Understanding Noncovalent Interactions: Ligand Binding Energy and Catalytic Efficiency from Ligand-Induced Reductions in Motion within Receptors and Enzymes

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Ligand Binding Energies

Understanding Noncovalent Interactions: Ligand Binding Energy and Catalytic Efficiency from Ligand-Induced Reductions in Motion within Receptors and Enzymes

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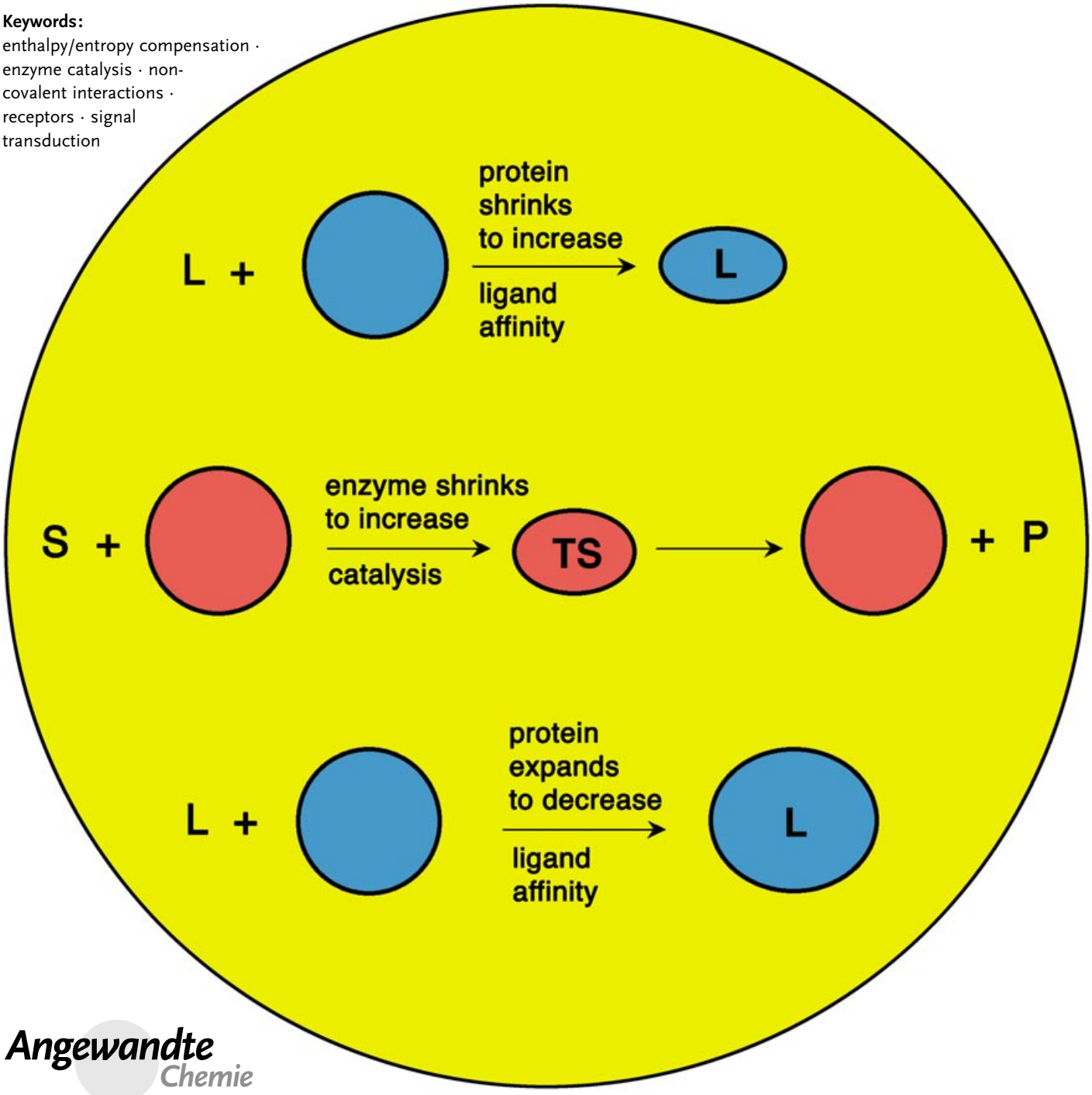

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Noncovalent interactions are sometimes treated as additive and this enables useful average binding energies for common interactions in aqueous solution to be derived. However, the additive approach is often not applicable, since noncovalent interactions are often either mutually reinforcing (positively cooperative) or mutually weakening (negatively cooperative). Ligand binding energy is derived (positively cooperative binding) when a ligand reduces motion within a receptor. Similarly, transition-state binding energy is derived in enzyme-catalyzed reactions when the substrate transition state reduces the motions within an enzyme. Ligands and substrates can in this way improve their affinities for these proteins. The further organization occurs with a benefit in bonding (enthalpy) and a limitation in dynamics (cost in entropy), but does not demand the making of new noncovalent interactions, simply the strengthening of existing ones. Negative cooperativity induces converse effects: less efficient packing, a cost in enthalpy, and a benefit in entropy.

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1. Introduction

With the completion of a large part of the sequence of numerous genomes, a frontier research interest has now developed in the field of proteomics. This field is concerned with the more complete identification of proteins (for example, the identification of 1484 proteins from the yeast strain *S. cerevisiae*),[1] with the interactions between these proteins, and with the way that they bind to ligands. The binding constants for these interactions depend on the differences in noncovalent bonding between free and bound states. Insofar as binding constants cannot be predicted, they are poorly understood, and in this sense the molecular basis of biological function is also poorly understood.

In this Review, an approach that treats systems of multiple interactions as the sum of their parts (the additive approach) is first described. It is a very useful approximation. However, in reality, the interactions are often (but not always) greater than the sum of their parts (positively cooperative) or less than the sum of their parts (negatively cooperative). Such cooperative interactions are covered in the second part of the Review. Some consequences for biology are described in Sections 9 and 11–14, with the implications for the understanding of ligand binding energy and enzyme catalysis being particularly important.

1.1. What Is It that Drives a Change?

The Second Law of Thermodynamics states that "in a spontaneous process, the entropy of the universe increases". The simplest description of entropy S is that it is a measure of disorder; thus entropy increases with increasing disorder (more accessible arrangements). The Second Law therefore tells us that in a spontaneous process there will be an overall increase in disorder (increase in S). If the entropy of the universe is S_0 before the change and S_1 after the change, then the overall entropy change is $\Delta S_{\text{overall}} = S_1 - S_0$. The more

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positive is $\Delta S_{overall}$, the greater is the extent to which the process will occur. In considering the magnitude of $\Delta S_{overall}$ for any process, it is fruitful to divide the entropy change into two parts, namely, the entropy change in the system (what we are studying) and the change in order in the surroundings (the rest of the universe). Since the surroundings are in practice essentially infinite, the change within them is effectively diluted to zero in any finite element, but it is there.

The change in order in the surroundings of the system undergoing change is determined by the change in the enthalpy (exothermic: negative ΔH, endothermic: positive ΔH) of the process, and by the temperature T at which the change occurs. An exothermic process leads to more disorder in the surroundings (by increasing the motion in the surrounding universe). The extent of this increase in disorder is inversely proportional to the temperature. The reason for this latter relationship can be qualitatively understood: surroundings possessing little kinetic energy (low T) become proportionately more random by a given exothermicity than are those possessing more kinetic energy (higher T). Since an exothermic process (ΔH negative) gives a positive contribution to the total entropy change, the increase in entropy from this term is $-\Delta H/T$.

The second part of the entropy change (the change in order within the system undergoing change) is denoted as ΔS_{sys}, and positive values of ΔS_{sys} promote the change. Therefore, taking the two terms together, the propensity for spontaneous change can be quantitated [Eq. (1); for simplicity, the subscript "sys" is normally dropped].

$$\text{Increase in disorder that occurs in a process} = -\Delta H/T + \Delta S_{sys} \quad (1)$$

It is a consequence of the crucial insights of Boltzmann and Gibbs that we have this relationship. Perhaps confusingly for generations of students, a new term ($-\Delta G/T$) was coined for this net increase in entropy in a spontaneous process. Thus, $-\Delta G/T = -\Delta H/T + \Delta S$, and leads to the famous Gibbs equation [Eq. (2)].

$$\Delta G = \Delta H - T\Delta S \quad (2)$$

The Gibbs equation states that ΔG (the free-energy change) is negative for a spontaneous change. Defining ΔG as the *negative* of the total entropy change for a process, multiplied by T, is not so strange as it may at first seem. Early in their careers, physical scientists tend to think in terms of spontaneous change being driven by a release of heat (exothermicity, which means that ΔH is negative). Although this is only one of the determining factors (see above), we therefore have early training to think of a driving force for a spontaneous change in terms of a parameter which has the units of energy and is negative in value. Continuity of thought is encouraged by taking the determinant of spontaneous change (a *positive* entropy change), changing its sign to make it negative, multiplying it by T to give it the units of energy, and calling it "free energy".

Not uncommonly, students are mystified by the spontaneous development of highly ordered organisms, which seems to them inconsistent with the Second Law. It is of course possible because, simultaneous with the development of the ordered organism, the surroundings of the organism are disordered to a greater degree.

Binding affinity can be expressed either in terms of the difference in free energy of free and bound states, or in terms of the equilibrium constant (K), where $\Delta G = -RT\ln K$ (where R is the gas constant and T is the temperature). The more negative ΔG is, the larger is the binding constant. For example, at 300 K, ΔG values of -5.7 and $-11.4\ \text{kJ mol}^{-1}$

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correspond to binding constants of 10 and 100 M^{-1}, repectively, etc. By using the Gibbs equation to understand binding we can monitor the effects of changes in bonding within the system (ΔH) and changes in the entropy of the system (in terms of $T\Delta S$). Although ΔS, rather than $T\Delta S$, is strictly the change in entropy, experiments carried out at one temperature show that the terms are proportional. $T\Delta S$ has the advantage that its effect upon ΔG can be directly compared with that of ΔH.

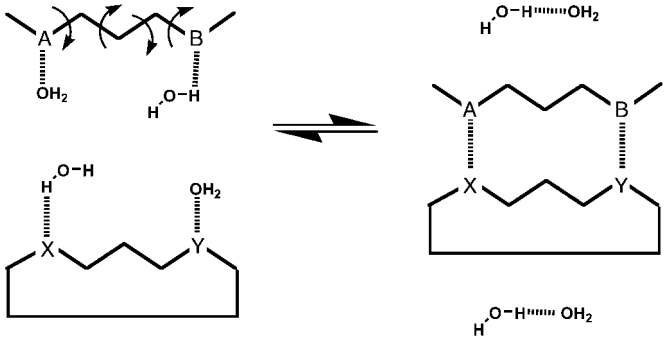

Figure 1. Complex formation between a ligand (containing polar functionalities **A** and **B**) and a receptor (containing polar functionalities **X** and **Y**) with exchange of four water molecules to the bulk solvent. (Broken lines indicate hydrogen bonds.)

1.2. Enthalpy/Entropy Compensation

A key point in what follows is that a decrease in motion implies a decrease in entropy, since it results in fewer accessible arrangements. Consider the formation of a specific noncovalent bond (for example, **A**···**B** for the transformation **A** + **B** → **A**···**B**). An increase in its strength (which corresponds to an increasing negative contribution to ΔH, and a more favorable binding process) will be accompanied by an increasing restriction in the relative motion of **A** and **B** in **A**···**B** (which corresponds to a negative contribution to ΔS, and so unfavorable to binding). This opposing interplay between enthalpy and entropy is known as enthalpy/entropy compensation, and is a fundamental property of noncovalent interactions.[2–4] Importantly, it is not confined to binding in aqueous solution,[5] nor should it be ascribed to errors of measurement.[6] It arises because bonding opposes motion and, also reciprocally, motion opposes bonding. The two effects can be traded off against each other because the strength of noncovalent bonds is, at room temperature, comparable to the thermal energies that oppose them. The idea of enthalpy/entropy compensation is therefore less important in the study of covalent bonds, which are typically too strong to be effectively opposed by thermal motions at room temperature.

2. The Additive Approach

When cooperativity is ignored, contributions of ΔG values to the total free energies of binding may be added together, and contributions of binding constants K are therefore multiplied.

2.1. Costs in the Formation of a Bimolecular Complex (L + R → L···R)

When a ligand **L** binds to a receptor **R** it loses some of its translational (t) and rotational (r) motion relative to the receptor. This cost of association is designated as ΔG_{t+r}, and is an adverse entropy term ($\Delta G_{t+r} = -T\Delta S_{t+r}$).[7–9] If the ligand lost *all* its motion relative to the receptor, then the cost of the association at room temperature for a ligand with a mass of a few hundred Daltons is approximately $+57$ kJ mol^{-1} (opposing binding by a factor of 10^{-10}).[7–9] However, in typical ligand/receptor complexes, the strengths of the noncovalent **A**···**X** and **B**···**Y** bonds (Figure 1) are comparable to thermal energies and hence the bonds are highly elastic. Thus, the motional restriction occurring on ligand binding costs much less than $+57$ kJ mol^{-1} (see Sections 3 and 4).

