Weak interactions and lessons from crystallization

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Ideas derived from the study of the process of crystallization may provide insights into molecular recognition in biological systems. Both processes exploit the cooperativity which arises from the formation of a large array of weak interactions.

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Linus Pauling pointed out [1] that crystallization is in many ways an instructive model for the molecular recognition processes that occur in biology. (Yet even for crystallization, the crystal structure is not normally predictable from the structure of the molecule from which it forms [2].) In both phenomena, weak interactions between molecules give rise to spontaneous order, and lessons applicable to molecular recognition can be learned from a study of crystallization.

The association of two molecules (A and B) is often formalized as in equation 1:

$$A + B \rightleftharpoons A{\bullet}B \qquad (1)$$

However, if one or more of the associating components is a folded polypeptide (essentially all biological receptors are in this category) or a polymer such as DNA or RNA that forms duplex or folded structures, then the binding energy of the two components cannot reliably be sought at the binding interface, even after consideration of the energetics of desolvation of this interface [3]. If we temporarily ignore the interactions with solvent, then in such cases a more appropriate form of the equilibrium would be:

$$A + B \rightleftharpoons A'{\bullet}B' \qquad (2)$$

Equation 2 recognizes that once A and B have associated, then typically, they no longer exist. Rather, they have been replaced by modified entities A' and B'. The crucial point, which the formalism of equation 2 emphasizes, is that the binding energy between the two entities is not simply a property of the interface between them, but also depends on the modifications of the internal structures of A and B (A → A' and B → B'). It might be argued that this is self-evident, but reference to the literature indicates that appreciation of this point is not universal. It is a common practice to rationalize the observed binding energy between A and B by examination of the interface between them, and to minimize or ignore the consequences of the changes A → A' and/or B → B'. These changes may at one extreme take the form of obvious structural modifications, but at the other extreme may, in principle, involve essentially no structural reorganization but simply a 'tightening' (or a 'loosening') of the internal structure of A when it is modified to A' (or of B when it is modified to B').

It is this subtle 'tightening' of biological structures that we will consider in terms of crystallization, in contrast to the well defined changes in the conformation of individual residues that can enhance binding by allosteric effects. First, we will discuss the origin of the 'tightening' of biological structures through an analysis of the melting of pure crystals and the 'melting' of RNA. We will then provide examples of associations which may derive substantial binding energy from changes within the associating structures rather than at their interface.

Kinetic barriers that give rise to order

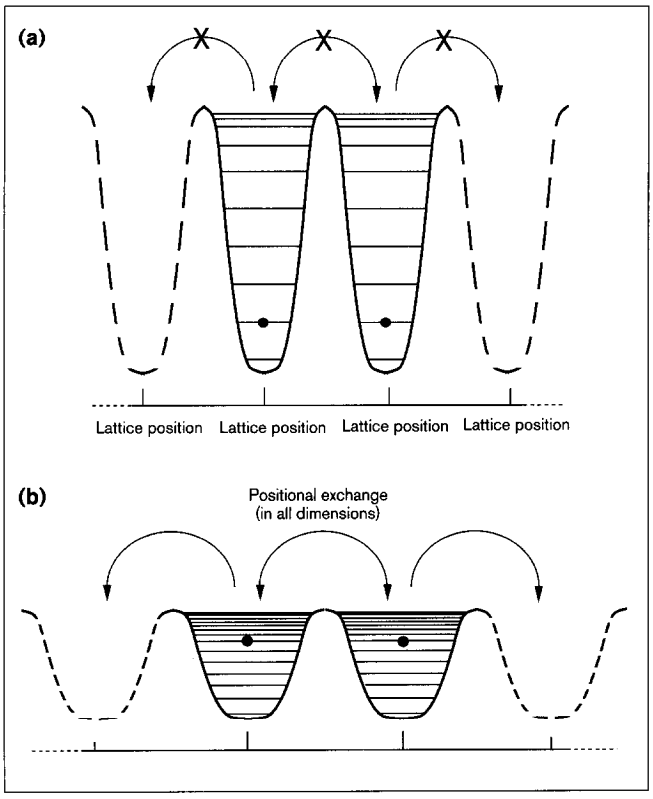
In a recent paper [4] we have pointed out that the melting points of a wide range of substances (from nitrogen, N_2 (T_m = 63 K) to calcium fluoride, CaF_2 (T_m = 1691 K)) correlate well with electrostatic binding energy. The correlation becomes evident only when molecules containing internal rotations (which can be freed, with entropic advantage, upon crystal melting, lowering the melting point) are excluded from the data set. To rationalize this correlation, we used a model for melting in which a barrier has to be overcome to allow a positional exchange of particles, and that this barrier is some fraction of the intermolecular potential [5]. Melting occurs when passage over this barrier occurs at a rate such that $\Delta H_f = T\Delta S_f$; in other words, the melting point is, of course, controlled by the thermodynamics of the system.

