Electrostatic aspects of protein–protein interactions

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Structural and mutational analyses reveal a central role for electrostatic interactions in protein–protein association. Experiment and theory both demonstrate that clusters of charged and polar residues that are located on protein–protein interfaces may enhance complex stability, although the total effect of electrostatics is generally net destabilizing. The past year also witnessed significant progress in our understanding of the effect of electrostatics on protein association kinetics, specifically in the characterization of a partially desolvated encounter complex.

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Abbreviation
FDPB finite difference Poisson–Boltzmann

Introduction

The ability of proteins to bind to one another in a highly specific manner is an important feature of a variety of biological processes. There has been considerable interest in the characterization of the forces that drive protein–protein interactions and in understanding how these forces are used to yield complexes with association constants that vary over many orders of magnitude and that can be regulated by different exogenous factors. In this review, we summarize recent studies on the role of electrostatic interactions in protein–protein association.

An important finding has been the presence of a significant population of charged and polar residues on protein–protein interfaces. Given the fact that the surfaces of proteins are quite polar, it is perhaps not surprising that the interfaces are polar as well and that complex formation results in the burial of a significant number of charged and polar residues. In principle, however, interfaces might have evolved to be nonpolar and, hence, to be similar to the interiors of the individual monomers. In fact, the contrast between protein–protein complexes and folded proteins, which have large hydrophobic cores and tend not to bury many charged and polar groups [1], is quite striking. The apparent differences in the balance of forces between protein folding and protein–protein association may be made possible, in part, by the fact that the configurational entropy penalty associated with protein folding is much larger than the loss of translational and rotational entropy, as well as the entropy loss of interfacial sidechains, associated with complex formation. Thus, the forces that drive protein–protein association need not be as large as those that drive protein folding and, for example, the relative contribution of hydrophobic interactions can be reduced in the latter case.

Since, due to desolvation effects, buried charges, ion pairs and hydrogen bonds appear to destabilize protein folding [2–6], it might be expected that polar groups buried in protein–protein interfaces oppose complex formation. As will be discussed, however, the accumulating evidence suggests that, besides contributing to the specificity of association, a role generally attributed to polar residues, these residues can contribute favorably to complex stabilization as well. Similarly, as will be discussed, it has been found that charged amino acids can significantly enhance diffusion-controlled association rates of protein–protein complexes, even though the rate-limiting step involves the partial desolvation of charged and polar groups.

Structural analyses reveal significant involvement of charged and polar groups in protein–protein association

A number of years ago, Jones and Thornton [7] analyzed average hydrophobicities of protein–protein interfaces and compared these with the hydrophobicities of protein cores and surfaces. The average hydrophobicity of a protein core is, of course, positive, whereas the hydrophobicities of protein surfaces were found to be negative. Homodimers, most of which exist only in an oligomeric state, were found to be the only class of protein complex to have interfaces with a positive mean hydrophobicity. Other complexes have interfaces that are far more polar; enzyme–inhibitor complexes have mean hydrophobicities close to zero, whereas antibody–antigen complexes have interfaces with mean hydrophobicities that are essentially indistinguishable from that of a typical protein surface.

In a recent survey of protein–protein interfaces, Janin and co-workers [8••] studied the recognition sites of protein complexes that have a more diverse range of activities than the enzyme–inhibitor and antibody–antigen complexes that had been the focus of much earlier work. The average size of these interfaces is approximately 1600 Å^2 (800 Å^2 per monomer), constituting about 10% of a typical monomer surface [8••]. An average interface was found to contain about 10 intermolecular hydrogen bonds (or approximately one hydrogen bond per 170 Å^2). Almost every third hydrogen bond involves at least one charged residue and 13% are formed between two charged groups, that is, one ion pair per interface. As the standard definition of a salt bridge involves a less strict geometric requirement than that normally associated with hydrogen bonds, the actual number of intermolecular salt bridges may be somewhat larger. If a simple distance criterion, of

oppositely charged heavy atoms being within 4.1 Å of one another, is used, about two salt bridges are found per interface (B Hitz, B Honig, unpublished data). Averages such as these have only limited meaning, because the variations among individual interfaces are quite large. For example, the number of intermolecular hydrogen bonds found by Lo Conte *et al.* [8••] ranges from one (complex of cytochrome *c* and cytochrome peroxidase [9]) to thirty-four (complex of $G_{t\beta\gamma}$ and phosducin [10]). The well-studied interface between barnase and its inhibitor, barstar [11], contains seven deeply buried charges, which form four intermolecular salt bridges.