If a molecule has internal rotations that become more restricted on passing from the free to the bound state, then there is an entropic cost of $-T\Delta S_r = \Delta G_r$ to be paid for each restricted rotation. For example, the ligand shown in Figure 1 (top left) has four internal rotors (indicated by arrows) that allow much internal motion of the ligand prior to binding, but this motion is restricted upon formation of the complex. Binding is opposed by a contribution $4\Delta G_r$, and in the general case by $n\Delta G_r$, where n is the number of restricted rotors.

Complete restriction of an internal rotation has a free-energy cost (ΔG_r) of about 5 kJ mol^{-1} at room temperature.[8,9] This value is also too large to be relevant to drug/receptor complexes, again because the energies of the interactions that are restraining the geometry of the complex are comparable to the thermal energies that tend to disrupt it. As a result, the internal rotors, although proscribed from undergoing full rotation, are able to undergo wagging motions in the bound state.

Molecules may bind to their receptors with the introduction of strain, or repulsive atom/atom interactions. Such effects are difficult to explore experimentally. For pragmatism, they are ignored in a simple approach to estimating binding affinities (Section 3), but are relevant in cases of negatively cooperative binding (Sections 6, 9, and 10).

2.2. Energy Benefits in the Formation of the Bimolecular Complex

The formation of a complex is favored when the ligand forms polar interactions to a receptor to give an overall negative change in free energy. The formation of two polar interactions (**A**···**X** and **B**···**Y**) in water is illustrated in Figure 1. Water molecules which solvate the polar groups **A**, **B**, **X**, and **Y** prior to association become part of the bulk water after formation of the complex. These benefits can be semiquantified by including a benefit in the free energy (ΔG_p) for each interaction (which includes the changes in entropy associated with changes in solvent mobility).

The first attempt at a semiquantitation of drug–receptor interactions in terms of the costs and benefits outlined above was by Andrews, Craig, and Martin.[10] We used a similar approach, but applied an additional term to account for the hydrophobic effect.[11]

The hydrophobic effect is classically defined as a benefit to binding that is applicable when binding occurs in water.[12,13] The benefit is a consequence of the removal of hydrocarbon surfaces from exposure to water upon formation of the complex. This effect can be measured by solvent-transfer experiments. For example, if **A** and **B** as well as **X** and **Y** are each separated by three methylene groups (Figure 1), the coming together of the hydrocarbon surfaces (with their concomitant removal from water exposure) will promote binding. The magnitude of the hydrophobic effect is proportional to the surface area of the hydrocarbon that is removed from exposure to water upon formation of the complex and can be estimated in terms of ΔG_h per unit of area A of hydrocarbon buried. The surface area of hydrocarbon that is removed from water exposure in the binding event is readily measured with the aid of computer graphics. Thus, the contribution to binding from the hydrophobic effect is $A\,\Delta G_h$.

The benefit of the removal of exposed hydrocarbon to water upon binding at room temperature derives from the fact that water molecules at hydrocarbon surfaces are more ordered than water molecules in bulk water.[14] Therefore, the hydrophobic effect is normally largely entropy driven at room temperature, although it has a favorable enthalpy component in the special cases where water is expelled from an internalized cavity upon ligand binding.[15]

3. An Equation for the Estimation of Binding Constants

Binding constants in aqueous solution can be estimated from Equation (3), which is derived from the above considerations.

$$\Delta G = \Delta G_{t+r} + n\,\Delta G_r + A\,\Delta G_h + \sum \Delta G_p \qquad (3)$$

ΔG is the observed free energy of binding, $\Sigma\Delta G_p$ is the sum of the free energies of binding for all the polar interactions made in the binding site,[11] and the other terms are as defined in Section 2. Böhm[16a] "trained" a variant of Equation (3) with a set of 45 interactions of experimentally known binding constants from the association of ligands of small molecular weight (66 to 1047 Daltons) with proteins through sets of known interactions (from X-ray structures). He divided the original ΔG_p values into two groups: those involving ionic interactions (ΔG_{ionic}) and those involving hydrogen bonds formed between neutral entities (the term ΔG_p was retained). Since the modified form of the equation has only five types of ΔG contributions and the 45 binding sites involve different combinations of these five types of ΔG contributions, average values for them can be obtained. The values obtained (Table 1) have proved very useful in the pharmaceutical industry.

Table 1: Average values for the parameters of Equation 3.

Parameter	Physical process	Value (kJ mol^{-1})	Factor[a]
ΔG_{t+r}	energy cost of bimolecular association	+5.4	(ca. 10)
ΔG_r	energy cost of restriction of an internal rotor	+1.4	(ca. 2[b])
ΔG_h	benefit of the hydrophobic effect (per Å^2 of buried hydrocarbon)	−0.17 (Å^{-2})	(ca. 1[c])
ΔG_p	benefit of making a neutral hydrogen bond of ideal geometry	−4.7	ca. 7
ΔG_{ionic}	benefit of making an ionic hydrogen bond of ideal geometry	−8.3	ca. 28

[a] Factor by which binding is promoted (opposed) at RT. [b] Per rotor. [c] Upon burial of 33 Å^2 of the hydrocarbon.

The use of the values given in Table 1 resulted in the equation reproducing the binding constants of the training set (which range experimentally from 40 M^{-1} to 2.5×10^{13} M^{-1}) with a standard deviation of 7.9 kJ mol^{-1}, which corresponds to a factor of 24 in binding affinity.[16a] The standard deviation was a factor of 50 in binding affinity for a set of drugs outside the training set. This result represents an impressive outcome for a limited data set, although it should be noted that the inclusion of a larger variety of drug structures (for example, more heavily weighted with heterocyclic structures) somewhat decreased the success of the algorithm.[16b] Several other relatively successful approaches for estimating binding constants have been published since this work, although the physical interpretation of the various terms (and coefficients) in these approaches is less simple.[17,18]

The apparent binding energies obtained for the interactions (Table 1) are in good agreement with those obtained by other approaches, for example, from the mutation of proteins, which are also based on the partitioning of binding-energy contributions.[19–21] Polar interactions (most commonly amide···amide hydrogen bonds and ionic interactions) are found to promote binding (negative values of ΔG) moderately in aqueous solution. The reason why the promotion of binding is relatively small is evident from Figure 1: the four hydrogen bonds of the dissociated state are replaced by four hydrogen bonds in the bound state.[19]

The value for the hydrophobic effect is in reasonable accord with the values most commonly found in other studies. It is somewhat larger than those values estimated from the (low) solubility of hydrocarbons in water (0.10–0.14 kJ mol^{-1} per Å^{-2} of the hydrophobic surface buried from exposure to solvent).[22–28] Yet it is smaller than those obtained by the deletion of methyl groups in binding sites, both within proteins or drug/receptor interactions (most frequently in the range 0.18–0.26 kJ mol^{-1} Å^{-2},[20,29–32] although a range extending from 0.06 to 0.64 kJ mol^{-1} Å^{-2} has been reported.[33–38]) Presumably, the large range found in the literature reflects, at least in part, variations in positive and negative cooperative effects.

The localization of the drug at the binding site and restriction of internal rotations upon binding (see Section 2.1) spontaneously emerge from Equation (3) as factors which oppose binding (Table 1, positive values of ΔG_{t+r} and ΔG_r, respectively). The average value $\Delta G_{t+r} = +5.4\ kJ\,mol^{-1}$ is remarkably small, and represents only about one tenth of the maximum theoretical entropy loss corresponding to complete immobilization of the ligand (see Section 2). This small value presumably reflects, at least in part, the large residual motion that the drugs can exercise relative to the receptor to which they are bound. The average cost of restricting the rotation of an internal bond in the drugs ($+1.4\ kJ\,mol^{-1}$) is slightly less than that found for the formation of crystals from neat liquids that contain internal rotors ($2–3\ kJ\,mol^{-1}$).[39] This finding probably reflects the fact that rotations are somewhat less restricted in these binding sites than they are in crystals. Most importantly, the application of the equation gives useful approximate binding constants in many cases.

4. Why is the Adverse ΔG_{t+r} Term Relatively Small?

Given the assumption that the average value of ΔG_{t+r} of $+5.4\ kJ\,mol^{-1}$ is representative of drugs as a whole, why is it so small? The stronger the noncovalent bonds that hold the complex together, the larger should be the restriction of overall motion (adverse entropy change, of which ΔG_{t+r} is a measure). To test this hypothesis, it is necessary to examine associations where the entropy change can be put down solely, or largely, to ΔG_{t+r}. Entropy changes arising from changes in solvation must be avoided, or minimized, which precludes the use of data for associations in water or other polar solvents. Additionally, the number of internal rotations that are restricted upon association must be zero, or limited, because interpretation of the entropy change must not be confused by the presence of a significant $n\Delta G_r$ term [Eq. (3)]. In the absence of these effects, the adverse entropy of association will be solely a consequence of, or dominated by, the ΔG_{t+r} term.[40]

The above considerations can be satisfied for simple bimolecular associations of relatively rigid entities occurring in the gas phase ($\mathbf{A}+\mathbf{B}\rightarrow\mathbf{A}\cdots\mathbf{B}$). Binding can only be promoted by a favorable enthalpy of interaction (ΔH), which measures the benefits of noncovalent bonding between the two portions of the complex. Binding can only be opposed by the unfavorable loss in translational and rotational entropy ($T\Delta S_{t+r}$). The anticipated correlation between the heat given out (ΔH) and the order imposed ($T\Delta S_{t+r}$, identical to $-\Delta G_{t+r}$) is shown in Figure 2.[2] There is a limit to the cost in entropy for bond formation in $\mathbf{A}\cdots\mathbf{B}$, which is reached when the bond strength is large compared to thermal energies, and at this point $\delta(\Delta H)/T\delta(\Delta S)$ is very large. However, when bond strengths are comparable to the thermal energy, the restraint is effectively opposed by the motion and $\delta(\Delta H)/T\delta(\Delta S)$ is smaller. Therefore, we can anticipate that the slope of the ΔH versus $T\Delta S$ graph (Figure 2) will increase as the bond strength increases through the "noncovalent region".

Dunitz has indeed shown that a curve closely following the form of Figure 2 can be derived from a semiquantitative approach based upon shallow energy wells.[3] For such wells, bond extension and increased dynamics should become important as the depth of the well decreases. This point is illustrated schematically in Figure 2 for decreasingly exothermic associations (green, red, and black points on the curve). The decreasingly exothermic associations not only give rise to "looser" associations, but also to ones that give a less-favorable free energy of binding ($\Delta G = \Delta H - T\Delta S$) for population of the organized state. This last consequence follows because the weakest noncovalent interaction (black point) is, of the three, the one with the most efficient enthalpy/entropy compensation.[3,4]

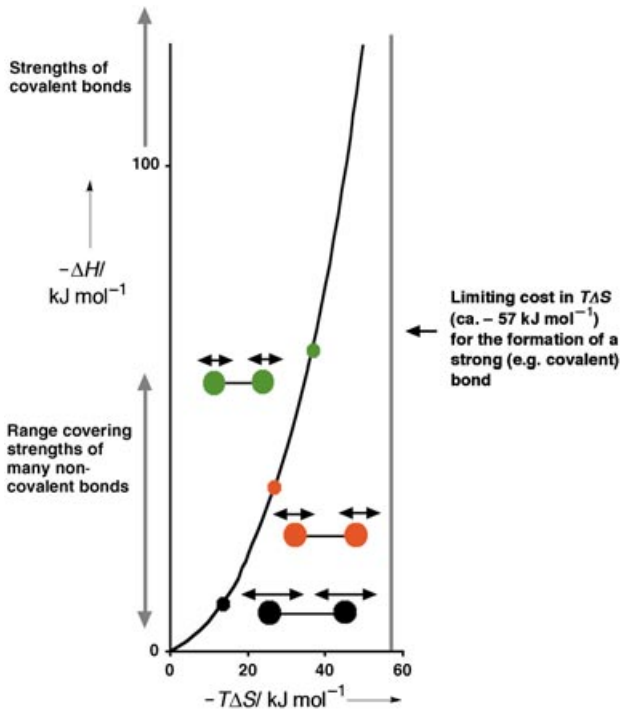

Figure 2. Anticipated relationship between the exothermicity (ΔH) and the cost in entropy ($T\Delta S$) for the complex formation $\mathbf{A}+\mathbf{B}\rightarrow\mathbf{A}\cdots\mathbf{B}$ in the gas phase or in a nonpolar solvent at 298 K (black curve). Under these conditions $-T\Delta S$ is a measure of ΔG_{t+r}. When the relative motion of **A** and **B** in $\mathbf{A}\cdots\mathbf{B}$ is highly restricted, the entropy costs approach the limiting value of $T\Delta S \approx -57\ kJ\,mol^{-1}$. At room temperature the limit is reached at enthalpies corresponding to the formation of a covalent bond. As the $\mathbf{A}\cdots\mathbf{B}$ bond becomes weaker, there is increasing residual motion of **A** relative to **B**. The increasing motion, and the accompanying increase in bond length, is illustrated in the three sets of "dumbbells", which correspond to the green, red, and black points on the curve. The adverse entropy of the association therefore becomes increasingly small.