In considering this model, one must appreciate how the thermodynamics of a system can be changed when the crucial parameter is the rate of passage over a barrier which allows positional exchange of particles. Whatever the mechanism of positional exchange, the product of exchange is taken to be identical to the starting state. The thermodynamic parameters that describe this state (its enthalpy and its entropy) depend upon the average bonding (enthalpy) and average motion (entropy) in the system. As the temperature is increased, the average degree of motion in the system increases (the entropy increases), and the average degree of bonding decreases (the absolute enthalpy increases). Since the thermodynamic description of the whole ensemble of particles

includes those which (with low probability) are in higher vibrational states, or even in the act of passing over the barrier, a faster rate of passage over the barrier increases the entropy of the ensemble and reduces the bonding in it. Thus the rate of passage over the barrier affects the thermodynamic parameters which describe the ensemble. Importantly, since all adjacent particles in the crystal help to hold each other in place, a critical level of particle motion and exchange can be reached (at the T_m) such that the barrier to exchange drops drastically (Fig. 1).

The model summarized in Figure 1 suggests a mode of binding that biological receptors might use. Suppose, for example, that a receptor in its free state has a binding cleft for a ligand, which behaves with respect to the receptor as a defect behaves in a crystal. The filling of the crystal defect stabilizes the crystal, and we might consider that in the same way the binding of a ligand to the receptor can induce a more stable receptor structure (perhaps even without obvious change in its geometry), and so increase the population of the ligand/receptor state (i.e., give rise to a large binding affinity). This notion leads us naturally to the consideration of the size of molecular arrays in which this kind of effect might be of significant magnitude.

Figure 1



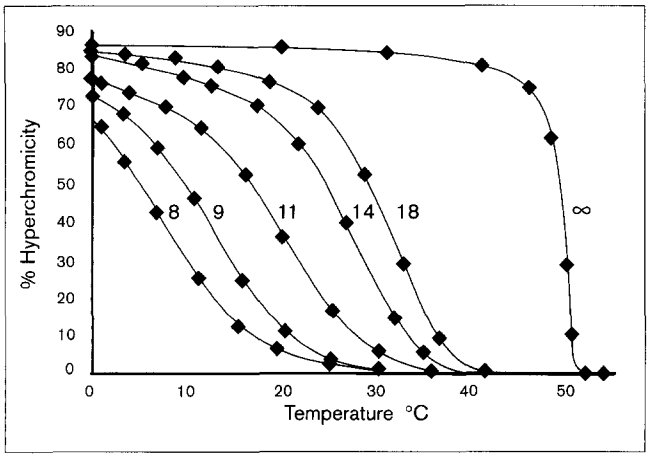
Enthalpy wells of adjacent molecules in a crystal marginally **(a)** below and **(b)** above T_m. Below the melting temperature (a), each particle (represented by a heavy dot) lies deep in an electrostatic well at a lattice site and positional exchange is not possible. At temperatures above T_m (b), there is more motion in the quasi-lattice and so each particle is not fixed on a lattice site. That is, each particle possesses more entropy but is bound less tightly such that it can move from one enthalpic well to the next. The horizontal lines denote vibrational energy levels.

