cellul. Biol.* **1989**, *80*, 193.
(28) Bryant, S. H.; Islam, S. A.; Weaver, D. L. *Proteins, Struct. Funct. Genet.* **1989**, *6*, 418. Miller, S.; Janin, J.; Lesk, A. M.; Chothia, C. *J. Mol. Biol.* **1987**, *196*, 641.
(29) Young, L.; Jernigan, R. L.; Covell, D. G. *Protein Sci.* **1994**, *3*, 717.
(30) Korn, A. P.; Burnett, R. M. *Proteins* **1991**, *9*, 37.
(31) Rashin, A. A.; Honig, B. *J. Mol. Biol.* **1984**, *173*, 515.
(32) Tsai, C. J.; Lin, S. L.; Wolfson, H. J.; Nussinov, R. *Protein Sci.* **1997**, *6*, 53.
(33) Padlan, E. A. *Proteins, Struct. Funct. Genet.* **1990**, *7*, 112.
(34) Mian, I. S.; Bradwell, A. R.; Olson, A. J. *J. Mol. Biol.* **1991**, *217*, 133.

(35) Pakula, A. A.; Sauer, R. T. *Nature* **1990**, 344, 363. Bowler, B. E.; May, K.; Zaragoza, T.; York, P.; Dong, A.; Caughey, W. *Biochemistry* **1993**, *32*, 183. Tamura, A.; Sturtevant, J. M. *J. Mol. Biol.* **1995**, *249*, 646. Herrmann, L.; Bowler, B. E.; Dong, A.; Caughey, W. S. *Biochemistry* **1995**, *34*, 3040.
(36) Fauchère, J. L.; Pliška, V. *J. Med. Chem.* **1983**, *18*, 369.
(37) Gellman, S. H. *Biochemistry* **1991**, *30*, 6633.
(38) O'Neil, K. T.; DeGrado, W. F. *Trends Biochem. Sci.* 1990, **15**, 59; O'Neil, K. T.; Erickson-Viitanen, S.; DeGrado, W. F. *J. Biol. Chem.* 1989, **264**, 14571.
(39) Morgan, R. S.; Tatsch, C. E.; Gushard, R. H.; McAdon, J. M.; Warme, P. K. *Int. J. Pept. Protein Res.* **1978**, *11*, 209. Bodner, B. L.; Jackman, L. M.; Morgan, R. S. *Biochem. Biophys. Res. Commun.* **1980**, *94*, 807. Némethy, G.; Scheraga, H. A. *Biochem. Biophys. Res. Commun.* **1981**, *98*, 482. Reid, K. S. C.; Lindley, P. F.; Thornton, J. M. *FEBS Lett.* **1985**, *190*, 209. Lebl, M.; Sugg, E. E.; Hruby, V. J. *Int. J. Pept. Protein Res.* **1987**, *29*, 40. Auld, D. S.; Young, G. B.; Saunders, A. J.; Doyle, D. F.; Betz, S. F.; Pielak, G. J. *Protein Sci.* **1993**, *2*, 2187. Viguera, A. R.; Serrano, L. *Biochemistry* **1995**, *34*, 8771. Stapley, B. J.; Rohl, C. A.; Doig, A. J. *Protein Sci.* **1995**, *4*, 2383. Spencer, D. S.; Stites, W. E. *J. Mol. Biol.* **1996**, *257*, 497.
(40) Smith-Gill, S. J. *Curr. Opinion Biotechnol.* **1991**, *2*, 568. Slingsby, C.; Bateman, O. A.; Simpson, A. *Mol. Biol. Rep.* **1993**, *17*, 185. Lin S. L.; Tsai, C. J.; Nussinov, R. *J. Mol. Biol.* **1995**, *248*, 151.
(41) Tsai, C. J.; Lin S. L.; Wolfson, H. J.; Nussinov, R. *J. Mol. Biol.* **1996**, *260*, 604.
(42) Miller, S. *Protein Eng.* **1989**, *3*, 77.
(43) Bennett, M. J.; Schlunegger, M. P.; Eisenberg, D. *Protein Sci.* **1995**, *4*, 2455.
