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Cooperativity and biological complexity

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Cooperative binding effects pervade biology. Only a few basic principles are at play, but in different biological contexts cooperativity appears in distinct guises to achieve different ends. Here I discuss some of the manifestations of cooperativity that are most important in biology and drug discovery as they pertain to systems at different levels of complexity and also highlight aspects of this broadly important phenomenon that remain poorly understood.

Levinthal's paradox—the observation that for a protein molecule to fold by randomly sampling all possible conformations would take not the few milliseconds typically seen but longer than the current age of the universe—is a compelling example of how nature uses cooperative interactions to accelerate or otherwise enhance specific processes. The use of cooperativity to circumvent the energetic, spatial and temporal constraints that would otherwise be imposed by physical chemical laws is essential to very many biological events when considered at the molecular level. Moreover, the higher the complexity of the molecular system, the greater the role cooperative interactions typically play in enabling and regulating its function. Cooperativity is thus a key 'emergent property' that links the molecular building blocks of life to the systems-level properties of macromolecules, pathways, cells and organisms.

The phenomenon of cooperativity is encountered early on in the education of students of the biological and chemical sciences. In biochemistry courses the concept is typically introduced through discussion of oxygen binding to hemoglobin, or perhaps the formation of the DNA double strand; in chemistry, cooperativity might be first encountered in the chelation of metal ions by EDTA. It is only upon more advanced study that it becomes clear that the above phenomena are actually rather different from each other. The same few underlying principles are at work, but they operate in a variety of ways to bring about different types of behavior. Once these distinctions are understood, it becomes apparent that these same principles are central to a very wide range of other biological phenomena, some of which might not at first appear to be closely related to the simple examples mentioned above.

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Two faces of cooperativity

Two distinct phenomena underlie different types of cooperative processes: allostery and configurational pre-organization. Allosterically cooperative systems (**Fig. 1a**) involve a 'receptor' (for example a protein molecule) that contains two or more ligand binding sites, in which the binding of a ligand to one site alters the affinity of a separate ligand molecule for binding at another site. This type of cooperativity requires allosteric communication between binding sites. Traditionally this has been considered in terms of a conformational change in the receptor—that is, ligand binding at each site stabilizes particular conformations of the receptor that possess altered binding properties at the other sites. More recently it has become recognized that communication between remote sites can be mediated through ligand-dependent changes in the dynamic properties of the receptor, and thus that allostery need not involve a detectable change in the average structure of the ligand[1,2].

The cooperativity involved in phenomena such as the chelation of metal ions by EDTA also entails initial binding events that render subsequent interactions more favorable. In these cases, however, the relative enhancement of binding for the later events has a different origin. The cooperativity seen in these cases involves polyvalent ligands—that is, ligands that contain multiple binding groups, such as the four carboxylates in EDTA. Cooperativity that arises from polyvalency is also sometimes referred to as 'avidity'. Polyvalent ligands interact with their binding partner to form a final complex in which the distinct binding elements on the ligand collectively form multiple discrete interactions. Once the first binding contact is made, subsequent interactions become intramolecular and potentially much more favorable (**Fig. 1b**). The initial binding event thus pre-organizes the other sites on the ligand for binding, reducing the number of nonproductive configurations the system can occupy and thereby reducing the entropic cost of bringing each additional binding element into its bound state. The formation of additional interactions may also cooperatively increase the enthalpic stability of each interaction through the so-called enthalpic chelate effect[3]. Overall, the greater the extent of pre-organization—that is, the greater the reduction in the freedom binding elements have to occupy positions distinct from those they will occupy when bound—the greater the cooperativity advantage.

Cooperative effects that occur within a single molecule, such as in protein folding, can also be considered as largely pre-organizational in origin. When a protein folds, an initial hydrophobic collapse greatly reduces the number of conformations that the polypeptide chain can occupy. Moreover, as different specific interactions involved in the folded structure are formed, for example through nucleation of an α-helix, the configurations of nearby residues become further pre-organized for subsequent interactions. Consideration of protein folding additionally illustrates that cooperativity is not solely an equilibrium phenomenon, but is also a strategy used by nature to increase reaction rates. Each step that reduces the number of possible nonproductive configurations decreases the entropy cost to reach the rate-limiting transition state.