The 'n' effect in molecular recognition

The sharpness of melting transitions can be understood in terms of the differences of the thermodynamics of 'small' and 'infinite' systems [6]. The sharpness of the transition increases with increasing numbers of identical interactions in the melting entity (the 'n' effect). This effect is simply illustrated by the following analysis. The classical chemical potential plot for phase changes predicts the co-existence of liquid and solid many degrees above and below the melting point, with a gradual change in the equilibrium constant between solid and liquid. For example, consider the case of the water to ice conversion, using the molar enthalpy of fusion (6.0 kJ mol^{-1}) and the molar entropy of fusion (22 J K^{-1} mol^{-1}) [7]. At 263 K, ΔG for freezing is calculated to be –0.2 kJ mol^{-1}; at 283 K, ΔG is calculated to be +0.2 kJ mol^{-1}. These free energy differences are so small that both species would be expected to be observed in equilibrium at these temperatures. Of course, this is contrary to the experimental facts and the phase rule, and it is concluded that the molar enthalpies and entropies can be considered to be irrelevant to the problem.

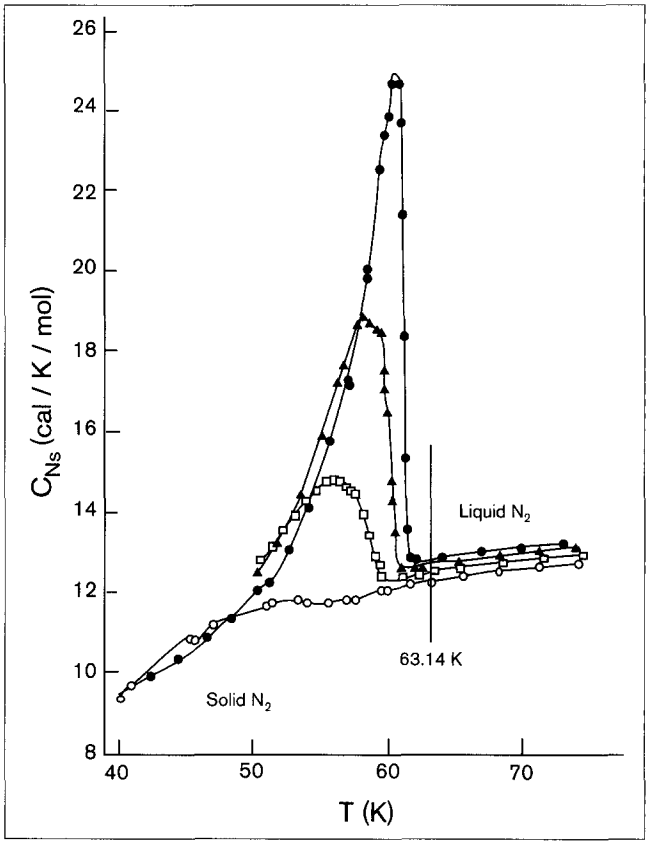
The entropy change that is relevant to the sharpness of the transition is not the molar entropy change but, by extended analogy with the mole concept, the relative molecular mass in grams of the extended bonded array. The system that behaves as a thermodynamic entity is the extended array of interacting particles. Here there is a close analogy with the increasing sharpness of the UV melting curves of RNA duplexes of increasing length. The melting curves for poly(A+U) from Porschke [8] are illustrated in Figure 2. As the molecular weight of the duplex increases, the molar entropy change upon melting becomes larger, and extremely large for a very long polymer (n > 100 repeat units). This is because the favorable entropy change associated with disordering the polynucleotide structure (associated largely with the disruption of base pairs) is multiplied more than 100 times over. Since ΔS is so large, a very small rise in T can tip the balance so that the equilibrium between duplex and disordered strands moves readily from ΔG positive to ΔG negative for melting (i.e. from $\Delta H > T\Delta S$ to $\Delta H < T\Delta S$). This example illustrates the irrelevance of the molar entropy of melting to the sharpness of the melting transition for a crystal made up of an effectively infinitely large number of interacting units. For the melting of a block of 18g of perfectly pure ice with a perfectly formed lattice, the relevant species to consider has a molecular mass given by $6.022 \times 10^{23} \times 18$ g. The approach we espouse here is followed when one considers the melting of an RNA homopolymer duplex; there, we take the mole as

Figure 2



UV melting curves of poly(A+U) duplexes. Hyperchromicity absorbance is used as a measure of helix melting, and plotted as a function of temperature and increasing chain length (which is indicated by the number on each curve). Adapted from Porschke [8], and reprinted with permission.