(44) Walls, P. H.; Sternberg, M. J. E. *J. Mol. Biol.* **1992**, *228*, 277.
(45) Padlan, E. A.; Cohen, G. H.; Davies, D. R. *Mol. Immunol.* **1986**, *23*, 951.
(46) Lawrence, M.; Colman, P. M. *J. Mol. Biol.* **1993**, *234*, 946.
(47) Hubbard, S. J.; Argos, P. *Protein Sci.* **1994**, *3*, 2194.
(48) Pawowski, K.; Bierzynski, A.; Godzik, A. *J. Mol. Biol.* **1996**, *258*, 349.
(49) Verdaguer, N.; Mateu, M. G.; Bravo, J.; Domingo, E.; Fita, I. *J. Mol. Biol.* **1995**, *256*, 364.
(50) Stanfield, R. L.; Takimoto-Kamimura, M.; Rini, J. M.; Profy, A. T.; Wilson, I. A. *Structure* **1993**, *1*, 83.
(51) Braden, B. C.; Fields, B. A.; Ysern, X.; Goldbaum, F. A.; Dall'Acqua, W.; Schwarz, F. P.; Poljak, R. J.; Mariuzza, R. A. *J. Mol. Biol.* **1996**, *257*, 889.
(52) MacCallum, R. M.; Martin, A. C. R.; Thornton, J. M. *J. Mol. Biol.* **1996**, *262*, 732.
(53) Naghibi, H.; Tamura, A.; Sturtevant, J. M. *Proc. Nat. Acad. Sci. U.S.A.* **1995**, *92*, 5597. Liu, Y.; Sturtevant, J. M. *Protein Sci.* **1995**, *4*, 2559.
(54) Weber, G. *J. Phys. Chem.* **1993**, *97*, 7108.
(55) Weber, G. *J. Phys. Chem.* **1995**, *99*, 1052. Weber, G. *Proc. Nat. Acad. Sci. U.S.A.* **1996**, *93*, 7452.
(56) Jelesarov, I.; Bosshard, H. R. *J. Mol. Biol.* **1996**, *263*, 344.
(57) Wiseman, T.; Williston, S.; Brandts, J. F.; Lin, L.-N. *Anal. Biochem.* **1989**, *179*, 131. Ladbury, J. E.; Chowdhry, B. Z. *Chem. Biol.* **1996**, *3*, 791.
(58) Ross, P. D. *Thermodynamic Data for Biochemistry and Biotechnology*; Hinz, H.-J., Ed.; Springer-Verlag: Berlin, 1986; p 227.
(59) Baugh, R. J.; Trowbridge, C. G. *J. Biol. Chem.* **1972**, *247*, 7498.
(60) Castro, M. J. M.; Anderson, S. *Biochemistry* **1996**, *35*, 11435.
(61) Fukada, H.; Takahashi, K.; Sturtevant, J. M. *Biochemistry* **1985**, *24*, 5109.
(62) Takahashi, K.; Fukada, H. *Biochemistry* **1985**, *24*, 297.
(63) Milos, M.; Schaer, J.-J.; Comte, M.; Cox, J. A. *J. Biol. Chem.* **1988**, *263*, 9218.
(64) Kelley, R. F.; O'Connell, M. P.; Carter, P.; Presta, L.; Eigenbrot, C.; Covarrubias, M.; Snedecor, B.; Bourell, J. H.; Vetterlein, D. *Biochemistry* **1992**, *31*, 5434.
(65) Hibbits, K. A.; Gill, D. S.; Willson, R. C. *Biochemistry* **1994**, *33*, 3584.
(66) Tsumoto, K.; Ueda, Y.; Maenaka, K.; Watanabe, K.; Ogasahara, K.; Yutani, K.; Kumagai, I. *J. Biol. Chem.* **1994**, *269*, 28777.