These conclusions are supported by experimental data. The adverse ΔG_{t+r} term (expressed in terms of $T\Delta S_{t+r}$) decreases as the favorable enthalpy of association is decreased (shallower well).[2,40] The adverse ΔG_{t+r} term is less than $13\ kJ\,mol^{-1}$ in the cases of weakly exothermic associations (less than $20\ kJ\,mol^{-1}$). Put simply, "weak springs" give more residual motion at the binding interface.

A similar relationship should hold for corresponding noncovalent associations in nonpolar solvents because, to a useful approximation, these are like those in the gas phase.

This follows since the favorable entropy of desolvation of the polar groups that interact when the complex is formed should be relatively small. Experimental data for 233 such associations[41] were examined to test this hypothesis.[42] Although 215 of the data points produced a good correlation of the form predicted, 18 appeared anomalous (Figure 3). These 18 data points were subsequently shown to arise because of various kinds of errors.[42].

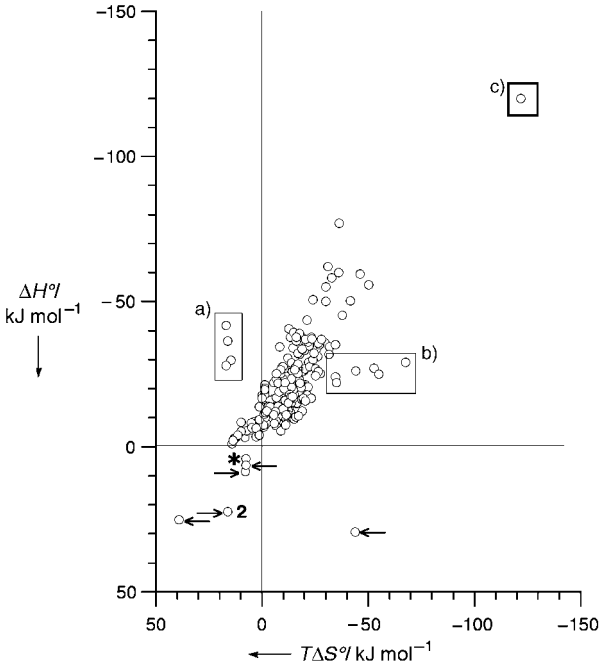

Figure 3. Enthalpy (ΔH) versus entropy ($T\Delta S$) diagram for the association of macrocycles with neutral molecules in a variety of nonpolar solvents.[41] There are six groups of data points that appear to be inconsistent with the general form of the enthalpy/entropy compensation curve, but all of these are associated with errors[42] (quite understandably arising in a valuable review[41] of large breadth). Three examples of which follow: a) Four data points were transcribed with an incorrect sign for ΔS, thus requiring their transposition from the north-west quadrant to the north-east quadrant). b) Six data points were transcribed in the review with a conversion factor for ΔS that was too large (by a factor of 4.18 Joules/calories), thus requiring their transposition into the general region of the correlation). c) A point that is not an error in transcription was indicated by our experimental value to be errorneous. Given the correction of these errors, and others indicated by the arrows and asterisk,[42] there is agreement between the general forms of Figures 2 and 3.

Many of the points lie in the north-west quadrant (Figure 3) for associations in nonpolar solvents that are less exothermic than 10 kJ mol^{-1}. This is because weakly exothermic associations lead to weakly ordered complexes **A**···**B** with only a very small cost in entropy. Hence, although the favorable entropy of desolvation will be small for associations in nonpolar solvents, it is large enough in the case of weakly exothermic associations to more than offset the small cost in entropy of the association.

There do not appear to be exceptions among the 233 data points to the general requirements of Figure 2, and the generalization has been used to correct errors in the literature. This last point is important, because unifying concepts are unlikely to emerge when the literature accumulates errors. The idea that forming a more exothermic noncovalent interaction proceeds with a greater cost in entropy (greater restriction in motion) is confirmed.

Collectively, the data for the noncovalent associations occurring in the gas phase and in nonpolar solvents also confirm the conclusion from the training set of drug–receptor data used in conjunction with Equation (3)—noncovalent associations can have relatively small adverse ΔG_{t+r} terms. On average, the drugs in the data set apparently have considerable motions relative to their receptors, that is, they are bound in a relatively dynamic manner.

5. Cooperative Binding Processes: Definitions

The aim in studying binding affinities is to understand the origins of the overall ΔG value in terms of structure. It is evident from the foregoing sections that the changes in the bonding (ΔH) and order ($T\Delta S$) terms for the binding of flexible molecules in aqueous solution are so numerous that the problem is a complex one. A useful approach is to separate the variables so that a deeper understanding can emerge. This has already been achieved at one level by separating the effects of noncovalent bonding in complex formation from those of desolvation (through the examination of complex formation in the gas phase and in nonpolar solvents, see Section 4). Cooperativity between noncovalent interactions has not so far been discussed. It will be seen that its effects upon the bonding (ΔH) and order ($T\Delta S$) terms can be predicted with some confidence, and so another layer of the complexity can be peeled away.

Noncovalent interactions are said to interact with each other in a positively cooperative manner when the binding energy that is derived from them acting together is greater than would be derived from the sum of their acting separately. Conversely, they are said to interact with each other in a negatively cooperative manner when the binding energy that is derived from them acting together is less than would be derived from the sum of their acting separately. Equation (3) was derived to remove part of the problem that arises from cooperativity. Equation (3) is based on the idea that the cost of an association (ΔG_{t+r}) has to be paid only once. It was therefore reasoned that if this adverse term could be factored out from the other free-energy contributions then, to a useful approximation, all the free-energy terms could then be treated additively.

Figures 2 and 3 illustrate the inadequacy of this approach in the general case, since the adverse ΔG_{t+r} term increases as association occurs with stronger bonding. The addition of an extra polar interaction at a binding interface can decrease the local dynamics and thereby increase the favorable free energy of adjacent interactions (see Section 6).[40] It is this gradual switching on of positively cooperative effects, as a result of the interplay between motion and bonding, that precludes rigorous analysis of binding affinities in terms of sums of the parts.

Nevertheless, in many systems (particularly synthetic ones) there may be no significant cooperativity, and in these cases the binding energy will correspond to the sum of the parts. For example, the affinity of polyamines to DNA can be described simply as a function of the number of possible contributions of each ammonium center.[43]

6. Cooperative Binding at a Single Interface

6.1. The Case of Positive Cooperativity

To illustrate how positive cooperativity can arise in a simple system of polar interactions, we consider a receptor that binds ligands **X**, **Y**, and **Z** with affinities ΔG_X, ΔG_Y, and ΔG_Z, respectively (Figure 4). Clearly, binding some combination of these ligands, each as separate entities, will result in an overall free energy change that is represented by the sum of the contributions from each individual ligand. Let us now consider the case where **Y** and **Z** are connected by a linker (**Y**–**Z**) (Figure 4b). There is now the benefit of the classic entropic chelate effect to binding described by Jencks and Page.[7–9] That is, if **Y** and **Z** each bound separately with an adverse $T\Delta S_{t+r}$ term of N, then **Y**–**Z** will bind with a cost of less than $2N$. We now add the cooperative effects addressed in this review to this benefit to binding. First, the restrictions in motion that **Y** and **Z** impose on each other when binding as **Y**–**Z** will *increase* the exothermicity of the noncovalent bonds that they make with the receptor; that is, there will be structural tightening of the receptor–ligand complex (Figure 4, $d_1 < d_0$). Second, the increased exothermicity associated with this first effect will in turn *reduce* the motions of **Y** and **Z** (in **Y**–**Z**) relative to the receptor. Therefore, these two effects will occur with a benefit in enthalpy and a cost in entropy relative to the situation occurring if there were no positive cooperativity of this nature. Analogous arguments allow us to conclude that if **X** is connected to **Y**–**Z** by a rigid strain-free linker to form **X**–**Y**–**Z** then further structural tightening will occur ($d_2 < d_1$) and lead to a further cooperative enhancement of binding (Figure 4c).

Studies with glycopeptide antibiotics established the reductions in distance modeled in Figure 4a–c.[44] Several peptide ligands, all containing the carboxylate group depicted lower right in Figure 5, were separately bound to the antibiotics. In all cases, a large downfield chemical shift ($\Delta\delta = 1$–3 ppm) of the antibiotic amide NH proton H^2 was observed upon binding of the ligand. A larger limiting downfield shift of this proton indicates a shorter and stronger carboxylate–NH hydrogen bond.[44–46] This hydrogen bond was formed to a higher degree as the number of hydrogen bonds and other motional restrictions adjacent to the carboxylate–NH interaction was increased. The motional restriction of the carboxylate group, afforded by these adjacent motional restrictions, strengthens the hydrogen bonds directly made to the carboxylate group.

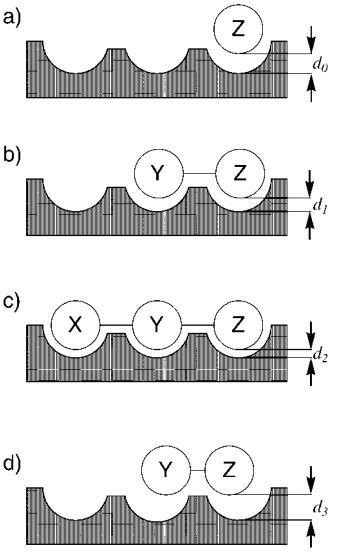

Figure 4. Schematic representation of a receptor that binds ligands **X**, **Y**, and **Z** with affinities ΔG_X, ΔG_Y, and ΔG_Z, respectively. a) Binding of **Z** results in a structure with an intermolecular distance d_0. b) When **Y** and **Z** are connected by a rigid, strain-free linker (**Y**–**Z**) they bind to the receptor with positive cooperativity ($\Delta G_{Y\text{-}Z}$ more negative than $\Delta G_Y + \Delta G_Z$) and there is structural tightening ($d_1 < d_0$). c) If **X** is connected to **Y**–**Z** by a rigid, strain-free linker to form **X**–**Y**–**Z** then further structural tightening will occur ($d_2 < d_1$) leading to a further cooperative enhancement. d) The shorter linker between **Y** and **Z** does not allow both these binding interactions to occur with optimal geometry. **Y**–**Z** binds the receptor with negative cooperativity ($\Delta G_{Y\text{-}Z}$ more positive than $\Delta G_Y + \Delta G_Z$) and there is structural loosening ($d_3 > d_0$).

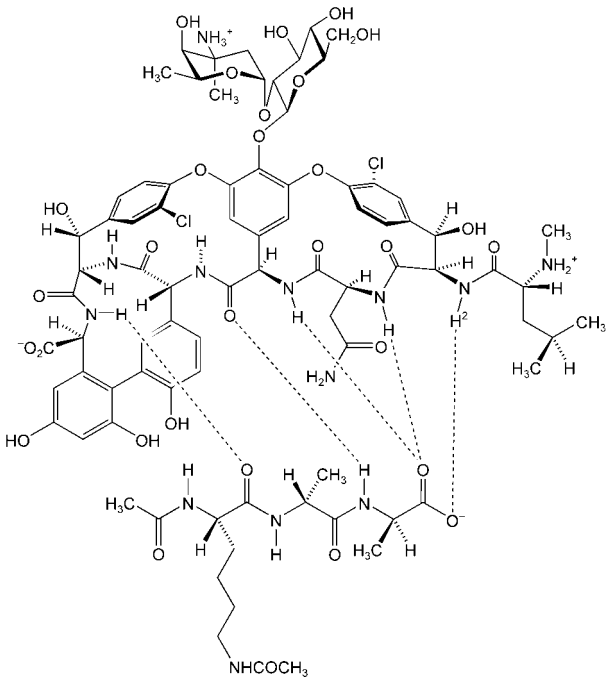

Figure 5. The binding interaction between the glycopeptide antibiotic vancomycin and the peptide ligand *N*-α-acetyl-Lys-(*N*-ε-acetyl)-D-Ala-D-Ala. Hydrogen bonds are indicated by dotted lines. The binding is also promoted by hydrophobic interactions, notably of the Ala methyl groups to the aromatic rings of the antibiotic. The amide NH proton H^2 discussed in the text is indicated.