Consistent with the findings of Jones and Thornton [7], the residue composition of most protein–protein interfaces appears to be more similar to that of protein surfaces, than to that of protein cores. A similar conclusion was reached by Tsai *et al.* [12], who found that, compared with protein cores, interfaces are enriched in both charged and polar residues. A recent study has identified another difference between protein–protein interfaces and protein cores. In a visual survey of 126 homodimers, which are more hydrophobic on average than other interfaces (see above), Larsen *et al.* [13] report that it is rare to observe a single large hydrophobic patch on the interface. Rather, hydrophobic residues tend to be scattered over the entire surface and form small patches that are interspersed with charged and polar residues.

Overall, protein interfaces tend to reflect the composition of protein surfaces, rather than protein interiors, although they are somewhat enriched in hydrophobic residues. This, however, is not always the case, as can be seen from the recent finding that the ligand binding face of CD2 is highly charged [14], even more so, in fact, than a typical protein surface. These observations raise general questions as to whether polar interactions in protein–protein interfaces can, in some cases, make a favorable contribution to complex stability or whether their major role is to enhance specificity. These questions can best be addressed through a combination of well-designed experimental and computational studies.

Analysis of mutagenesis data

The essential test of a theoretical approach is its ability to reproduce experimental data. The most relevant data for the calculation of free energies are the binding affinities of protein–protein complexes of known three-dimensional structure and the effect of mutations on these affinities. As will be discussed, in addition to inherent theoretical uncertainties, comparisons between theory and experiment are often complicated by experimental issues, such as the resolution of the structure determination, the absence of structures of the unbound monomers and, most importantly, the generally undefined changes in structure that accompany mutation.

A compilation of the results of alanine-scanning mutagenesis of protein–protein complexes [15•] demonstrates that, in the majority of cases, mutations of polar and charged residues result in complex destabilization. As has been discussed previously in the context of protein folding [16], the removal of one member of an isolated hydrogen-bonded pair or ion pair is generally expected to result in destabilization, even if the hydrogen bond (or ion pair) is itself destabilizing. Thus, the mutagenesis results reflect the effects of the removal of a particular charge, but do not, by themselves, indicate whether polar interactions stabilize or destabilize protein–protein complexes. For example, if one member of an ion pair is removed and a complex is destabilized, this demonstrates that the residue in question is stabilizing when the other residue is present. It does not indicate whether the ion pair itself is stabilizing relative to a reference state consisting of the two isolated monomers.

Hendsch and Tidor [17•] recently discussed two different partitioning schemes to describe the effect on complex stability of the removal of either a full or a partial charge from a given residue. In the first scheme, the electrostatic contribution of a particular residue consists of the sum of its desolvation penalty and half the value of the change in its electrostatic interactions with other residues upon complex formation. In the second, all interactions with other residues are fully attributed to the contributions of the given residue. In the first method, the total electrostatic free energy of association is a sum of individual residue contributions. The major disadvantage is that the free energy contribution of a given residue does not correspond to the effect of mutating that residue into a neutral residue. This is more accurately represented in the second scheme, for which the individual residue contribution represents the effects of mutating that residue and, hence, the relative importance of that residue to the stability of an individual complex. In this case, however, the total electrostatic contribution to binding is not the sum of individual residue contributions, so that, for example, the contribution of an ion pair to complex stability cannot be extracted from the sum of the single residue values. The same consideration holds for experimental mutagenesis studies on protein complex stability.