Figure 3



The sharpness of the phase transition increases with the amount of N_2 adsorbed onto a surface. The diagram shows the molar heat capacity of adsorbed nitrogen (N_2) in the transition temperature region for varying thickness of nitrogen layer. ○, 2.2 V_m; □, 3.1 V_m; ▲, 4.0 V_m; ●, 4.8 V_m, where V_m is the volume of a monolayer over a fixed surface area, and C_{Ns} is the molar heat capacity at constant amount absorbed; 1 $calK^{-1}mol^{-1} = 4.184\ J\ K^{-1}\ mol^{-1}$. Adapted from Morrison *et al.* [9].

that of the covalent entity and not that of the individual bases. The way in which increasing levels of an impurity in a crystal increasingly broaden the melting transition is, of course, accommodated; it is due to the decreasing molecular mass of each perfectly extended array in the crystal.

The usefulness of the view that the extended array should be considered as the effective mole is emphasized by the enthalpies of melting (ΔH_{vH}) which can be obtained from van't Hoff plots. Recall that ΔH_{vH} is obtained from the slope of a line in a plot of ln K against 1/T. It is clear from Figure 2 that ΔH_{vH} will increase with increasing duplex length, as naturally expected on a molar basis. However, it is equally clear that the van't Hoff enthalpy of melting of ice is essentially infinite (ln K varies essentially infinitely over a minute temperature range), in sharp contrast to the previously cited value of 6.0 kJ mol^{-1}. The origin of this apparent anomaly is evident from the model for crystal melting described above — the ensemble that behaves as an entity is essentially an infinite one, and therefore the relevant mole is essentially infinite. Thus, for a system of n identical interacting units in a perfect array, then as $n \rightarrow \infty$, $\Delta H_{vH} \rightarrow \infty$.

The potential for extended arrays to act as sharp switches that may be turned on by relatively subtle effects can be illustrated by some very early work by Morrison *et al.* [9] (Fig. 3). Their data show that as the layers of N_2 molecules adbsorbed on to a graphite substrate of a given area become more numerous, the stability of the ensemble becomes greater, and the sharpness of the transition temperature (melting point) increases. The greater the number of layers, the greater the kinetic barriers to particle exchange and the greater the bonding per molecule.

To summarize the argument so far, then, a ligand may in principle tighten a receptor structure and so stabilize it, in the same way as filling a defect can stabilize a crystal. The tightening would be associated with the increase of local kinetic barriers to disrupting the structure, and so improve the local bonding with a benefit to ΔG which is greater than the associated cost in entropy. Because of the 'n' effect, sharp transitions providing much potential for binding energy can best be found in extended arrays; that is, receptors can benefit from being a large network of weak interactions.

Cooperativity beyond the interface

A clear illustration of enhanced binding energy originating from a tightening of structure remote from the ligand binding interface is given by the vancomycin group of glycopeptide antibiotics. While some glycopeptide antibiotics show no measurable propensity to dimerize (e.g., teicoplanin), some dimerize strongly (e.g., eremomycin) [10] . In general, the antibiotics dimerize more strongly in