The dimers of the glycopeptide antibiotics (Figure 6) exhibit the same effect. The proton resonance H^4 at the dimer interface undergoes a downfield shift when the dimer is formed from the monomer. The extent of this downfield shift

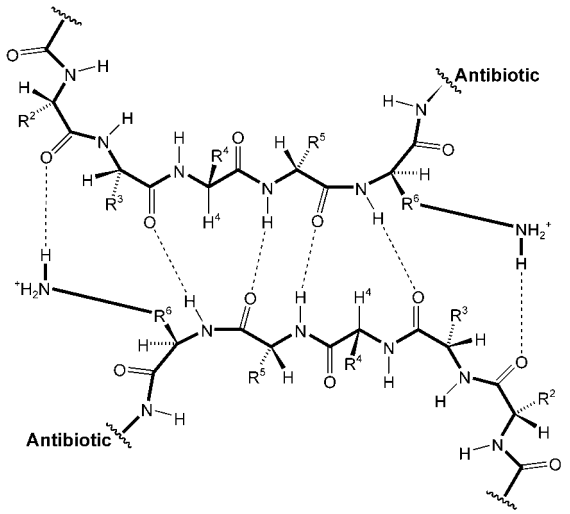

Figure 6. Backbone structure of the dimer of the glycopeptide shown in Figure 5. Hydrogen bonds are indicated by dashed lines. The α-proton H^4 mentioned in the text is indicated. The central four hydrogen bonds at the dimer interface are common to all glycopeptide dimers, whereas the two outer hydrogen bonds can only be made by antibiotics that possess an amino sugar attached to R^6.

increases as additional bonding restraints, more distant from H^4 than the four central hydrogen bonds depicted in Figure 6, are added at the dimer interface. In analogy with the correlation observed between increased downfield changes in the chemical shifts of the α protons of β-strand structures as the interstrand distance decreases,[47,48] these data indicate that remote bonding restraints reduce the interfacial distances in the dimer in the vicinity of H^4.

Thus, structural tightening occurs in the bound state as a consequence of positive cooperativity.[49] Furthermore, thermodynamic measurements indicate that the structural tightening occurs with a benefit in enthalpy (ΔH) and a smaller cost in entropy (in terms of $T\Delta S$).[49]

6.2. The Case of Negative Cooperativity

The model of Figure 4 can also be used to illustrate how negative cooperativity may arise. Consider the case where **Y** and **Z** are rigidly held in a conformation that does not allow both binding interactions to occur with the preferred geometry shown in Figure 4b. This situation could be induced, for example, by introducing a linker between **Y** and **Z** that is too short (Figure 4d). The "pull" of **Y** towards its preferred binding geometry will adversely affect the binding of **Z** by forcing it away from its preferred binding geometry, and vice versa, with the consequence that ΔG_{Y-Z} will be less negative than the sum of $\Delta G_Y + \Delta G_Z$. **Y** binds with negative cooperativity with respect to **Z**, and vice versa. Based on the arguments presented above, a consequence of this negative cooperativity should be loosening of the interaction of **Z** into its receptor cup (Figure 4d, $d_3 > d_0$), at a cost in enthalpy but with a benefit in entropy.

Studies on the melting of DNA duplexes support the models of negatively cooperative binding. For example, the self-complementary duplex **1** is formed in aqueous solution from its constituent single strands with an exothermicity of $-430\ kJ\,mol^{-1}$.[50] The magnitude of the exothermicity of duplex formation drops by $220\ kJ\,mol^{-1}$ upon the introduction of only two mismatches into the sequence (formation of **2** with $X = {}^{m6}G$, namely, methylation at the purine O6 site). This extremely large fall in the exothermicity of duplex formation is associated with a correspondingly dramatic fall in the adverse entropy for duplex formation ($-1164\ J\,mol^{-1}\,K^{-1}$ for **1** versus $-577\ J\,mol^{-1}\,K^{-1}$ for **2**).

CGCGAATTCGCG	CGCXAATTTGCG
GCGCTTAAGCGC	GCGTTTAAXCGC
1	**2**

Since the negative cooperativity associated with the introduction of the two mismatches reduces the melting temperature of the duplex, a correction must be made for the heat capacity change upon melting of the DNA.[51–53] After allowing for heat capacity effects, it is found[54] that the introduction of just two mismatches reduces the favorable enthalpy of duplex formation by $123 \pm 53\ kJ\,mol^{-1}$. Thus, there is a large reduction in the bonding in the duplex structure, even though formally it involves the removal of only two C=O···HN hydrogen bonds and the introduction of two repulsive $C{=}O{\cdots}OR_2$ interactions. Therefore, it appears likely that the introduction of the mismatched T···X interactions in **2** loosens adjacent interactions. A similar calculation for the entropy term indicates that the large reduction in bonding is accompanied by a large increase in the motional dynamics of **2** relative to **1**. There is enthalpy/entropy compensation, with the consequence that the introduction of the two mismatches has a much smaller effect on the relative stabilities ($\Delta\Delta G = 46\ kJ\,mol^{-1}$) of the two duplexes than would otherwise be the case.

7. Cooperative Binding over Multiple Interfaces

Although positively or negatively cooperative binding between interactions expressed at the same interface (Section 6) is important, such effects expressed over multiple interfaces are of supreme importance in biology. We first consider the free energy of positively and negatively cooperative binding when expressed over two interfaces.

Figure 7 shows a thermodynamic cycle for the formation of a dimer, which can occur either in the presence, or absence, of a ligand. The dimer can bind two molecules of ligand.[55]

The transformation **A**→**C** represents the free energy of dimerization and **A**→**B** represents the free energy of binding two molecules of ligand to two of monomer. The transformation **C**→**D** represents the free energy of binding two ligands to dimer in the absence of cooperativity, which by

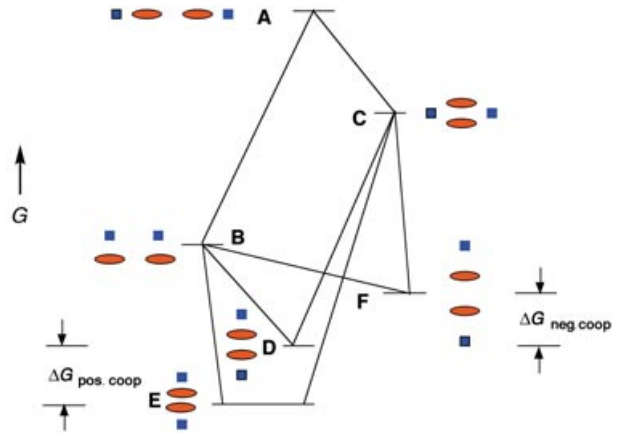

Figure 7. Formation of a fully coordinated dimer from the constituent elements of two ligand molecules (blue) and two antibiotic monomers (red). Vertically arranged species are bound, horizontally arranged species are not bound. Starting from the free components (**A**) the association can occur to give two ligand-bound monomers **B** or a ligand-free dimer **C**. The coordinated dimers may be formed without cooperativity (**D**), with positive cooperativity (**E**), or with negative cooperativity (**F**).

definition is therefore the same as the free-energy change for **A**→**B**. The transformation **B**→**D** represents the free-energy change for dimerization of ligand-bound monomer in the absence of cooperativity, and is therefore by definition the same as the free-energy change for **A**→**C**.

If the dimer and ligand interfaces are formed in a positively cooperative manner, then a more stable species **E** is formed instead of **D**. The difference between free energy levels **E** and **D** ($\Delta G_{\text{pos.coop}}$) represents the free energy benefit of the positive cooperativity. If the dimer and ligand interfaces are formed in a negatively cooperative manner, then a less-stable species **F** is formed instead of **D**. The difference between free energy levels **F** and **D** ($\Delta G_{\text{neg.coop}}$) represents the free energy cost of the negative cooperativity.

Suppose the dimerization constant for the formation of a symmetrical dimer is increased by some factor x (for example, 100) by the occupation of both its ligand binding sites (positively cooperative binding, Figure 7). Then the binding constant for each ligand into the two identical binding sites of the dimer must be greater by $\sqrt{x}$ (for example, 10) over binding to the monomer. More generally, if a receptor system is stabilized upon ligand binding, the ligand affinity for the receptor must thereby be increased. This important point is discussed in the general context in Section 11.

8. Positive Cooperativity over Multiple Interfaces

8.1. Monomeric Protein Receptors

A structural model[56] to understand this phenomenon of positively cooperative binding is given in Figure 8a, and shows a portion of a protein receptor prior to (top) and after (bottom) binding to a ligand. First consider the free receptor (top): the formation of its two depicted hydrogen bonds is opposed by the relative motions of its two peptide backbones. The internal motions of the chain that is presented to the ligand can be reduced by the formation of hydrogen bonds from it to the ligand (Figure 8a, bottom). Since motion opposes bonding, the restriction of the internal motions of this chain upon ligand binding should result in the strengthening of the hydrogen bonds within the receptor.

The "damping down" of the motions of the depicted receptor residues upon ligand binding can, in turn, improve noncovalent bonding more deeply within the receptor. Such transmission will result in an improvement in noncovalent bonding at all sites within the receptor that are coupled with

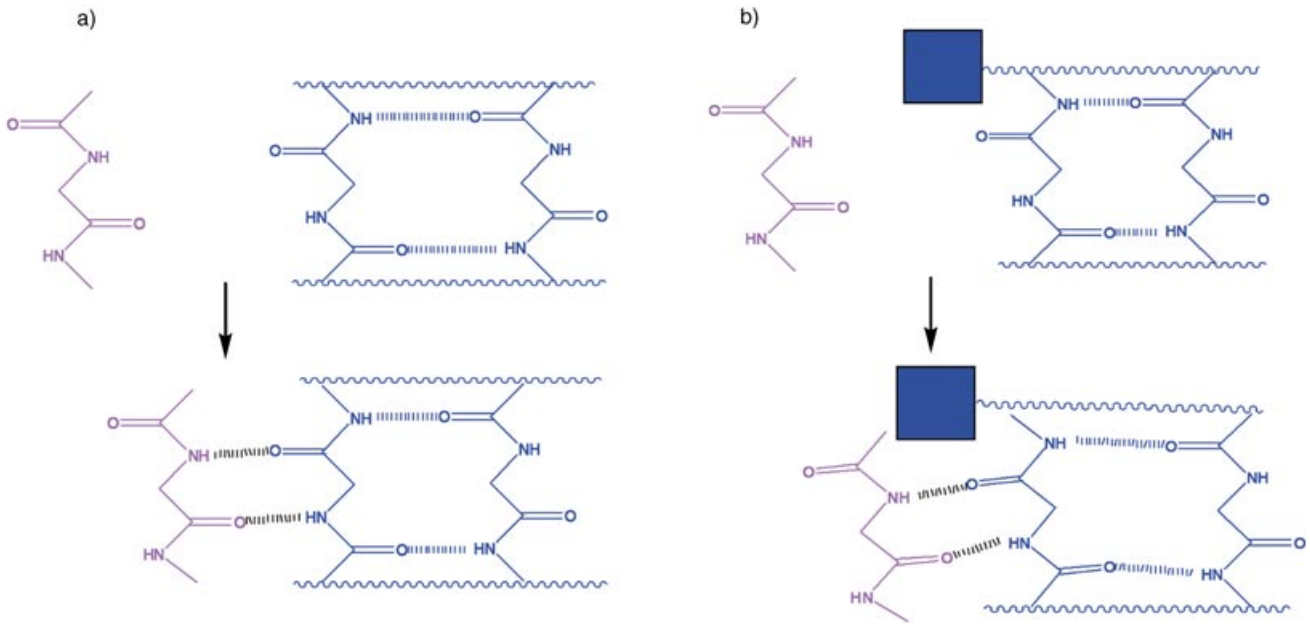

Figure 8. Structural models for positively cooperative binding (a) and for negatively cooperative binding (see text for details).

positive cooperativity to ligand binding. Ligand binding to the receptor is improved because the receptor is stabilized upon ligand binding.

A consequence of improved bonding within a receptor following positively cooperative ligand binding will be a reduced degree of H/D exchange of selected amide NH protons of the receptor (see Section 11).

8.2. Dimeric Receptors

The model of Figure 8a is equally useful in considering the case where the portion in blue represents not a single protein receptor, but rather two peptide chains that can come together through a bimolecular association (the wavy lines are ignored). Under these circumstances, the formation of the blue hydrogen bonds (for example, when a receptor dimer is formed) lead to the presentation of a more organized template to the ligand. For this reason, the cost in entropy in binding the ligand to the dimer will be less than in binding the ligand to the monomer. Molecular dynamics simulations illustrate that this contribution to positive cooperativity is a benefit to entropy.[57]

It must now be considered how the above "templating" advantage of a dimeric over a monomeric receptor structure will cause "knock-on" effects on the structure. Since it is motion that opposes bonding, the presentation of a more-ordered surface (of a dimeric receptor) to a ligand will allow an improvement in interfacial ligand/receptor bonding (Figure 8a). In fact, there will tend to be mutual strengthening of all the hydrogen bonds between the chains in strain-free binding. A consequence of this strengthening of the hydrogen bonds must be to reduce the relative translational motions of the chains, and to further restrict their internal wagging motions, at a cost in entropy.