(67) Bhat, T. N.; Bentley, G. A.; Boulet, G.; Greene, M. I.; Tello, D.; Dall'Acqua, W.; Souchon, H.; Schwarz, F. P.; Mariuzza, R. A.; Poljak, R. J. *Proc. Nat. Acad. Sci. U.S.A.* **1994**, *91*, 1089. See also: Ito, W.; Iba, Y.; Kurosawa, Y. *J. Biol. Chem.* **1993**, *268*, 16639.
(68) Schwarz, F. P.; Tello, D.; Goldbaum, F. A.; Mariuzza, R. A.; Poljak, R. J. *Eur. J. Biochem.* **1995**, *228*, 388. Tello, D.; Goldbaum, F. A.; Mariuzza, R. A.; Ysern, X.; Schwarz, F. P.; Poljak, R. J. *Biochem. Soc. Trans.* **1993**, *21*, 943.
(69) Tello, D.; Eisenstein, E.; Schwarz, F. P.; Goldbaum, F. A.; Fields, B. A.; Mariuzza, R. A.; Poljak, R. J. *J. Mol. Recognit.* **1994**, *7*, 57
(70) Jelesarov, I.; Bosshard, H. R. *Biochemistry* **1994**, *33*, 13321.
(71) Evans, L. J. A.; Cooper, A.; Lakey, J. H. *J. Mol. Biol.* **1996**, *255*, 559.
(72) Martínez, J. C.; Filimonov, V. V.; Mateo, P. L.; Schreiber, G.; Fersht, A. R. *Biochemistry* **1995**, *34*, 5224.
(73) Kelley, R. F.; Costas, K. E.; O'Connell, M. P.; Lazarus, R. A. *Biochemistry* **1995**, *34*, 10383.
(74) Kresheck, G. C.; Vitello, L. B.; Erman, J. E. *Biochemistry* **1995**, *34*, 8398.
(75) McLean, M. A.; Sligar, S. G. *Biochem. Biophys. Res. Commun.* **1995**, *215*, 316.
(76) Murphy, K. P.; Freire, E.; Paterson, Y. *Proteins* **1995**, *21*, 83.
(77) Raman, C. S.; Allen, M. J.; Nall, B. T. *Biochemistry* **1995**, *34*, 5831.
(78) Li, J.; Swanson, R. V.; Simon, M. I.; Weis, R. M. *Biochemistry* **1995**, *34*, 14626.
(79) Johanson, K.; Appelbaum, E.; Doyle, M.; Hensley, P.; Zhao, B.; Abdel-Meguid, S. S.; Young, P.; Cook, R.; Carr, S.; Matico, R.; Cusimano, D.; Dul, E.; Angelichio, M.; Brooks, I.; Winborne, E.; McDonnell, P.; Morton, T.; Bennett, D.; Sokoloski, T.; McNulty, D.; Rosenberg, M.; Chaiken, I. *J. Biol. Chem.* **1995**, *270*, 9459.
(80) Philo, J. S.; Aoki, K. H.; Arakawa, T.; Narhi, L. O.; Wen, J. *Biochemistry* **1996**, *35*, 1681.
(81) Pearce, K. H., Jr.; Ultsch, M. H.; Kelley, R. F.; de Vos, A. M.; Wells, J. A. *Biochemistry* **1996**, *36*, 10300.
(82) Chauvin, F.; Fomenkov, A.; Johnson, C. R.; Roseman, S. *Proc. Nat. Acad. Sci. U.S.A.* **1996**, *93*, 7028.
(83) Philo, J. S.; Wen, J.; Wypych, J.; Schwartz, M. G.; Mendiaz, E. A.; Langley, K. E. *J. Biol. Chem.* **1996**, *271*, 6895.
(84) Lee, C. H.; Leung, B.; Lemmon, M. A.; Zheng, J.; Cowburn, D.; Kuriyan, J.; Saksela, K. *EMBO J.* **1995**, *14*, 5006.
(85) Renzoni, D. A.; Pugh, D. J. R.; Siligardi, G.; Das, P.; Morton, C. J.; Rossi, C.; Waterfield, M. D.; Campbell, I. D.; Ladbury, J. E. *Biochemistry* **1996**, *35*, 15646.
(86) Baker, B. M.; Murphy, K. P. *J. Mol. Biol.* **1997**, *268*, 557.