Computational studies of binding free energies

Most treatments of electrostatic contributions to binding have been based on the methods of continuum electrostatics [6]. The internal dielectric constant of a macromolecule, ε_{in}, is a crucial variable associated with calculations of this type and there has been much discussion and controversy regarding the most appropriate value of this parameter. It is essential to understand, in this regard, that a dielectric constant is a means of accounting for responses to an electric field that are not treated explicitly. Thus, for example, a dielectric constant of four approximately accounts for the response of the dipolar groups of the polypeptide backbone [18], but higher values may well be appropriate for the regions of the protein surface, especially those near charged residues. Indeed, values of ε_{in} as large as 20 have been used in the calculation of pK_as [19], but as structural responses, such as the redistribution of

protons and conformational change, upon changes in ionization state have been modeled explicitly [20•–22•], there has been a return to the use of smaller dielectric constants.

The same issues pertain to the calculation of the binding free energies of protein–protein complexes. There will, in general, be conformational changes associated with binding or with the mutation of one or more residues, and the dielectric constant that is used should depend on how these changes are treated. For example, Schapira *et al.* [23] used a value of eight in their study of protein–protein, protein–peptide and protein–ligand binding. Muegge *et al.* [24] suggested the use of a high value of ε_{in} for treating mutations of ionizable interfacial residues and a lower value for neutral interfacial residues. Although such approaches are certainly reasonable, the variety of values that have been used for ε_{in} is somewhat disconcerting. Even once it is understood that the range of values simply reflects the fact that the structural change associated with a certain process (e.g. binding, ionization) is in itself case-dependent, one is left with the problem of having to predict what dielectric constant is most appropriate to a particular problem. This essentially requires that the conformational change associated with that process be predicted or at least estimated, so that one is left with the problem of dealing with that change in an explicit fashion.

Faced with this dilemma, our own response has been to adopt a somewhat conservative approach, which allows different terms to be clearly defined. Specifically, we assume that all conformational changes (i.e. all nuclear motion) will either be treated explicitly or will be ignored (in the sense that they will not be accounted for with a dielectric constant). We use a dielectric constant of two for a given fixed conformation, for which the only dielectric response corresponds to electronic polarizability. On the basis of this approach, we describe protein–protein association as a two-step process [25,26]:

$$P_1 + P_2 \rightarrow P_1^* + P_2^* \quad (1a)$$

$$P_1^* + P_2^* \rightarrow P_1^* P_2^* \quad (1b)$$

where the first step corresponds to the uncomplexed proteins adopting 'strained' conformations identical to those they adopt in the bound complex. The second step corresponds to the rigid-body association of the two 'strained' molecules. The free energy change associated with the first process is defined as ΔG_{strain} and corresponds to any conformational enthalpy and entropy changes of the individual monomers that result from binding. ΔG_{strain} must always be positive. Calculations based on Step 1b should thus yield binding free energies that are too low. The electrostatic free energy change associated with binding is limited to the second step and, for this rigid-body association, the use of dielectric constant of about two is appropriate. We note, in this context, that protein–protein association often involves only modest conformational change [8••,27], suggesting that a rigid-body approximation might be applicable in many cases.

An analysis of the binding of a series of peptides to MHC class I compounds revealed that, as expected, electrostatic interactions are net destabilizing and that nonpolar interactions, as exemplified by free energy surface area relationships, drive binding [26]. This general feature is also observed in the studies by Schapira *et al.* [23] and Reddy *et al.* [28•]. Hendsch and Tidor [17•] found that the net effect of electrostatics was to oppose dimer formation by the GCN4 leucine zipper, but did note instances where individual ion pairs enhanced complex stability. They also point out that intramolecular electrostatic interactions are enhanced upon binding. For example, opposite charges on the same subunit whose interaction is shielded by solvent in the free monomer find themselves in a low dielectric environment in the complex, so their attraction is enhanced. This effect can be comparable in magnitude to 'direct' intermolecular interactions and provides one way in which pairwise interactions can be enhanced so as to compensate for the desolvation penalty associated with charge burial.