In summary, positively cooperative binding of a ligand to a dimeric receptor should be beneficial in enthalpy, that is, with a shortening of the noncovalent bonds involved. However, the overall entropy changes cannot be predicted from the model. Strain-free templating of binding gives an entropic benefit, whereas entropy/enthalpy compensation should give an entropic cost. Experiments are required to address this problem.

The structural and thermodynamic features of positive cooperativity expressed over multiple interfaces have been demonstrated experimentally. Dimers of glycopeptide antibiotics of the vancomycin group are typically further stabilized and, without reported exception, become less dynamic when they bind two molecules of the bacterial cell-wall analogues (Figure 9). This reduced dynamic behavior of the dimer receptor system occurs with distance reductions at the dimer interface, as indicated by NMR data.[46] The reduction in the dynamic behavior of the dimeric receptor is greater for strongly binding ligands than for weakly binding ligands.[58] The positive cooperativity is typically associated with a benefit in enthalpy and a cost in entropy.[59] In such cases, the motional restriction that accompanies improved bonding outweighs the benefit in entropy of binding to a more-ordered template.

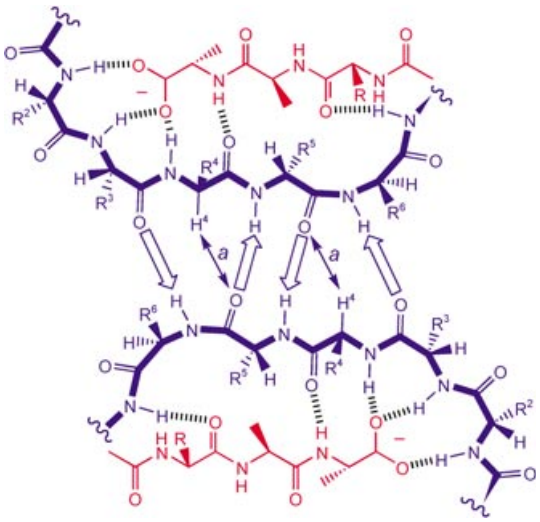

Figure 9. Peptide backbone of a glycopeptide antibiotic dimer (blue) that is simultaneously bound to two molecules of a bacterial cell peptide precursor analogue (red). The binding of the analogue occurs with positive cooperativity, such that the dimer system is stabilized and shortens the distances marked *a*. The effect affords a benefit in enthalpy and a cost in entropy.

The structural changes occurring upon positively cooperative binding bear analogy to the changes occurring upon cooling a substance. On reducing the temperature of a pure substance, whether liquid or solid, it is universally observed that the change is favorable in enthalpy and adverse in entropy.[60] It is also almost universally observed that reducing the temperature leads to a volume reduction (the ice/water transition is an example of a relatively rare exception). The physical basis for this analogy is evident: both in the exercise of positive cooperativity and in cooling a substance, a reduction in dynamic behavior improves noncovalent bonding and shortens the average noncovalent bond lengths.

9. Negative Cooperativity over Multiple Interfaces—Hemoglobin

Since cooperative binding is a phenomenon of great importance in chemical biology, it might be assumed that a unified definition of the phenomenon would already be in use. This is not the case. For multiple ligand binding sites,[61,62] positively cooperative binding is said to occur when binding ligands are successively bound with increasing affinities. Conversely, negatively cooperative binding is said to occur when binding ligands are successively bound with decreasing affinities.

Importantly, ligand binding can be positively cooperative if the above definition for multiple binding sites is used, while being negatively cooperative if the definition employed here is used (see Section 5). The advantage of the definition of positive cooperativity used in this Review is that it requires that the sets of noncovalent interactions are, when made together, mutually *reinforcing*. Such a condition would seem

to be a mandatory requirement for interactions that are "positively cooperative". We now illustrate the nontrivial consequences of these considerations by reference to the binding of O_2 to hemoglobin.

A structural model to illustrate negatively cooperative binding over two interfaces is given in Figure 8b. Here, the surface chain of the protein receptor must incorporate a structural feature (for example, steric inhibition by the blue square) that inhibits ligand binding to the structure of the isolated receptor. As required for a structurally meaningful definition of "negatively cooperative", the two sets of interactions (the preferred noncovalent interactions at the ligand/receptor interface and those preferred within the receptor) are mutually incompatible. Therefore, when ligand-binding occurs, the ground-state structure of the receptor must be distorted from its preferred geometry (Figure 8b, top) to a state (bottom) in which its internal noncovalent bonding is weakened. Thus, ligand binding that is negatively cooperative should cause receptors to loosen their structures and become more dynamic. Consequently, the extent of H/D exchange of the amide–NH protons of the peptide backbone of the receptor (upon exposure to D_2O) should increase upon ligand binding. In addition, the reduction in bonding efficiency within the receptor upon negatively cooperative ligand binding will make ligand binding less favorable in enthalpy, and more favorable in entropy than would otherwise be the case. Evidence to support these conclusions is presented below.

Hemoglobin, the protein that carries oxygen in the blood stream, exists as a tetramer. The classic work of Monod, Wyman, and Changeux (MWC)[62] allows a deep insight into the changes that occur when O_2 binds to hemoglobin. The initially available form of the tetramer is described as a "tense" (T) form (Figure 10a). The geometry of this form does not allow efficient binding of O_2 to it. The initially available T form is distorted to give a more "relaxed" structure (the R form, Figure 10a) to enable O_2 to bind more efficiently. The binding of just one O_2 molecule to the tetramer is envisioned as distorting all four subunits towards the R structure (Figure 10b), so that the subsequently binding O_2 molecules bind with higher affinity. The O_2 binding is therefore defined in the MWC model as positively cooperative. The energy required for the T→R conversion is largely carried out by the first O_2 molecule that binds. Therefore, subsequently binding O_2 molecules have the advantage of accessing a relatively high population of the R state, and bind with greater affinity.[63]

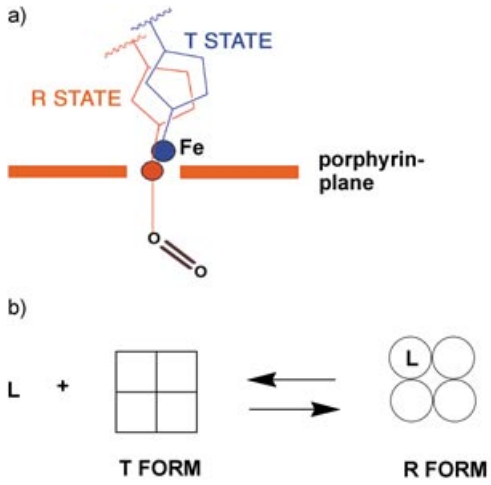

Figure 10. a) Negatively cooperative binding of O_2 to hemoglobin. In the T state (blue) the hemoglobin is unable to bind O_2 efficiently because the access of the O_2 to the T state is hindered by the plane of the porphyrin ring. The structure of the hemoglobin is therefore distorted (loosened, with a decrease in efficiency of its internal noncovalent bonding) to give the R state (red), which is better able to bind O_2. b) MWC model for the binding of the first molecule of ligand (L) to a tetrameric protein existing in tense (T) and relaxed (R) forms.

However, in terms of the definition used here, the binding of O_2 to hemoglobin is negatively cooperative. The T state does not allow efficient binding of O_2, and therefore the binding of O_2 distorts it to the R state (Figure 10a). This situation is analogous to the situation shown in Figure 8b.

The negatively cooperative binding does indeed force a loosening of the T state of the tetramer, through the breaking of ionic interactions between subunits.[64] However, and importantly for the structural consequence of negatively cooperative binding developed here, widespread structural changes in the T to R transition seem possible: all noncovalent interactions within a receptor system that are coupled with negative cooperativity to ligand binding should loosen.

To test the above conclusion we determined[65] the change in the dynamic behavior of the (horse) hemoglobin tetramer polypeptide backbone when it binds O_2 by ESI-mass spectrometry. Through the binding of oxygen, a further 7–8 exchangeable amide hydrogen atoms per α chain (5.2–6% of the total number) and a further 16 per β chain (11.4% of the total number) underwent solvent exchange. Thus, complete saturation of the hemoglobin tetramer by O_2 binding results in an increase of 46–48 backbone NH atoms undergoing exchange. The dramatic increase in amide NH exchange is in agreement with the predictions regarding the changes associated with negatively cooperative binding. This increase in dynamic behavior of the amide backbones of the hemoglobin subunits had not been uncovered by previous X-ray studies. Presumably, the changes in dynamic behavior, which are important for an understanding of binding interactions, are masked by crystal-packing forces.

The requirement that negative cooperativity, as exercised on the T state, will be accompanied by enthalpy/entropy compensation (in the sense of a benefit in entropy and a cost in enthalpy) is also satisfied. Thus, as O_2 binding promotes the T to R transition, there should be an uptake of heat by, and increase in disorder within, the hemoglobin tetramer. The following thermodynamic parameters (per subunit) were obtained for the case of O_2 binding to trout hemoglobin:[66] The first O_2 molecule binds with $\Delta H = 0\ kJ\,mol^{-1}$ and a favorable $T\Delta S$ term of $+21\ kJ\,mol^{-1}$. In contrast, the fourth O_2 molecule binds exothermically ($\Delta H = -32\ kJ\,mol^{-1}$) and with a slightly unfavorable entropy term ($T\Delta S = -3\ kJ\,mol^{-1}$). The binding of O_2 to the iron atom of hemoglobin in isolation can confidently be assumed to be exothermic. However, since O_2 binding drives the T→R

conversion (Figure 10b), the associated loss in bonding within the hemoglobin structure offsets this exothermic gain. Hence, $\Delta H = 0\ \mathrm{kJ\,mol^{-1}}$ for O_2 binding starting from the T form is understandable. The favorable $T\Delta S$ term of $+21\ \mathrm{kJ\,mol^{-1}}$ reflects the loosening of the hemoglobin structure that accompanies its loss of internal bonding. Thus, it may be a useful approximation to isolate the binding event of O_2 to the R form of hemoglobin as being reflected by $\Delta H = -32\ \mathrm{kJ\,mol^{-1}}$ and $T\Delta S = -3\ \mathrm{kJ\,mol^{-1}}$. Indeed, O_2 binding *in isolation* should involve only a small adverse entropy change (reflecting the dynamic binding of a ligand of small mass to a receptor). This approximation isolates the thermodynamics of the T→R transition in terms of $\Delta H = +32\ \mathrm{kJ\,mol^{-1}}$ and $T\Delta S = +24\ \mathrm{kJ\,mol^{-1}}$. These are plausible numbers for the generation of a structure that is less well bonded and exhibits more dynamic behavior (see the exchange data for horse hemoglobin above for comparison). Additionally, since $\Delta G = \Delta H - T\Delta S$, the T→R transition is $\Delta G \approx +8\ \mathrm{kJ\,mol^{-1}}$. Thus, at room temperature the free T state of trout hemoglobin should be more highly populated than the free R state by a factor of about 25. Seen in this light, the thermodynamic parameters make physical sense.

Analogous experiments on myoglobin are clearly of interest, since it exists as a monomeric species. In the original classic paper of Monod, Wyman, and Changeux it was assumed that "the subunits of the R form are closer to the conformation of the [hemoglobin] monomer" (cf. myoglobin) and that "myoglobin may be thought of as a relaxed subunit of hemoglobin".[62] According to this assumption, oxygen binds strongly to myoglobin, at least in part, because it does not have to pay the price of the T to R conversion (Figure 10).

However, it is important to recall that the internal structure of the R form has been shown above to be loosened relative to the T state, and this internal loosening must be driven by O_2 binding. Thus, the myoglobin structure might also be loosened internally by O_2 binding. The extent of H/D exchange of the amide protons on the peptide backbone of myoglobin in the absence, and presence, of bound oxygen was determined by ESI-MS. A further 13–15 exchangeable backbone amide hydrogen atoms were exposed to solvent exchange through the binding of oxygen.[56] Myoglobin thus possesses a tight (or "tense") internal structure, and not a relaxed one as originally seemed likely. This conclusion also makes physical sense because, as Perutz commented,[67] it is the binding of O_2 that perturbs the protein structure. Both myoglobin and the T form of hemoglobin bind O_2 with negative cooperativity as defined by the model used here (see Figures 8b and 10a). Consistent with the model, the reduction in binding energy of the O_2 molecule to the T form of hemoglobin is widely spread, not only at the tetramer interfaces, but also among the internal network of noncovalent interactions of each monomeric unit.