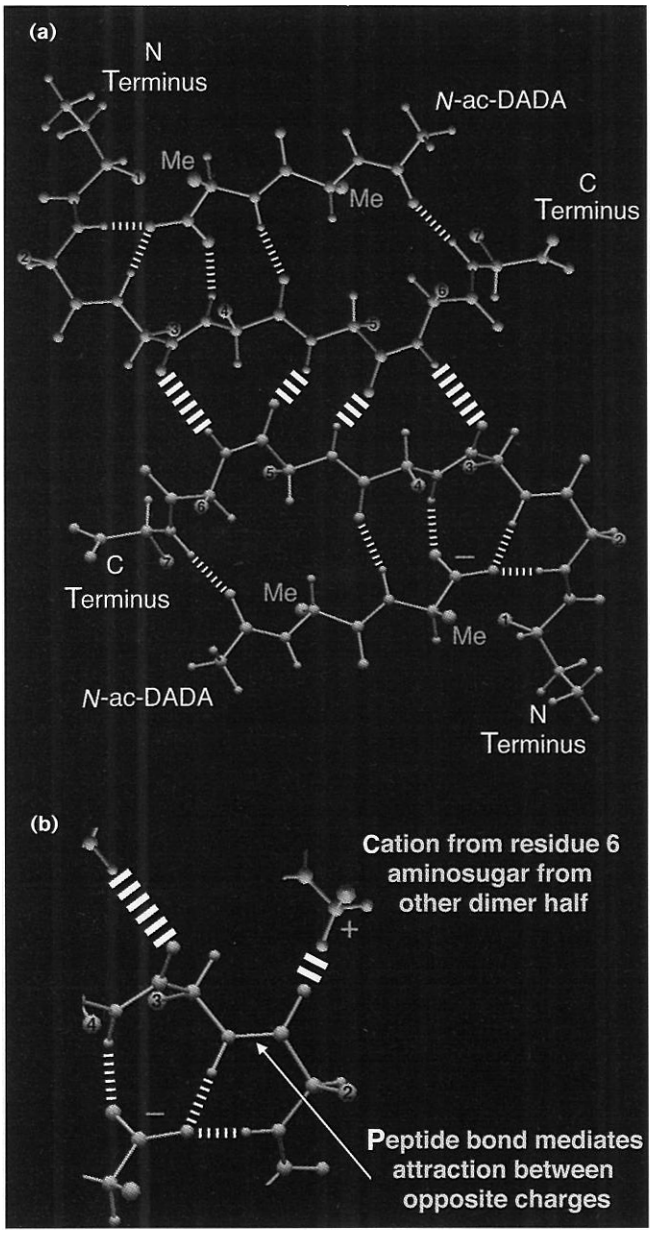
the presence of bacterial cell wall mucopeptide precursor analogues than in their absence [10]. For example, the glycopeptide antibiotic eremomycin has a K_{dim} of $3 \times 10^6\ M^{-1}$ in the absence of di-*N*-acetyl-Lys-D-Ala-D-Ala, but a K_{dim} of $3 \times 10^8\ M^{-1}$ in its presence. It follows (from a thermodynamic cycle) that di-*N*-acetyl-Lys-D-Ala-D-Ala is bound more strongly by the dimer than by the monomer by a factor of 10 (each site of the dimer binds the cell-wall analogue with the same affinity [11]). The basis for at least part of this cooperativity can be inferred from the structure of the ligand-bound dimer (Fig. 4) The antibiotics showing the largest dimerization constants have, in addition to the four hydrogen bonds at the dimer interface (heavy dashed lines in Fig. 4a), two additional hydrogen bonds. These two bonds are from the alkylammonium ions of the amino sugars [10,12] (which are found in the strongly dimerizing antibiotics) to amide carbonyl groups in the other half of the dimer (Fig. 4b). The alkylammonium ions, one at each end of the head-to-tail dimer, are brought into the proximity of the cell-wall analogue carboxylate anion (one in each half of the dimer; Fig. 4b). The resulting Coulombic attraction can tighten binding at both the ligand/antibiotic and dimer interfaces (Fig. 4). One way of looking at this cooperativity is that strong dimer formation also makes for the formation of a salt bridge which is mediated through an intervening amide bond (arrowed in Fig. 4b). The evolution of this sophisticated interaction suggests that dimers may work more efficiently than monomers in antibacterial action, and indeed this has been shown to be the case [12]. But in this context, the relevant questions are:

(1) what is the origin of the extra binding energy that increases the dimerization constant of eremomycin when ligand is bound?

(2) what is the origin of the extra binding energy that increases the affinity of binding of di-*N*-acetyl-Lys-D-Ala-D-Ala to the dimer of eremomycin?