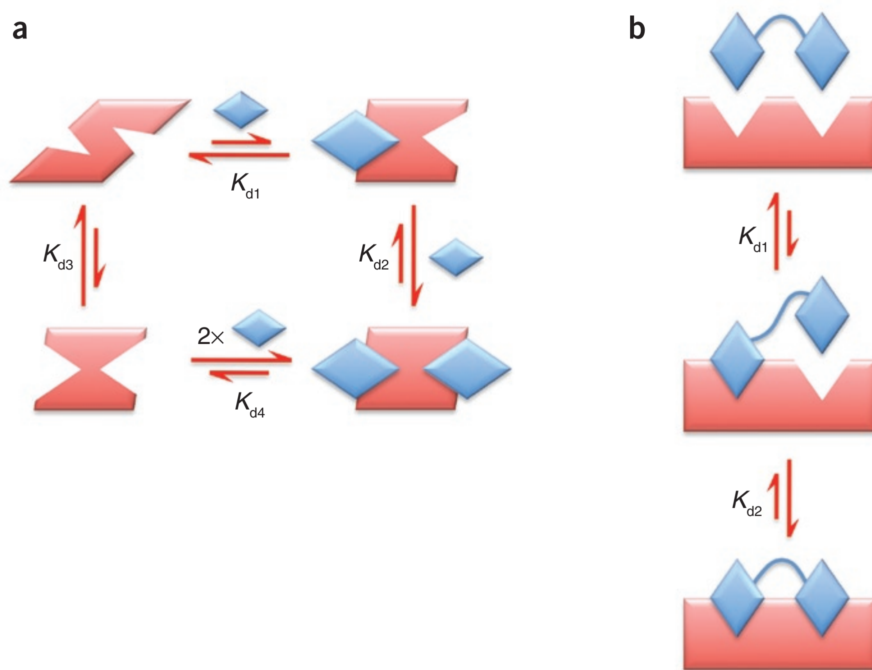

Figure 1 Allosteric versus configurational cooperativity. (**a**) Allosteric cooperativity. Binding of the first ligand molecule (shown in blue) stabilizes a modified conformation or state of the receptor (in red) that alters remote binding sites for additional ligand molecules. The interacting ligands can be identical or different, and any number of ligands can be involved. Binding of the first ligand can either increase ($K_{d2} < K_{d1}$, 'positive cooperativity') or decrease ($K_{d2} > K_{d1}$, 'negative cooperativity') the binding affinity of subsequent ligands. The individual steps are thermodynamically coupled, such that $K_{d1} \times K_{d2} = K_{d3} \times K_{d4}$. (**b**) Configurational cooperativity. A polyvalent ligand makes multiple distinct binding contacts with the receptor. After the initial binding event, subsequent binding steps are intramolecular, which results in enhanced binding (that is, $K_{d2} < K_{d1}$), provided that the geometry of the complex is appropriate for the additional binding events to take place without excessive strain. The polyvalent ligand can contain any number of distinct binding elements, which can be identical or dissimilar.

In nature very little is free. Cooperativity, too, has its costs. In allosteric cooperativity, binding of the first ligand induces the receptor to adopt a conformation or dynamic state that is higher in energy than the ground state. This change is paid for by the binding energy generated upon binding of the first ligand. Only a portion of this binding energy is expressed as the observed affinity, with the remainder being used to alter the state of the receptor. In configurational cooperativity, pre-organizing the system by reducing the number of available nonproductive configurations also has an energetic cost. This cost is partly paid during the synthesis of the polyvalent system (for example, the energetic cost of colocalizing multiple amino acids into a polypeptide) and partly derives from the binding energy generated as each successive interaction in the polyvalent complex is formed. Fortunately, binding energy is readily available in nature, and most biomolecular systems bind less strongly than they could if they had evolved to maximize affinity. Cooperativity thus represents a means to trade excess energy for a higher level of organization, and as such it is a critical enabling mechanism for life.

A full appreciation of the varied manifestations of cooperativity in chemistry and biology requires a definition of this phenomenon that is broad enough to encompass both allosteric and configurational modes of operation, that includes effects on reaction rates as well as on equilibrium affinities, and that is not restricted to any particular class of molecules. For the purpose of this discussion, therefore, I will consider cooperativity to include any set of molecular interactions in which the occurrence of some affects the rate or affinity of others through allostery or configurational pre-organization. When considered in this broad light, cooperativity can be seen to operate at many different levels of organization, from single molecules through large multimolecular complexes, pathways and networks, to interactions involving multiple cells. The following sections describe selected examples of cooperative processes, chosen to illustrate different aspects of cooperativity as it is manifested in biological systems at different levels of complexity.

Cooperativity in drug discovery

In recent years some benefits have emerged from thinking of small-molecule (that is, synthetic organic) drugs—and particularly of the early-stage hits and leads that are the precursors of drugs—as an array of linked binding elements that act cooperatively. This change in perspective was coincident with the advent of fragment-based approaches to lead discovery[4], in which lead molecules are identified by screening the target against a library of small molecular fragments rather than against a collection of drug-sized molecules (**Fig. 2**). The great advantage of fragment-based lead identification is that it tests more potential binding interactions in fewer assays than does conventional screening, and thus provides a much more efficient means of surveying chemical space to identify hits and leads[5]. However, because fragment hits contain only one or two elements of binding functionality, they tend to bind to their target only weakly (typical affinities range from 0.1 mM to 10 mM). Considerable elaboration of these hits is thus required to incorporate a sufficient array of binding functionality to achieve even the 1–10 μM binding affinities that would represent a typical starting point for lead validation and optimization. The question of how to combine or otherwise elaborate fragment hits so that individual elements of binding functionality combine with maximal configurational cooperativity to achieve strong binding has thus been a subject of considerable research.