The advantages of the negatively cooperative model for O_2 binding to hemoglobin are threefold: 1) it is part of a model for cooperativity (Figure 8) that is consistent with the sense in which the term "cooperativity" is most frequently used. Mutual *enhancement* of compatible sets of interactions is described as *positively* cooperative binding (Figure 8a, for example, as in protein folding[68,69] and crystallization). Mutual *weakening* of initially incompatible sets of interactions is described as *negatively* cooperative binding (Figure 8b, for example, as in the case of O_2 binding to hemoglobin (Figure 10a)). 2) The model postulates specific structural consequences. For example, since all noncovalent interactions within a receptor system that are coupled with negative cooperativity to ligand binding should loosen, it requires that the internal structure of each hemoglobin subunit be extensively loosened upon O_2 binding (this has been established by H/D exchange experiments[65,70]). 3) The use of the terms T and R states[62] is naturally accommodated, since negatively cooperative binding of O_2 forces a reduction in bonding in the T state (cost in enthalpy) to give the R state which is more dynamic (benefit in entropy).

If the binding of O_2 to hemoglobin were positively cooperative in a structural sense (in terms of noncovalent bonding), then the initial O_2 binding event would represent "normality", and subsequent O_2 binding events would be accompanied by mutual enhancement of noncovalent interactions within the O_2/hemoglobin system. This is not the case. Rather, nature has built the greatest adversity into the first O_2-binding step, where the restriction of access of O_2 to the iron atom is the result of the steric hindrance of the porphyrin ring (Figure 10a). The successively stronger O_2-binding steps that are described as positively cooperative in the Monod, Wyman, Changeux model are in fact successive steps of decreasing negative cooperativity.

Other literature data are consistent with the general properties proposed here for negatively cooperative binding. The binding of the cofactor (6*R*)-L-erythro-5,6,7,8-tetrahydrobioptrin to the tetrameric recombinant human tyrosine hydroxylase isoform 1 occurs with negative cooperativity.[71] The cofactor-bound form of the enzyme shows, relative to the free form of the enzyme, a decreased resistance to limited tryptic proteolysis—as would be expected from a loosening of the enzyme structure. Additionally, a decreasing incremental affinity is observed for the binding of ligands with one, two, or three carbohydrate units to lectins.[72,73] That is, the binding is negatively cooperative. The ligands with several carbohydrate units coordinate with more positive entropy of binding relative to the analogues with one unit,[74] presumably reflecting the disorder produced in the lectins by the negatively cooperative binding of the second and third carbohydrate epitopes.

Lastly, a decrease in protein stability is induced by ligand binding which increases the flexibility of the protein.[75]

10. Negative Cooperativity that Breaks Interfaces

If the free-energy change associated with negatively cooperative binding were sufficiently large, the free energy of **F** would lie above that of **B** (Figure 7). Under these circumstances, a ligand binds with negative cooperativity to a receptor that is in its dimeric state in the absence of ligand, and thereby induces dissociation of the dimer. Such cases are established,[76] and, indeed, Monod, Wyman, and Changeux[62] considered dissociation of an oligomeric species as the limit of structure loosening.

11. Reduced Dynamics within Protein Receptors as a Source of Ligand Binding Energy

In Sections 7 and 8 it was shown that in the positively cooperative binding of a ligand to a receptor (there a small dimer) the free-energy benefit of the positively cooperative binding is correlated with a tightening (that is, a local volume reduction) within the receptor system. A thermodynamic cycle shows that where a receptor system becomes more stable upon ligand binding it is axiomatic that ligand binding is thereby improved.[55] This conclusion is represented schematically in Figure 11.

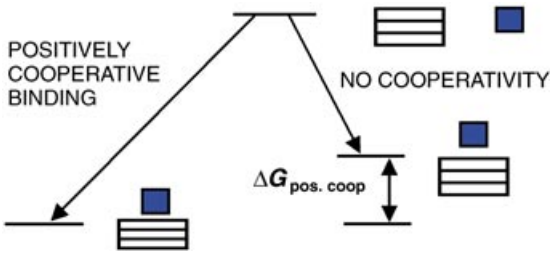

Figure 11. Schematic illustration showing that stabilization of a receptor system (oblong) upon ligand binding (blue square) increases ligand binding energy. In the absence of positively cooperative binding (right), the ligand binds without modification (tightening) of the receptor structure. In the presence of positively cooperative binding (left), the ligand binds with overall tightening of the receptor structure. The increase in ligand binding energy arising from positive cooperativity is because of increased stability of the ligand/receptor system. It may in principle be expressed at any of the noncovalent interfaces of the ligand/receptor system.

Has evolution exploited reduced dynamics within protein receptors as a source of ligand-binding energy? Clearly, the search for such effects should be through the examination of systems in which a localized view of noncovalent interactions would seem inadequate in understanding the observed affinities.

The binding of biotin to the streptavidin (STV) tetramer is so remarkably strong ($K = 10^{13.4}\,\text{M}^{-1}$) that it finds widespread use in biology. It is poorly understood and typically found to be an "outlier" in comparison with other affinities.[16,77] Specifically, it is about 1000 times stronger than expected on the basis that the binding should correspond to the sum of the parts.[16,77] We used mass spectrometry to measure the extent of H/D exchange of the amide NH protons in STV both in the absence and presence of biotin. It was found that 22 backbone amide NH protons per STV monomer unit are protected from H/D exchange (RT, pH 8, 2 h) upon binding of biotin.[65] The location of the backbone NH protons protected by biotin binding was obtained from a pepsin digest. The data show that the binding of biotin reduces the solvent accessibility of streptavidin backbone NH protons in much of the structure (Figure 12).

It is noteable that the effects are much more marked in some parts of the structure than in others: thus, the improved packing induced within the receptor upon binding of biotin is not that which might be engendered upon simply carrying out

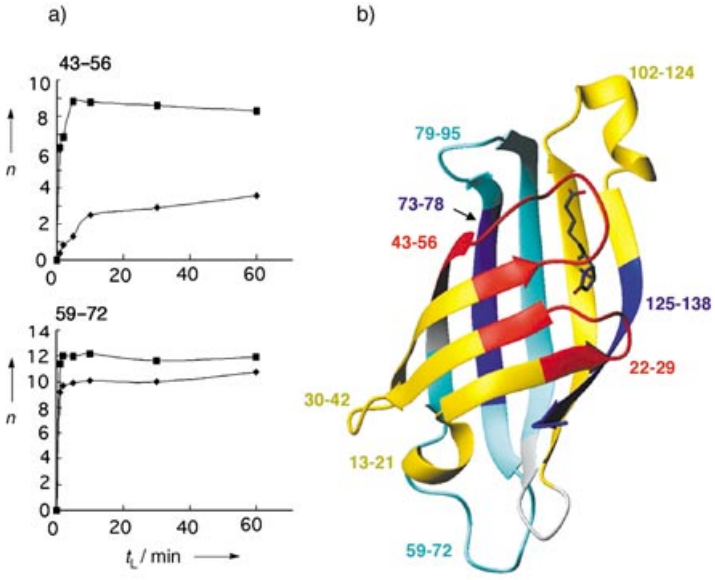

Figure 12. a) Deuterium incorporation into streptavidin in the absence (■) and presence (◆) of biotin as a function of time t_L. The level of deuterium incorporation in two peptides (43–56 and 59–72) obtained by pepsin digestion was investigated. b) Position of the peptides in a ribbon model of a STV subunit with bound biotin (the figure was prepared with the program MOLMOL[106]). The degree of increased NH protection in the presence of biotin is indicated by color: red $>30\%$, yellow 20–30%, light blue 10–20%, and dark blue $<10\%$.

binding at a higher pressure. Rather, it is exercised only in the parts of the receptor where positively cooperative binding is expressed. It is clear that the binding energy of biotin to streptavidin is widely delocalized. X-ray crystallographic studies of the free and bound tetramer[78–81] do not illustrate the widespread greater dynamic behavior of the free tetramer, presumably because this is damped down by crystal-packing forces. In this respect, we note that liquid to crystal transitions universally occur with improvements in noncovalent bonding that restrict dynamic behavior.

The streptavidin/biotin system provides a clear example where the binding affinity is the property of the whole system, rather than something that can be understood by examination of the ligand/receptor interface. This point is consistent with other, less structure specific, measurements. The effect of biotin binding on the thermal stability of STV has been evaluated by using differential scanning calorimetry (DSC).[79,81] Biotin binding increases the thermally induced denaturation of STV in phosphate buffer from $T_m = 75$ to 112 °C. Thus, the biotin/STV system is much more resistant to thermal unfolding than is STV in the absence of biotin, and clearly more stable than is the isolated STV. Additionally, the binding of biotin to STV is remarkably exothermic and adverse in entropy ($\Delta H = -134\,\text{kJ mol}^{-1}$ and $T\Delta S = -57\,\text{kJ mol}^{-1}$,[81] also reported in another study as $\Delta H = -102\,\text{kJ mol}^{-1}$ and $T\Delta S = -26\,\text{kJ mol}^{-1}$).[79] All the data satisfy the proposed effects of positively cooperative binding in which ligand binding energy is provided in part because the ligand reduces the dynamic behavior of the receptor. The formation of new noncovalent interactions within the receptor is not required, but simply the strengthening of existing

ones. In this sense, distinct conformational changes are not required.

The above conclusions also receive support from independent findings that an increase in protein stability is induced by ligand binding which reduces the flexibility of the protein.[75] They are also in accord with the long-standing observation of Sturtevant that ligand binding to proteins commonly occurs with a reduction in heat capacity, which is consistent with the loss of many internal vibrational degrees of freedom.[82] Notably, Cooper and Dryden have also concluded[83] that positive cooperativity in ligand binding will induce a "stiffening" in the protein (corresponding to the "tightening" shown here). We note that although the H/D exchange experiments indicate the strengthening of hydrogen bonds, all the binding interactions that are positively coupled to the strength of these bonds will be enhanced.

An additional comment is necessary here. It is widely perceived that if there is a change in structure from the observed state of a free protein to a more packed state upon ligand binding, then there must be a cost in the free energy associated with the structural change of the protein. This idea is incorrect: once the ligand has bound to the protein, a new system (ligand·protein) has been generated. Since the ligand restricts the dynamic motion of the protein in positively cooperative binding (see above data), a more compact state is now the most favored in terms of free energy.

12. Ligand/Receptor Interactions that are Strongly Enthalpy or Entropy Driven

Since positive cooperativity promotes enthalpy-driven binding, and negative cooperativity promotes entropy-driven binding, these relationships can be used plausibly to infer the manner in which drugs are likely to order, or disorder, the receptors to which they bind. The thermodynamic parameters of the binding of 136 drugs to biological receptors has been plotted in a graph of ΔH versus ΔS (Figure 13).[84] Some associations are endothermic by quantities in the region of 30 kcal mol^{-1} (125 kJ mol^{-1}), while at the other extreme, some

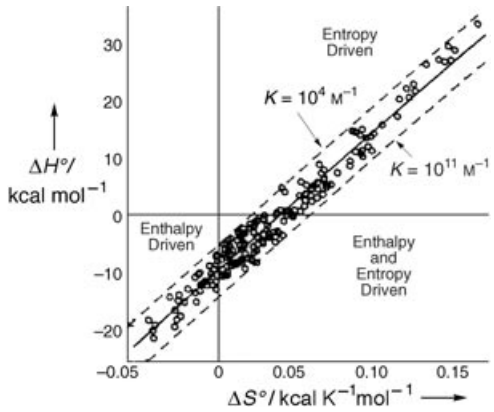

Figure 13. Plot of the standard enthalpies (ΔH^0) versus standard entropies (ΔS^0) for the binding equilibria of 136 different ligands to 10 biological receptors, one DNA, and two enzymes. The two dashed lines correspond to the ligand–macromolecule association constants K of 10^4 M^{-1} and 10^{11} M^{-1}. (Reproduced with permission from Ref. [84].)

are exothermic by about 20 kcal mol^{-1} (84 kJ mol^{-1}). The differences in these extremes are massive (ca. 200 kJ mol^{-1}) and can be ascribed to two scenarios:

1) The highly endothermic binding is associated with negative cooperativity and with associated decreased bonding within the receptor (in its drug-bound, relative to drug-free, state). The decreased bonding in the receptor upon binding the drug increases its internal motion, thus accounting for the very favorable positive entropy change ($T\Delta S$ is in the region of 160 kJ mol^{-1} at room temperature).
2) The highly exothermic binding is associated with positive cooperativity, and with associated improved bonding within the receptor (in its drug-bound, relative to drug-free, state). The increased bonding in the receptor upon binding the drug decreases its internal motion, thus accounting for the very unfavorable negative entropy change ($T\Delta S$ is in the region of -47 kJ mol^{-1} at room temperature).

It is of course possible that both positively and negatively cooperative interactions will occur within the same system, and the thermodynamic parameters will then reflect the net effect. Cases where enthalpy/entropy compensation occurs upon ligand binding to proteins have been highlighted, and examples where binding of ligand causes both increases and decreases in flexibility within the same protein are reported.[85] Additionally, we note that we are only able to consider cases where cooperativity acts to loosen or tighten receptor structures. The situation becomes more complex than can be treated here when ligand binding induces the formation of receptor structures that contain new sets of noncovalent interactions within the receptor.