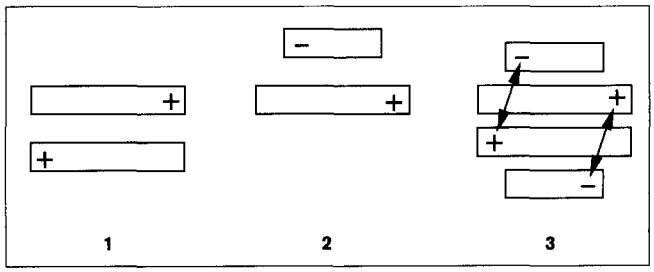
These questions have not yet been addressed by experiment. Yet it is possible to construct what seems to be a physically reasonable model (shown schematically in Fig. 5,

Figure 4



Interactions between glycopeptide antibiotic dimers and their ligands. **(a)** The structure of the antibiotic dimer (backbone only) bound to ligand (*N*-acetyl-D-Ala-D-Ala, labelled *N*-ac-DADA). The hydrogen bonds at the dimer interface are represented as broad dashed lines and those in the ligand binding pocket as narrower dashed lines. **(b)** The strongly dimerizing antibiotics have an amino sugar (not shown in Fig. 4a) on residue 6 which has a Coulombic interaction with the carboxylate of the ligand. This is essentially a salt bridge mediated by the peptide bond between residues 2 and 3.

Figure 5



A model for the interactions between eremomycin and di-*N*-acetyl-Lys-D-Ala-D-Ala. The salt bridges that can form only when the antibiotic is dimerized are shown as double-headed arrows. These interactions cause a tightening of all three intermolecular interfaces, shown as a decreased distance between the molecules. Although the reduction in the degrees of freedom of the atoms in the interface will lead to a more unfavorable entropy of binding of the dimer, the enthalpy of binding is presumably increased to a larger extent.

with the dimer represented by **1**, ligand-bound monomer by **2**, and ligand-bound dimer by **3**). In these diagrams, the + signs represent the ammonium ions of the amino sugar, and the – signs represent the negative charge of the carboxylate ion of the cell-wall analogue ligand. Since there is little doubt that at least part of the cooperativity is due to the Coulombic attraction between these two opposite charges, this model suggests that part of the extra stability of the dimer structure **3** over the dimer structure **1** comes from the stronger binding of the ligand in **3** relative to its binding in **2**. Thus, when we compare the process of two **2** entities coming together to give **3** with the process of the formation of **1** from two antibiotic monomers, some of the increased energy of association comes from the fact that ligand is bound more tightly in **3** than **2**. In other words, some of the favorable free energy that increases the dimerization constant by a factor of 100 would come from the 'tightening' of the ligand/antibiotic interface upon dimerization. The Coulombic attractions that are unique to **3** (indicated by double-headed arrows) can be expected to lead to strengthening of the weak interactions at all three interfaces present in **3**. It is for this reason that we have schematically inferred bond shortening at all three interfaces in **3** relative to **1** and **2**.

Similarly, the increased affinity of the association of the ligand with the dimeric receptor (giving **3**) over that of its association with the monomeric receptor (giving **2**) cannot be solely ascribed to the strengthening of the weak interactions at the ligand/receptor interface in **3** relative to **2**. It will also be due, in part, to the strengthening of the weak interactions at the dimer interface.

Remote effects on binding affinities in other systems

The above models illustrate in a simple way the possible origins of binding affinity that does not involve the binding interface. We now consider how such remote interactions can, if extended in a three-dimensional network, add to the stability of biological structures; the notion is the same in principle, but more subtle in practice. This argument relies on a consideration of the behaviour of crystals to develop the idea that extended sets of interactions possessing kinetic barriers to local motion can, if extended to three-dimensional networks of receptors, provide the potential to bind a ligand to a receptor with very large affinities. The origins of the binding energy in these systems may be not at all evident at the binding interface.

Although the thermodynamics of extended systems have important implications for the study of biological receptors, and for the study of binding affinities by the pharmaceutical industry, these implications currently have to be ignored, because of our inability to deal quantitatively with these problems. In this section, we consider experimental evidence for the kind of effects considered above, and comment on the difficulties of approaches which, for reasons of pragmatism, have to ignore these effects.

The trp repressor–operator system

Ladbury *et al.* [13] have investigated the differences in the crystal structures of the free trp repressor and the trp repressor when complexed to the trp operator by measuring the mean values of the temperature factors (B) for each residue. A low value of B indicates that there is relatively little motion of the residue within the crystal. When ΔB ($= B_{complex} - B_{repressor}$) is calculated for each residue of the repressor, it is clear that for most of the residues ΔB is negative, so that there is less motion of most residues in the crystals of the complex than in the crystals of the free repressor. This occurs through most of the protein and not just at the repressor–operator interface. Hence, it is clear that on the formation of a complex involving a protein, the change in the motion throughout the whole protein must be considered. Any spontaneous change in the motion of individual residues must be energetically favorable overall and therefore should contribute to the observed free energy of binding.