(87) Murphy, K. P.; Xie, D.; Garcia, K. C.; Amzel, L. M.; Freire, E. *Proteins* **1993**, *15*, 113.
(88) Gómez, J.; Freire, E. *J. Mol. Biol.* **1995**, *252*, 337.
(89) Page, J. D.; You, J.-L.; Harris, R. B.; Colman, R. W. *Arch. Biochem. Biophys.* **1994**, *314*, 159.
(90) Lemmon, M. A.; Ladbury, J. E.; Mandiyan, V.; Zhou, M.; Schlessinger, J. *J. Biol. Chem.* **1994**, *269*, 31653. Roughly similar results for the binding of the SH3 domain of murine Grb2 to several peptides derived from murine Sos have been reported by others. Specifically; ΔG, −5.9; ΔH, −9.7; ΔS, −12.5 at 30 °C, 25 mM sodium phosphate, 10 mM DTT, 0.01% sodium azide, pH 7.0. Wittekind, M.; Mapelli, C.; Farmer, B. T.; Suen, K. L.; Goldfarb, V.; Tsao, J.; Lavoie, T.; Barbacid, M.; Meyers, C. A.; Mueller, L. *Biochemistry* **1994**, *33*, 13531.
(91) Ladbury, J. E.; Lemmon, M. A.; Zhou, M.; Green, J.; Botfield, M. C.; Schlessinger, J. *Proc. Nat. Acad. Sci. U.S.A.* **1995**, *92*, 3199.
(92) Ladbury, J. E.; Hensmann, M.; Panayotou, F.; Campbell, I. D. *Biochemistry* **1996**, *35*, 11062.
(93) Mandiyan, V.; O'Brien, R.; Zhou, M.; Margolis, B.; Lemmon, M. A.; Sturtevant, J. M.; Schlessinger, J. *J. Biol. Chem.* **1996**, *271*, 4770.
(94) Leder, L.; Berger, C.; Bornhauser, S.; Wendt, H.; Ackerman, F.; Jelesarov, I.; Bosshard, H. R. *Biochemistry* **1995**, *34*, 16509.
(95) Milos, M.; Schaer, J.-J.; Comte, M.; Cox, J. A. *J. Biol. Chem.* **1987**, *262*, 2746.
(96) Varadarajan, R.; Connelly, P. R.; Sturtevant, J. M.; Richards, F. M. *Biochemistry* **1992**, *31*, 1421.
(97) Weber, P. C.; Pantoliano, M. W.; Thompson, L. D. *Biochemistry* **1992**, *31*, 9350.
(98) Schmidt, T. G. M.; Koepke, J.; Frank, R.; Skerra, A. *J. Mol. Biol.* **1996**, *255*, 753.
(99) Wu, J.; Li, J.; Li, G.; Long, D. G.; Weis, R. M. *Biochemistry* **1996**, *35*, 4984.
(100) Petrella, E. C.; Machesky, L. M.; Kaiser, D. A.; Pollard, T. D. *Biochemistry* **1996**, *35*, 16535.
(101) Lin, Z.; Schwarz, F. P.; Eisenstein, E. *J. Biol. Chem.* **1995**, *270*, 1011.
(102) Makhatadze, G. I.; Privalov, P. L. *Adv. Protein Chem.* **1995**, *47*, 307.
(103) Sturtevant, J. M. *Proc. Nat. Acad. Sci. U.S.A.* **1977**, *74*, 2236.
(104) Fisher, H. F.; Singh, N. *Methods Enzymol.* **1995**, *259*, 194.
(105) Lee, C. H.; Saksela, K.; Mirza, U. A.; Chait, B. T.; Kuriyan, J. *Cell* **1996**, *85*, 931.
(106) Gill, S. J.; Wadsö, I. *Proc. Nat. Acad. Sci. U.S.A.* **1976**, *73*, 2955. Spolar, R. S.; Livingstone, J. R.; Record, M. T., Jr. *Biochemistry* **1992**, *31*, 3947. Blokzijl, W.; Engberts, J. B. F. N. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 1545. Ross, P. D.; Rekharsky, M. V. *Biophys. J.* **1996**, *71*, 2144.