13. Changes in the Aggregation of Receptors in Signal Transduction

The extremes of enthalpy-driven versus entropy-driven binding were interpreted above as reflecting a tightening or loosening of the receptor system. A common feature of cell-signaling pathways is that membrane-bound receptors are induced to change the extent of their aggregation upon binding to the natural ligand (Figure 14).[86] In some cases, agonists (which activate a receptor when they bind to it[87]) induce aggregation of receptors.[86] Such agonist binding should therefore be particularly beneficial in bonding (negative contribution to ΔH) and adverse in entropy (negative contribution to $T\Delta S$). These consequences (although not previously interpreted in this way) are seen in Figure 15 for the binding of agonists (●) to the β-adrenergic receptor.[88]

In other cases agonists induce the dissociation of receptor multimers.[86] Such agonist binding should therefore be relatively adverse in overall bonding and favorable in entropy. These consequences are seen in Figure 16 for the binding of agonists (●) to adenosine A_1 and A_2 receptors.[89–91]

Antagonists are compounds that bind to such receptors but do not activate them.[87] Therefore, antagonists must avoid induction of the same order of the receptor system that is

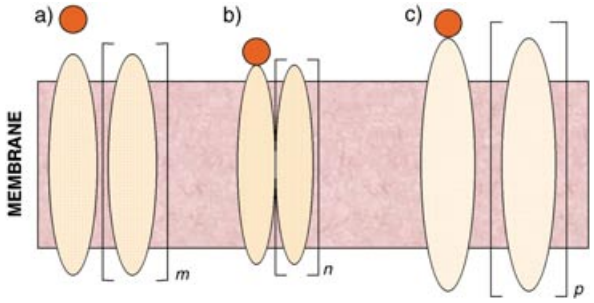

Figure 14. The binding of a ligand (red circle) to a receptor (ellipses) in a membrane: a) ligand-free receptor, b), c) receptors with ligands. b) The binding of a ligand induces greater oligomerization ($n > m$) and possibly also closer packing of the oligomers, c) the binding of the ligand induces dissociation of the receptor ($p < m$) and possibly also looser packing of the oligomers. The receptors may be partially bound (as shown in b and c) or fully saturated.

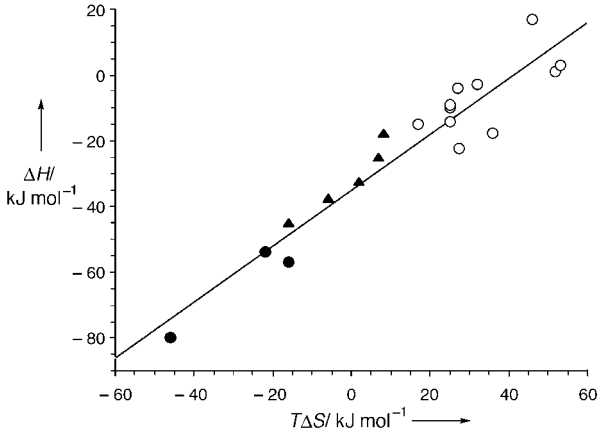

Figure 15. Plots of ΔH versus $T\Delta S$ for the binding of agonists (●), antagonists (○), and partial agonists (▲) to the β-adrenergic receptor.

induced by agonists. Thus, where the agonist induces receptor oligomerization (for example, the β-adrenergic receptor), the antagonist may avoid induction of oligomerization, or even induce the formation of monomeric receptor. Such antagonist binding should therefore be (in comparison to agonist binding) relatively adverse in overall bonding and favorable in entropy. This conclusion is consistent with the data points for antagonist binding to the β-adrenergic receptor (○ in Figure 15).

In contrast, if agonists induce the dissociation of receptor multimers (for example, the adenosine A_1 and A_2 receptors), antagonists may avoid the induction of this dissociation or even induce the formation of receptor oligomers. Such antagonist binding should therefore be (in comparison to agonist binding) relatively favorable in overall bonding and unfavorable in entropy. This conclusion is consistent with the data points for antagonist binding to the adenosine A_1 and A_2 receptors (○ in Figure 16).

Analogous effects are found in numerous systems.[89–91] Thus, agonists and antagonists appear to frequently act differently in their effects on enthalpy/entropy compensation, and in such cases thermodynamic studies can be used to probe the changes in the oligomeric states of receptors upon ligand binding.

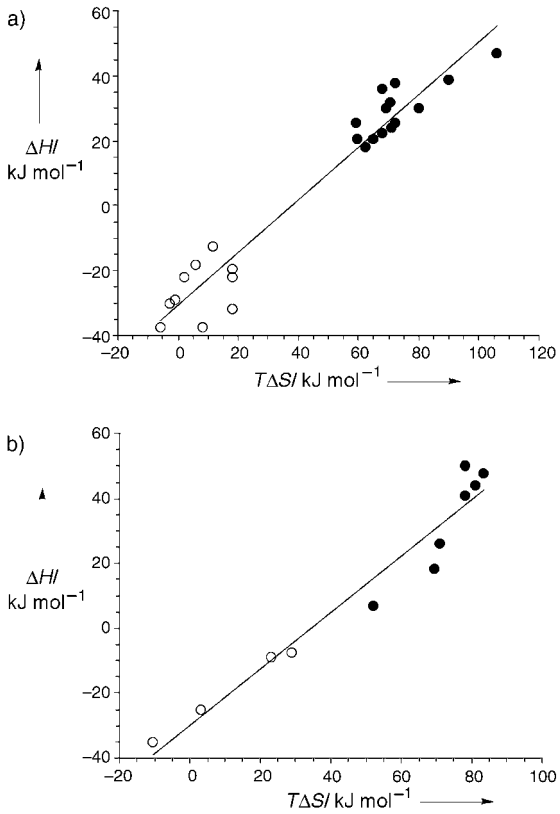

Figure 16. Plots of ΔH versus $T\Delta S$ for the binding of agonists (●) and antagonists (○) to the a) adenosine A_1 receptor and b) adenosine A_2 receptor.[91]

14. Volume Reductions within Enzymes can Promote Catalysis

14.1. Benefits of Improved Bonding in Enzyme Catalysis

A reaction of substrate→product (S→P) catalyzed by an enzyme (E) benefits, relative to the reaction in free solution, because the adverse entropy of the reaction in free solution is reduced by the preorganization of the catalytic groups in relation to the substrate [Eq. (4)].[63] Catalysis will also be promoted if the enzyme binds the substrate transition state ($S^{\neq}$) with positive cooperativity.

$$E + S \rightarrow E \cdot S \rightarrow E \cdot S^{\neq} \rightarrow E \cdot P \rightarrow E + P \qquad (4)$$

According to the model presented here, such positively cooperative binding will occur with a cost in entropy (because of reduced dynamics) and a benefit in enthalpy (because of noncovalent bond contractions) within parts of the enzyme structure in the transition state for reaction. The prediction is therefore that this latter cost in entropy will offset the advantage of the preorganization, but that a large benefit in enthalpy should be apparent in enzyme catalysis.

Relevant data are available for the reaction catalyzed by cytidine deaminase.[92] The effect of enzyme catalysis is to increase the reaction rate by 10^{16}, as a result of a benefit in enthalpy ($\Delta\Delta H^{\neq}$) of $-84\ kJ\,mol^{-1}$ and a benefit in entropy

($T\Delta\Delta S^{\neq}$) of only 7 kJ mol^{-1}. From the Boltzmann equation, −5.7 kJ mol^{-1} corresponds to the rate of reaction at room temperature increasing by a factor of 10^1. Thus, the benefit of improved bonding to the enzyme-catalyzed reaction is a factor of about 10^{15}, whereas the benefit from improved order is only a factor of about 10^1 (Figure 17). The data are consistent with catalysis being derived in part by improving the bonds *within the enzyme*, as a consequence of the transition-state structure of the substrate.

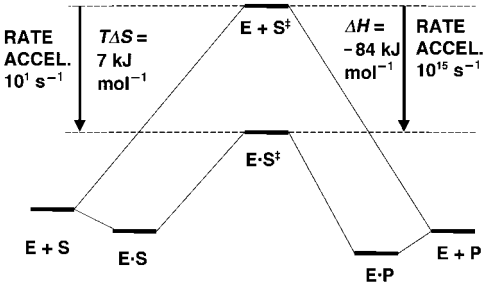

Figure 17. Free energy profiles for the uncatalyzed (upper profile, E + S→E + S$^{\neq}$→E + P) and catalyzed (lower profile, E + S→ES→ES$^{\neq}$→EP→E + P) deamination of cytidine (modified from [92] The rate enhancement of the enzyme-catalyzed reaction (10^{16}) is obtained by deconvolution of the free energy benefit of catalysis and arises from overall improvements in bonding (10^{15}) and in order (10^1).

The relative reaction rates of six reactions (enzyme-catalyzed versus noncatalyzed, $k_{cat.}/k_{uncat.}$) have been measured by Wolfenden and co-workers.[93,94] Strikingly, all the enzyme-catalyzed reactions are greatly accelerated as a result of the large improvement in bonding in the transition state (Table 2). One apparent source of this improved bonding is between the substrate transition state and the enzyme. The additional source proposed here is the strengthening of existing noncovalent bonds within the enzyme.

Table 2: Benefit in enthalpy ($\Delta\Delta H^{\neq}$) between some uncatalyzed and enzyme-catalyzed reactions.[88,89]

Entry	Enzyme	$\Delta\Delta H^{\neq}$ [kJ mol^{-1}]	$k_{cat.}/k_{uncat.}$[a]
1	chorismate dismutase	−33	10^6
2	chymotrypsin	−66	10^{12}
3	staphylococcal nuclease	−63	10^{11}
4	bacterial α-glucosidase	−80	10^{14}
5	urease	−93	10^{16}
6	yeast OMP decarboxylase	−143	10^{25}

[a] Acceleration of the enzyme-catalyzed reaction relative to the uncatalyzed reaction as a consequence of $\Delta\Delta H^{\neq}$.

14.2. Reduced Dynamic Behavior of Enzyme Transition State Structures

If the above large benefits in bonding do indeed reflect changes within the enzyme to an important extent, rather than simply the formation of strong bonds between the enzyme and the substrate transition state, then enzymes should undergo markedly less H/D exchange when in the form that binds this transition state. Two recent studies give convincing support to this idea.

Hydrogen/deuterium (H/D) exchange into backbone amide bonds in hypoxanthine-guanine phosphoribosyl transferase (HGPRT) was used to compare the dynamic properties of human HGPRT alone, in forms with bound reactant/product, and in a form with a bound transition-state analogue.[95] It was found that out of a possible 207 amide H/D exchange sites in the enzyme, after 1 h at RT in D_2O, HGPRT alone exchanged 160, an equilibrium reactant/product complex exchanged 139, and the transition-state analogue complex exchanged 126 of these amide protons. Thus, the enzyme structure becomes better packed overall to provide binding energy for the reactant/product, and then the packing is improved further to provide even greater binding energy for the transition-state analogue.

Equally striking results are found for the binding of a transition-state analogue to a trimeric purine nucleoside phosphorylase (PNP).[96] The transition-state analogue (immucillin-H) binds to the enzyme extremely strongly ($K_d = 23$ pM) when only one of the three catalytic sites is occupied. Deuterium exchange occurred at 167 slow-exchange sites in 2 h when no ligands were present at the catalytic site. A substrate analogue and product prevented H/D exchange at 10 of these sites. When only one of the three sites of the homotrimer was filled with the transition-state analogue immucillin-H, 27 of the slow-exchange protons were protected from exchange in all of the three subunits. The decisive function of the positively cooperative binding of the transition-state analogue is to reduce the dynamic behavior of the receptor (trimer) system to such a degree that a further 81 backbone NH protons are protected from exchange. The reduction in dynamic behavior occurs almost throughout the trimer,[96] and the binding energy of the transition-state analogue can therefore be derived in a highly delocalized manner.

As in the case of positively cooperative binding of ligands to receptors (Section 11), the improved packing induced within enzyme/substrate transition states is different from that which can be induced by high pressures. Instead, it occurs selectively at those noncovalent interfaces where positively cooperative binding is induced by the substrate transition state (Figure 18).