The polar nature of protein interfaces provides other means by which buried charged and polar groups can be stabilized. In a study of 19 protein–protein complexes, Nussinov and collaborators [29] reported a positive and statistically significant correlation between binding affinity and the number of ionic interactions spanning an interface. Using the methods of continuum electrostatics, the authors identified two salt bridges, out of four examined, that appeared to enhance binding affinity. They point out that the polar nature of protein–protein interfaces allows greater electrostatic stabilization of complexes than is possible, for example, in the interior of proteins. This stabilization might involve networks of electrostatic interactions, such as those that have been found in hyperthermophilic proteins and that account, in part, for their enhanced thermal stability [30•]. A similar effect is likely to play a role in protein–protein association. For example, in a random sample of 10 complexes representing different functional classes, we found that ionic and hydrogen-bonding groups often form networks, with a single residue participating in several interactions across the interface (FB Sheinerman, R Norel, B Honig, unpublished data).

The bifurcated ion pair formed between Asp39 of barstar and Arg83 and Arg87 of barnase provides an example of how a simple network of electrostatic interactions can enhance complex stability. Our calculations using the finite difference Poisson–Boltzmann (FDPB) method [31,32] indicate that, although each of the two salt bridges would be unstable in isolation, the association of all three charges is energetically favorable (FB Sheinerman, B Honig, unpublished data). To test the generality of this observation, we examined all interfacial salt bridges in three complexes that have been well-characterized experimentally: barnase–barstar [11], human growth

hormone–receptor [33] and neuraminidase N9–NC41 antibody [34]. In all cases, the interfacial salt bridges are stabilized by the adjacent ionic or hydrogen-bonding interactions and favor binding (FB Sheinerman, B Honig, unpublished data). However, the same calculations indicate that the overall effect of electrostatics is to destabilize the human growth hormone–receptor and neuraminidase–antibody complexes, and is quite small for the barnase–barstar complex (relative to the uncomplexed state).

Despite the increasing success of theoretical methods to account for binding affinities, the accuracy of these calculations is likely to remain limited for some time by a number of factors. The well-known limitations of the models that are used, such as uncertainties in the potential functions and/or dielectric constants, are likely to be with us for some time. In addition, calculations are extremely sensitive to geometry and it is essential that highly accurate structures be used. This is, of course, very difficult to achieve in cases for which significant conformational changes accompany binding. Similarly, the conformational changes in a complex upon mutation will also, in general, not be known, a complexity that is underlined by recent structural studies that reveal significant conformational rearrangements occurring upon mutation [35•,36•]. Finally, it is often important to consider a possible change in the ionization state of charged residues at the mutated interface [37•]. The effect is particularly pronounced if the residue that is substituted is charged and the mutation is not conservative.

In a practical sense, uncertainties concerning conformational change clearly complicate the accurate calculation of binding free energies or changes in binding free energies upon mutation. Our approach with regard to the study of mutational effects has been to assume that there is no conformational change in the protein upon mutation (FB Sheinerman, B Honig, unpublished data). This guarantees that the effects we calculate will be overestimates but, as we have shown, the residues with the largest calculated effects correlate nicely with those that have the largest observed effects. In this way, we can identify 'hot spots' [38] on protein surfaces using the relatively fast FDPB calculations that do not deal with conformational change. An even more efficient approach to the treatment of electrostatic interactions is to use simplified electrostatic scoring functions that capture the essential features of the interaction [39]. Such functions are also finding increasing applications in docking exercises, where electrostatics serves as an effective screen against complex structures that are reasonable in the sense that they satisfy geometric criteria, but that appear to have highly unfavorable electrostatic contributions to binding [40,41].

Rate-determining factors in protein–protein association

In addition to affecting binding free energies, it has been known for some time that electric fields around proteins can affect association rates as well, leading to 'on constants' that can be substantially larger than expected from a diffusion-controlled reaction in the absence of an electric field. A combination of Brownian dynamics simulations and the FDPB method has been used to study the association rates of small substrates to proteins for some time [42]. The simulations have been able to reproduce observed salt effects on association and, for example, early predictions on the effects of mutations on superoxide dismutase [42] have been subsequently verified using site-directed mutagenesis [43]. Similarly, extensive experimental and theoretical studies on acetylcholine esterase and triose phosphate isomerase have revealed that the binding of ligands involves the diffusion-controlled association of the substrate in the electric field generated by the enzyme [44–46]. The channeling of substrates between enzyme active sites in multienzyme complexes, under the influence of electric fields, has also been observed [47], suggesting that electrostatically mediated diffusion might be a phenomenon of quite general applicability in biological systems (see also [48]). An important finding in all of these studies has been that the charge distribution of the enzyme is a crucial factor affecting association rates. In addition, the phenomenon of electrostatic focusing of electric fields, caused by the shape of the dielectric boundary between the protein and aqueous phase, is an important factor in determining the magnitude of the electric fields produced by a protein [49].