The streptavidin–biotin and avidin–biotin systems

The origin of the remarkably strong binding affinity of the small molecule biotin to streptavidin ($K \approx 10^{13}\ M^{-1}$) and to avidin ($K \approx 10^{15}\ M^{-1}$) [14], is not evident from an examination of the binding interface between the associating entities. It might of course be considered that this is because there is an obvious structural reorganization of the streptavidin or avidin receptor upon binding biotin — an approach which is widely considered in analogous cases. But the points which we have emphasized above allow for another possibility — that there is no obvious structural alteration to these proteins on binding biotin; instead there are subtle changes in the 'tightness' of their structures.

Both streptavidin and avidin exist in solution as tetramers with four binding sites with equal affinity for biotin. It has been shown that biotin binds to the tetramers much more strongly than to the individual monomers, and part of this enhanced binding is attributed to a Trp residue in one of the tetramer subunits participating in the binding site of an adjacent subunit [15]. However, there is no obvious conformational change in the protein when biotin binds and there is no evidence for biotin binding more strongly to a partially bound (strept)avidin tetramer [16]. In the well characterized case of oxygen binding to the haemoglobin ($\alpha_2\beta_2$) tetramer, enhanced binding can be attributed to the distinct motion of key residues on the binding of the first molecule of oxygen which enhances binding to other haemoglobin subunits [17,18]. This is not obviously the case for the binding of biotin to (strept)avidin and so we must look for a mechanism other than allosteric interactions that is responsible for the extremely large binding constant.

The binding of biotin to streptavidin has a very large favourable exothermicity and a large adverse entropy ($\Delta H = -134$, $T\Delta S = -57$, $\Delta G = -77$ kJ mol^{-1}) [19]. This is consistent with a tightening of much of the protein structure so that the motion of many of the residues becomes more restricted (giving rise to the adverse entropy) and the bonding between them becomes stronger (giving rise to the favorable enthalpy). Thus large changes in exothermicity and large adverse entropies may arise from very many small changes in numerous interactions within the protein. Possibly no distinct change in the structure would be observed, as very small changes in many interactions could readily account for a large overall change in the thermodynamics.

Mutants and protein structure

A single point mutation in phospholipase A_2 (Asp99→Asn) causes massive non-local changes in the structure of the enzyme resulting in a molten globule-like state [20]. This result raises the question of how such a conservative local change can cause large global changes in structure. Another example of a single point mutation that produces clear, non-local changes in structure is the moderately conservative Glu43→Asp mutation in staphylococcal nuclease [21,22]. This mutation causes perturbations around the active site that may originate from the lowering of the local barriers to local motion, analogous to crystal melting and to the observations with the phospholipase A_2 mutants.

In the above examples, there are no obvious requirements for pronounced allosteric changes, and it is possible that changes in the local barriers to local motion largely control the thermodynamic stability of these proteins. As in crystal melting, a small perturbation may cause the local barriers to drop, in turn causing nearby barriers to drop, so that the effect is propagated throughout the structure, reducing the bonding and increasing the motion. The net effect is to reduce the favorable free energy of the mutant proteins on folding.

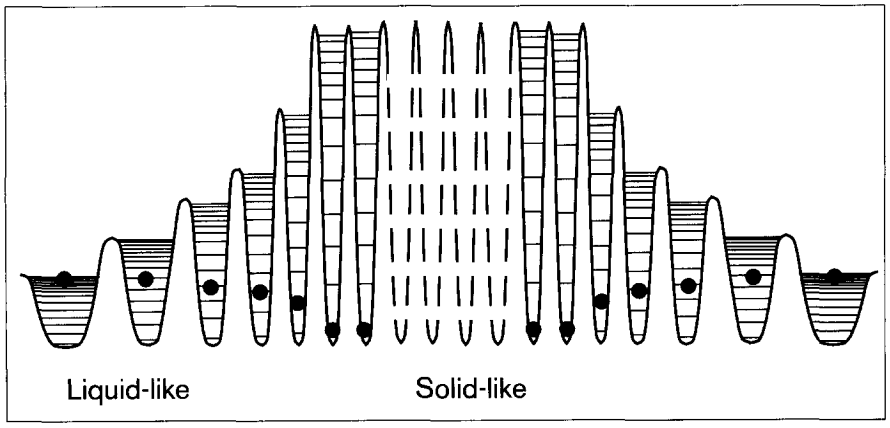
Helices as models for the increase in binding in 'internalized' structures