(107) Murphy, K. P.; Gill, S. J. *J. Mol. Biol.* **1991**, *222*, 699.
(108) Privalov, P. L.; Gill, S. J. *Adv. Protein Chem.* **1988**, *39*, 191. Spolar, R. S.; Ha, J.-H.; Record, Jr., M. T. *Proc. Nat. Acad. Sci. U.S.A.* **1989**, *86*, 8382.
(109) Murphy, K. P.; Freire, E. *Adv. Protein Chem.* **1992**, *43*, 313.
(110) Durham, B.; Millett, F. S. *Comprehensive Supramolecular Chemistry*; Atwood, J. L., Davies, J. E. D., MacNicol, D. D., Vögtle, F., Eds.; Pergamon: Oxford, 1996; Vol. 5, p 219.
(111) Spolar, R. S.; Record, M. T., Jr. *Science* **1994**, *263*, 777.
(112) Kelley, R. F.; O'Connell, M. P. *Biochemistry* **1993**, *32*, 6828.
(113) Guinto, E. R.; Di Cera, E. *Biochemistry* **1996**, *35*, 8800.

(114) Christensen, J. J.; Hansen, L. D.; Izatt, R. M. *Handbook of Proton Ionization Heats*; J. Wiley & Sons: New York, 1976. Morin, P. E.; Freire, E. *Biochemistry* **1991**, *30*, 8494.
(115) Doyle, M. L.; Louie, G.; Dal Monte, P. R.; Sokoloski, T. D. *Meth. Enzymol.* **1995**, *259*, 183. Baker, B. M.; Murphy, K. P. *Biophys. J.* **1996**, *71*, 2049.
(116) Yakovlev, G. I.; Moiseyev, G. P.; Protasevich, I. I.; Ranjbar, B.; Bocharov, A. L.; Kirpichnikov, M. P.; Gilli, R. M.; Briand, C. M.; Hartley, R. W.; Makarov, A. A. *FEBS Lett.* **1995**, *366*, 156.
(117) McLane, K. E.; Wu, X.; Conti-Tronconi, B. M. *Biochem. Biophys. Res. Commun.* **1991**, *176*, 11.
(118) Frankel, A. D.; Chen, L.; Cotter, R. J.; Pabo, C. O. *Proc. Nat. Acad. Sci. U.S.A.* **1988**, *85*, 6297.
(119) Schramm, H. J.; Billich, A.; Jaeger, E.; Rucknagel, K. P.; Arnold, G.; Schramm, W. *Biochem. Biophys. Res. Commun.* **1993**, *194*, 595.
(120) Jones, D. H.; McMillan, A. J.; Fersht, A. R. *Biochemistry* **1985**, *24*, 5852.
(121) Ward, W. H. J.; Jones, D. H.; Fersht, A. R. *Biochemistry* **1987**, *26*, 4131. Ward, W. H. J.; Jones, D. H.; Fersht, A. R. *J. Biol. Chem.* **1986**, *261*, 9576.
(122) Brange, J.; Ribel, U.; Hansen, J. F.; Dodson, G.; Hansen, M. T.; Havelund, S.; Melberg, S. G.; Norris, F.; Norris, K.; Snel, L.; Sørensen, A. R.; Voigt, H. O. *Nature* **1988**, *333*, 679.
(123) Lee, E. H.; Soper, T. S.; Mural, R. J.; Stringer, C. D.; Hartman, F. C. *Biochemistry* **1987**, *26*, 4599.
(124) Stebbins, J. W.; Kantrowitz, E. R. *J. Biol. Chem.* **1989**, *264*, 14860.
(125) Roberts, S.; Cheetham, J. C.; Rees, A. R. *Nature* **1987**, *328*, 731.
(126) Mossing, M. C.; Sauer, R. T. *Science* **1990**, *250*, 1712.
(127) Borchert, T. V.; Abagyan, R.; Radha Kishan, K. V.; Zeelen, J. P.; Wierenga, R. K. *Structure* **1993**, *1*, 205.