Protein–protein association is a harder process to simulate than ligand–protein association, as both interacting molecules are large and will, in general, have complex shapes and charge distributions. Hence, Brownian dynamics simulations of protein–protein association have often employed simplified protein models (e.g. [50]). An important finding has been that electrostatic interactions increase the amount of time that two proteins stay close to one another, allowing them sufficient opportunity to rotate into a proper orientation for binding. During the past year, there has been major progress reported in the quantitative description of protein–protein association. Two approaches have been used. The first involves Brownian dynamics simulations and the second is based on transition-state theory. In the first, it is necessary to define an 'encounter complex', which is the state that corresponds to the end point of an association reaction. The transition-state approach requires that the nature of the transition state for association be defined. Whether the encounter complex and transition state are formally identical depends on their precise definition; however, both are influenced by long-range, as well as short-range, interactions and, in both cases, the final complex is formed quickly after the encounter complex (transition state) has been reached.

Gabdoulline and Wade [51•] used Brownian dynamics to study the association of barnase and barstar, and compared their calculations with the experimental results of Schreiber and Fersht [52,53]. They used a definition of an encounter

complex involving at least two native intermolecular contacts. Vijayakumar *et al.* [54•] used transition-state theory and an expression due to Zhou and Szabo [55] to study the same experimental data [52,53] and also obtained good agreement with experiment. In these calculations, the electrostatic free energy of the transition state is calculated directly, assuming that, in this state, the interacting proteins are separated by one layer of solvent. In a study of a number of protein–protein complexes, Selzer and Schreiber [56] have shown that the electrostatic energy of interaction between proteins in a complex correlates strongly with the rate of association.

Recent Brownian dynamics simulations of the binding of barnase to barstar [51•] and fasciculin to acetylcholine esterase [57••] have found that the best agreement with experiment is obtained if it is assumed that partial desolvation of polar groups accompanies the formation of the encounter complex. The observation that the extent of desolvation is less in the encounter complex than in the final structure accounts for the fact that electrostatics can enhance association rates while destabilizing the final complex [57••]. The nature of the encounter complex obtained in these studies is related to the transition state defined by Vijayakumar *et al.* [54•], which, despite having a different structure, will also produce moderate desolvation effects. It is also consistent with the study of Camacho *et al.* [58•], who demonstrated that partial desolvation is an important feature of encounter complexes and that it becomes dominant for complexes with at least one neutral reactant.

Conclusions

Taxonomic studies of protein complexes reveal that protein–protein interfaces are quite polar and are more closely related to the surfaces of proteins than to their interiors. This requires that interfaces be designed so as to exploit electrostatic interactions, which generally destabilize folded proteins. Desolvation effects are partially compensated in interfaces through the formation of networks of ion pairs and hydrogen bonds, which are positioned so as to interact favorably with one another. Theoretical calculations can account, in a qualitative sense, for observed binding free energies and for the observed effects of mutations on complex stability. Accurate quantitative agreement is difficult to obtain, in part as a result of poorly defined conformational changes that accompany binding and mutation.

The rate of protein–protein association is also strongly affected by electrostatic interactions. An important finding of the past year is that the encounter complex formed prior to the appearance of the final complex involves partial desolvation of the interacting proteins. This allows attractive long-range interactions to dominate the kinetics of binding and accounts for the observation that electrostatics opposes binding in a thermodynamic sense, but drives association kinetically.

In summary, it appears that protein interfaces can be designed so as to optimize interactions between charged and polar groups, and that these interactions, under certain circumstances, can be stronger than the desolvation penalty associated with their burial. This, in turn, can produce interactions that are stabilizing, highly directional and distant-dependent, allowing the remarkable specificity that characterizes recognition processes involving biological macromolecules.

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