In considering the interplay between thermodynamics and kinetics in ordered assemblies of weakly interacting particles, the model developed for crystal melting (Fig. 1) gives useful insights as the size of the molecular assembly is gradually reduced. In Figure 1, the large and small free energy barriers to particle exchange existing in crystal and liquid forms, respectively, are represented. In a very small assembly of identical particles, the free energy difference between solid and liquid states (per mole of the assembly) is smaller than for the essentially infinite assembly. Solid and liquid can now co-exist over a finite temperature range. The barrier to particle exchange is less, and the free energy of melting per (conventional) mole is less than for the infinite assembly (cf. Fig. 3). As the size of the finite assembly is gradually increased towards the infinite limit, then at the given temperature, the stability of the solid state relative to the liquid state increases, as does also the kinetic barrier to particle exchange in the solid. In Figure 6, analogous arguments are applied to helices (e.g., α-helices or DNA duplexes). Where the helices are relatively unstable (near the ends), the kinetic barriers to disruption of the helix are relatively small, whereas at the centre of a long helix, the kinetic barriers to helix disruption are larger and the helix is more stable. Hence NH exchanges with water are observed to be slowest at the centre of DNA duplexes, and fastest at the ends [23]. In a sense (although the analogy is not perfect), the helix is akin to a liquid at its ends and more akin to a crystal at the center. Thus, the restriction of motion, by whatever means, can increase kinetic barriers and improve the enthalpy of binding.

Enhanced binding affinity by mutations remote from the binding site

Somatic mutations in antibodies remote from the binding site have been observed to increase the affinity for the hapten [24,25]. It has been suggested by Chen *et al.* [24] that this increase in affinity is due to increasing binding site flexibility due to long-range effects, whereas Patten *et*

Figure 6



Schematic of the electrostatic potential wells along the length of a helix. For instance, each well may represent the bonding provided by a hydrogen bond of an α-helix, or by the base-pair interactions along a DNA duplex. At the ends of the helix the structure is essentially liquid-like, while in the centre, where adjacent interactions hold their neighbors in place, the structure is analogous to a solid (see Fig. 1). The heavy dots represent the time-averaged positions of the atoms in the wells.

al. [25] have ascribed it to a reorganization of the active site or reduction in flexibility. It may be the case that the mutations which cause the increased binding affinity do so by promoting an overall tightening of the structure; no obvious structural differences are observed between the crystal structures of the native antibody–hapten complex and mutant antibody–hapten complex [25]. As in the examples above, the tightening of the overall structure on ligand binding may contribute to the observed free energy of binding.

Conclusions

The concepts in this review are garnered from several different fields of endeavor. Collectively considered, they can enhance our understanding of binding energies. Even where biological molecules appear to undergo little structural alteration, the source of the binding energy of association may not be discovered by analysis of the binding interface and the interactions that the interfacial components make with the solvent before association. Large receptors can, in principle, afford much binding affinity for a ligand while themselves only undergoing very subtle changes in the bond lengths of the weak interactions. Devices for restricting motion, which are added remotely from the binding site, can improve the electrostatics of binding without direct modification of the binding site. For example, we have recently shown that membrane anchoring of bacterial cell wall analogs can improve the electrostatics of their bonding to glycopeptide antibiotics [26]. The use of such remote devices to reduce motion in the binding site can usefully be regarded as equivalent to lowering the effective temperature in the binding site. The increase of binding affinities by restriction of motion at an adjacent site can be explored by a combination of the usual methods to measure these affinities (e.g., calorimetry) and to probe motion versus bonding (e.g., NMR and X-ray crystallography).

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