(128) Beernink, P. T.; Tolan, D. R. *Protein Sci.* **1994**, *3*, 1383.
(129) Sheolson, S. E.; Lu, Z.-X.; Parlautan, L.; Lynch, C. S.; Weiss, M. A. *Biochemistry* **1992**, *31*, 1757. Brems, B. N.; Alter, L. A.; Beckage, M. J.; Chance, R. E.; DiMarchi, R. D.; Green, L. K.; Long, H. B.; Pekar, A. H.; Sheilds, J. E.; Frank, B. H. *Protein Eng.* **1993**, *5*, 527.
(130) Jørgensen, A. M. M.; Olsen, H. B.; Balschmidt, P.; Led, J. J. *J. Mol. Biol.* **1996**, *257*, 684.
(131) Jackson, R. M.; Gelpi, J.; Cortes, A.; Emery, D.; Wilks, H.; Moreton, K.; Hlasall, D.; Sleigh, R.; Behan-Martin, M.; Jones, G.; Clarke, A.; Holbrook, J. *Biochemistry* **1992**, *31*, 8307.
(132) Steffan, J. S.; McAlister-Henn, L. *Arch. Biochem. Biophys.* **1991**, *287*, 276.
(133) Brieter, D. R.; Resnik, E.; Banaszak, L. J. *Protein Sci.* **1994**, *3*, 2023.
(134) Mainfroid, V.; Terpstra, P.; Beauregard, N.; Frère, J.-M.; Mande, S. C.; Hol, W. G. J.; Martial, J. A.; Goraj, K. *J. Mol. Biol.* **1996**, *257*, 441.
(135) Mainfroid, V.; Mande, S. C.; Hol, W. G. J.; Martial, J. A.; Goraj, K. *Biochemistry* **1996**, *35*, 4110.
(136) Leistler, B.; Perham, R. N. *Biochemistry* **1994**, *33*, 2773.
(137) Wells, J. A. *Methods Enzymol.* **1991**, *202*, 390.
(138) Jin, L.; Fendly, B. M.; Wells, J. A. *J. Mol. Biol.* **1992**, *226*, 851.
(139) Hawkins, R. E.; Russell, S. J.; Baier, M.; Winter, G. *J. Mol. Biol.* **1993**, *234*, 958.
(140) Nuss, J. M.; Whitaker, P. B.; Air, G. M. *Proteins Struct. Funct. Genet.* **1993**, *15*, 121.
(141) Wells, J. A.; de Vos, A. M. *Annu. Rev. Biochem.* **1996**, *65*, 609.
(142) Cunningham, B. C.; Wells, J. A. *Science* **1989**, *244*, 1081. Cunningham, B. C.; Wells, J. A. *J. Mol. Biol.* **1991**, *234*, 554.
(143) Bass, S. H.; Mulkerrin, M. G.; Wells, J. A. *Proc. Nat. Acad. Sci. U.S.A.* **1991**, *88*, 4498.
(144) Clackson, T.; Wells, J. A. *Science* **1995**, *267*, 383.
(145) Novotny, J.; Bruccoleri, R. E.; Saul, F. A. *Biochemistry* **1989**, *28*, 4735.
(146) Kelley, R. F. *Structure of Antigens*; Van Regenmortel, M. H. V., Ed.; CRC Press: Boca Raton, 1996, Vol. 3, p 1.
(147) Kam-Morgan, L. N. W.; Smith-Gill, S. J.; Taylor, M. G.; Zhang, L.; Wilson, A. C.; Kirsch, J. F. *Proc. Nat. Acad. Sci. U.S.A.* **1993**, *90*, 3958.
(148) Tsumoto, K.; Ogasahara, K.; Ueda, Y.; Watanabe, K.; Yutani, K.; Kumagai, I. *J. Biol. Chem.* **1995**, *270*, 18551.
(149) Tsumoto, K.; Ogasahara, K.; Ueda, Y.; Watanabe, K.; Yutani, K.; Kumagai, I. *J. Biol. Chem.* **1996**, *271*, 32612.

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