14.3. Stabilization of the Enzyme in an Enzyme-Bound Intermediate

It can be expected that enzyme-bound intermediates (EBIs) will often bear structural similarities to the transition states for the reaction. Where this is the case, then catalysis should be aided if an EBI has 1) a higher melting temperature (T_m) and 2) a reduced susceptibility to trypsin digestion (relative to the enzyme from which it is formed). These properties have been demonstrated for a haloalkane dehalogenase (DhlA) that catalyzes the hydrolysis of haloalkanes via an alkyl–enzyme intermediate, which is then cleaved by a water molecule that is activated by His 289.[97] When His 289 is replaced by Gln, the mutant enzyme (His289Gln-DhlA)

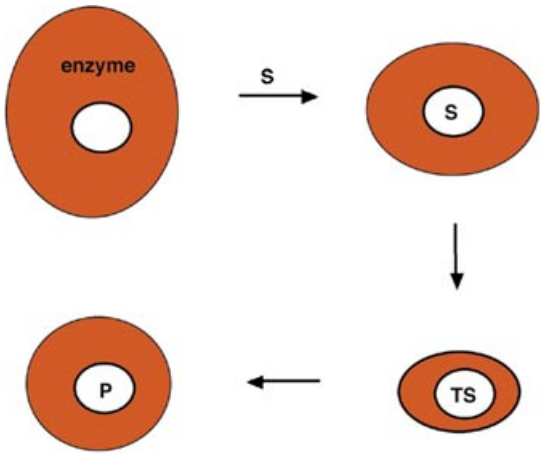

Figure 18. Improved packing of parts of an enzyme structure upon binding the substrate (S), the substrate transition state (TS), and the product (P). The magnitudes of the size and shape changes are grossly exaggerated to illustrate the principle.

accumulates the alkyl–enzyme intermediate. Both the mutant enzyme and the alkyl–enzyme intermediate generated from it through reaction with 1,2-dibromoethane retain the overall conformation of the wild-type protein. Unsurprisingly, the His289Gln-DhlA mutant is thermally less stable ($T_m = 41\,°C$) than the native enzyme ($T_m = 48\,°C$). However, the alkyl–enzyme intermediate is considerably more thermally stable ($T_m = 51\,°C$) than the His289Gln-DhlA mutant from which it was derived. In accord with the conclusion that higher T_m values reflect less dynamic and more stable structures, a number of trypsin cleavage sites became far less susceptible in the enzyme intermediate than in the enzyme from which it was derived. The stabilization of the enzyme-intermediate structure is consistent with catalytic efficiency being derived in part through reduction in the dynamic behavior of the enzyme-intermediate structure.

15. The General Structural Consequences of Positively Cooperative Binding

We have argued here that when two sets of noncovalent bonds are made in a manner where each can restrict the motion associated with the other, there is a benefit in free energy to the ordered state. This conclusion is consistent with analogous (positively cooperative) changes that occur when the size of a homogeneous system is increased from n to $n+m$ molecules. The classical treatment of this effect was established by Hill.[98] Consider a cluster of n molecules with a repeating geometry in the solid state. As the cluster increases in size, typically 1) the solid becomes thermodynamically more stable and 2) the transition to convert the solid into a liquid becomes sharper.[99,100]

Table 3 shows the case of increasing numbers of layers of N_2 molecules absorbed on a surface.[99] The physical basis for this effect is that as the assemblies increase in size, any given molecule within the solid has other molecules extending out to greater distances that help to hold it in place. In larger assemblies, any given molecule has reduced dynamic behavior because the other $n-1$ molecules that hold it in place are increased in number. This reduced dynamic behavior means it is better bonded to its immediate neighbors.

Table 3: Stabilities of N_2 layers as a function of the number of layers.

Number of layers	Melting temperature
2.2	ca. 52 K (very broad)
3.1	56 K (broad)
4.0	58 K (mod. broad)
4.8	61 K (mod. sharp)
infinite	63.14 K (very sharp)

The van't Hoff equation [Eq. (5)] requires that the greater the change in the equilibrium constant for a melting process in a given temperature range, the greater must be its enthalpy change.

$$\ln K = -\Delta H/RT + \Delta S/R \qquad (5)$$

Thus, the sharper melting transitions undergone by systems as they increase in size (Table 3) reflect larger enthalpy changes. These larger enthalpy changes derive from two effects: 1) the increased size of the system (for example, 2.2 versus 4.8 layers) and 2) the more effective bonding of each molecule to its neighbors in the larger system. The addition of an extra layer of nitrogen molecules to the system is analogous to the binding of a ligand to a receptor in a strain-free manner. The contractions within such a system as it increases in size are illustrated schematically in Figure 19.

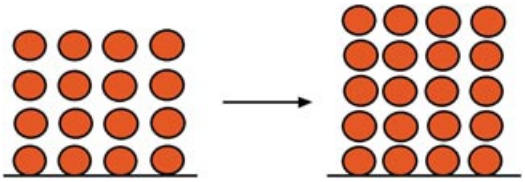

Figure 19. Improved packing and bonding within an ordered system of molecules upon addition of an extra layer.

A corollary of the above argument is that as systems of homogeneous solids become smaller the supramolecular assemblies that behave as an entity become smaller, and their van't Hoff enthalpies of fusion become smaller. It is for this reason that crystals usually exhibit lower (and broader) melting transitions as they are contaminated with increasing amounts of an impurity. In Table 3, the "impurities" are at the boundaries of the N_2 molecules below the surface and the gas above. In biological systems, the water at the surface of a receptor can behave as an "impurity" relative to more positively cooperative binding in the ligand–receptor system.

Consistent with the above generalizations, the incorporation of increasing quantities of cholesterol (as an impurity) progressively reduces the temperature, enthalpy, and cooperativity of the gel-to-liquid-crystalline phase transition of lipid bilayers constituted from phosphatidylserine.[101]

16. Conclusion

An approach in which the simplification is made that noncovalent interactions have characteristic binding energies is useful in the estimation of binding energies. However, in reality each interaction is context-dependent. This context ddependency is associated with enthalpy/entropy compensation.

Enthalpy/entropy compensation, sometimes proposed to be an artifact of experimental error, is a fundamental property of noncovalent interactions (Houk et al. have written a recent summary where either the physical validity or presumed lack of validity of enthalpy/entropy compensation is presented[102]). It occurs in any solid/liquid transition, or when the temperature of any solid or liquid containing noncovalent bonds is changed. It is not only predicted from fundamental theoretical considerations,[2–4] but is also in many experiments far too large to be an artifact arising from errors (see, for example, Figures 13 and 16). In this connection, it should be noted that the uncertainties in the determination of ΔG and ΔH values by calorimetry are not very different.[59]

The correlations presented here between enthalpy/entropy compensation and the changes in dynamic behavior of proteins (as established by H/D exchange) establish the physical nature of a source of enthalpy/entropy compensation in biological systems. The enthalpy well for a local interaction

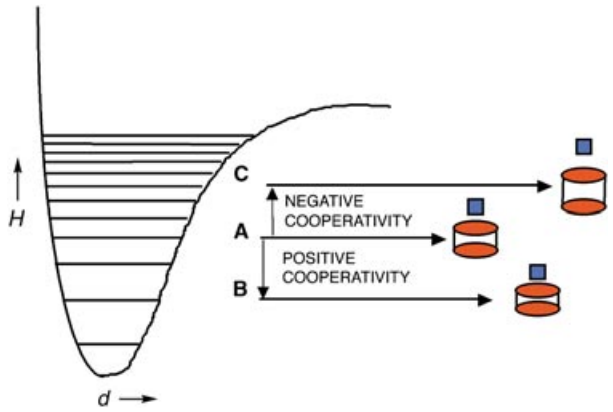

Figure 20. Relative vibrational levels occupied when the strength of a specified hydrogen bond of the receptor (red) and the coordination of the ligand (blue) are A) not cooperatively coupled, B) are coupled with positive cooperativity, and C) are coupled with negative cooperativity. The enthalpy of the interaction is more favorable (more negative) when it lies more deeply in the well. The asymmetry of the well means that when the interaction lies more deeply in the well its bond length is shorter.

influenced in this way is indicated in Figure 20. Promoting an interaction to lie more deeply in an enthalpy well provides a benefit in enthalpy at a cost in entropy; such a change is positively cooperative since ligands are able to bind better to organized templates. Conversely, inducing an interaction to lie more shallowly in the well gives a benefit in entropy at a cost in enthalpy; such a change is negatively cooperative in the general case since here ligands must distort their receptors, which thereby become more dynamic. When the enthalpy barrier to breaking an interaction is very small, the available thermal energy will sometimes break the bond (that is, the interaction will be broken by "hopping" out of the well). Thus, when the bonding is extremely weak, the increases in bond length (that are associated with negative cooperativity or the removal of a positively cooperative effect) will also be associated with fraying.[103]

Where ligand binding directly increases the electrostatic polarization of an interaction within the receptor (for example, of a receptor amide/amide hydrogen bond), the well shown in Figure 20 is additionally deepened, thus providing a further source of bond shortening. It is also clearly possible that distortion of a receptor upon ligand binding may occur in such a manner that the well of Figure 20 becomes less deep, and this effect further promotes the bond lengthening associated with negative cooperativity.

The total changes in the free energies in the organized systems (Figure 20) would be determined by summing over all the noncovalent interactions that are influenced in the manner indicated. Note that in a bound state, lying more deeply or shallowly inside a specified well (Figure 20) has the same consequences as lying in a deeper or shallower well (Figure 2).

Thus, evolution can lead to the use of better packing within parts of receptor structures, which occurs upon ligand binding, to aid ligand binding. It is perhaps useful to envision that the ligand causes a "pseudocrystallization" of parts of the receptor.[56] In other cases, the loosening of parts of receptor structures occurs to weaken ligand binding. These changes are most simply represented as in Figures 18 and 21. The occurrence of the volume reductions indicated in Figure 18 and the upper panel of Figure 21 (grossly exaggerated) upon the positively cooperative binding of ligands to proteins is supported by the direct measurement of volume changes

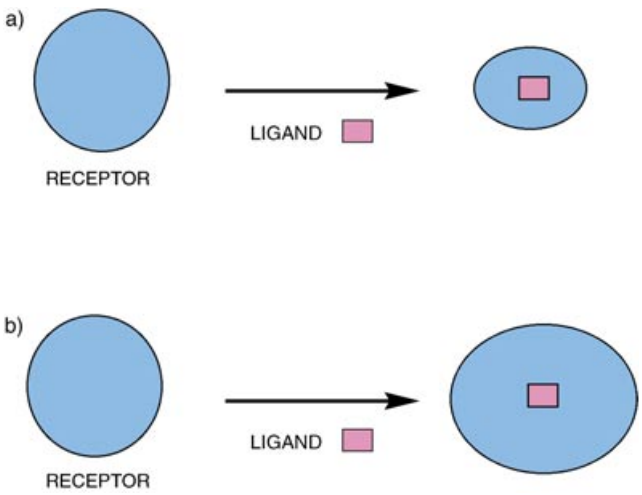

Figure 21. Changes in packing of parts of a receptor structure upon binding its ligand with a) positive cooperativity and b) negative cooperativity. The magnitudes of the size and shape changes are grossly exaggerated to illustrate the principle. The nonsymmetrical induced changes in the shape of the receptor illustrate that cooperativity will typically be exercised more in some parts of the structure than in others. The effects cannot therefore be equated with the ones that would simply be induced by changes in pressure.

occurring in such processes. Thus, it has been reported that decreases in volume (on the order of 1%) occur when *N*-acetyl-D-glucosamine oligomers bind to lysozyme.[104] Importantly, the largest decreases in volume are observed for the ligand that exhibits the highest affinity.

Binding of a small molecule is promoted where the receptor protein shows some or all of the following properties:

1) reduced H/D exchange of the amide protons,[59,87,88]
2) a raised T_m value,[59,73]
3) improved internal bonding and reduced dynamic behavior (more negative values of ΔH and ΔS for ligand binding),[65,81]
4) greater resistance to enzymic digestion and possibly also promotion of the stability of oligomeric forms of the protein.[105]

In the examples cited here, negatively cooperative binding promotes converse effects.[65,71]

Catalytic efficiency is promoted when substrate transition states extensively reduce the dynamic behavior of enzymes. Such a mechanism suggests a reason why enzymes are relatively large structures.

Addendum

It is clearly important to determine whether large binding energies can be provided in the delocalized manner envisioned in this Review. To establish this point, we have recently determined the reduction in the dynamic behavior of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) upon successively binding four molecules of its cofactor (NAD^+).[107] We find that in the binding of the first NAD^+ to the enzyme tetramer, 60 backbone amide NH protons are protected from H/D exchange, whereas in the binding of the fourth NAD^+ molecule, there is no measurable protection of amide backbone NH protons from H/D exchange. The binding constant for the first NAD^+ molecule is greater than that for the fourth NAD^+ molecule by a factor of about 10^6. Thus, it is clear that the binding constant can, in this case, be increased by a factor of a million because the dynamic behavior of the enzyme/cofactor system is reduced upon binding the first NAD^+, but not upon binding the fourth NAD^+ molecule.

We have also shown[107] that under circumstances in which streptavidin is concluded to decrease its average amide hydrogen bond length by about 1%, (on decreasing its temperature from 55 to 5 °C),[108] its increased stability and reduced dynamic behavior decrease its extent of backbone amide H/D exchange from 99 to 61 (same conditions as reported in Section 11). This experiment, taken in conjunction with the data of Section 11 and the above GAPDH data, suggests that very large ligand binding energies, and increased catalytic efficiencies (of the order of a million-fold), can be derived by decreasing the lengths of the numerous hydrogen bonds of a protein (upon binding a small molecule) by as little as about 1%. It is therefore not surprising that X-ray studies have not uncovered the structural basis of the effects proposed here.

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