Early approaches to advancing fragment hits were based on the idea that two weak-binding fragments could simply be linked together to generate a bivalent binder with enhanced affinity (**Fig. 2**)[6]. Because proteins and other biomacromolecules are intrinsically flexible, however, fragment binding can alter the local structure of the protein in small ways by selecting from among the energetically accessible conformational microstates those that contain the most complementary binding site. Different fragment hits may thus bind to subtly different conformations of the protein. Linking the two fragments together will result in substantially stronger binding only if (i) a linkage can be found that allows the fragments to adopt a mutual distance and orientation that positions each of them precisely at its optimal binding site, while constraining their relative motions sufficiently to benefit from configurational cooperativity and yet avoiding significant strain, and (ii) the two fragments bind to the same or compatible conformational microstates of the macromolecular target. Because the tolerance for geometric error is small, the affinity gain achieved by linking fragments is often disappointing. As a result, most successes in advancing fragment hits have come not from linking but from 'growing' a single promising fragment. Improved approaches for linking

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fragments for maximal configurational cooperativity would help accelerate the fragment-to-hit process (which can be a slow step) and would additionally enable fuller exploitation of the rich information that fragment screens provide about the small-molecule binding potential of the target.

The conformational obstacles to linking fragments represent an example of undesired allosteric cooperativity—in this case negative cooperativity. The binding of each fragment alters the average conformation of the protein in a way that weakens binding of the other fragment. This observation illustrates the important point that functionally useful cooperativity is an emergent property of a macromolecular or cellular system that represents a form of higher order organization. Putting components together will typically not result in a productive interaction between them, and productive cooperativity thus cannot be linearly extrapolated from the properties of the component interactions considered in isolation.

Despite decades of intensive effort, we still do not fully understand what magnitude of affinity advantage to expect from the precise configurational pre-organization of two or more binding elements into a single rigid molecule. The method one occasionally sees of simply multiplying the equilibrium constants (for example by assuming that linking two fragments that each bind with $K_d = 10^{-3}$ M will give a combined molecule with $K_d \sim 10^{-6}$ M) has no validity whatsoever[7]. One venerable attempt to quantify this advantage[8] estimated that freezing out the translational and rotational motions of a reactant to perfectly pre-organize it for reaction would confer upon it an "effective molarity" of ~10^8 M, far exceeding for example the molarity of water molecules in liquid water (~55 M). Others have challenged this estimate as being either too high or too low. In real-world binding systems, the affinity advantage from linking two ligand fragments together rarely exceeds ~10^5 M and is often much smaller, which suggests that achieving the theoretical maximum benefit from dimerization or multimerization is difficult. Accurately determining a theoretical upper limit to the binding enhancement achievable from dimerization remains an important and useful goal. Moreover, a quantitative understanding of such relatively simple cases is presumably a prerequisite to fully understanding the impact of cooperativity in more complex biological systems.

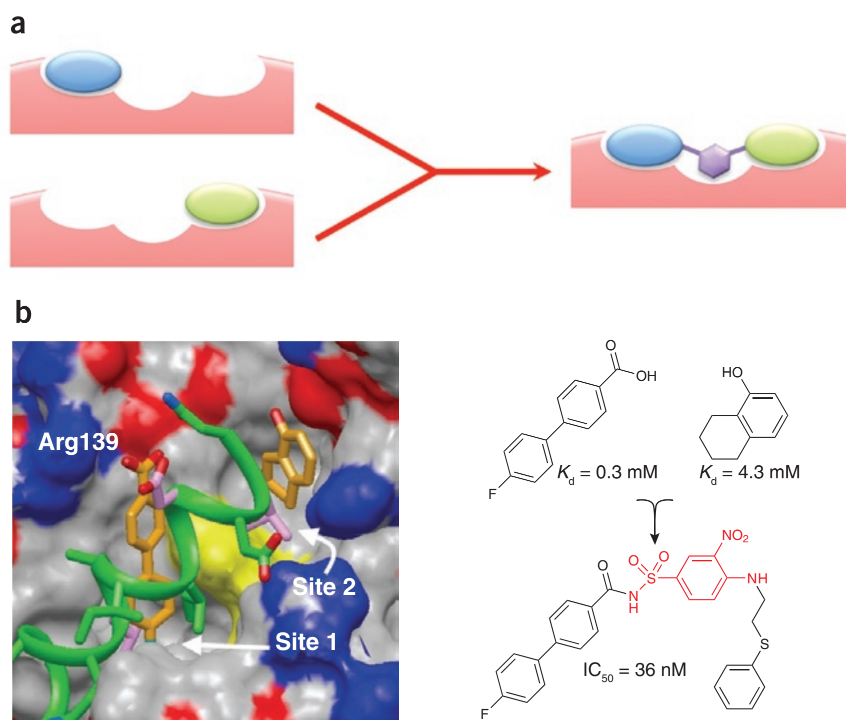

Figure 2 Advancing fragment hits by linking. (**a**) Fragment-based screening identifies small (molecular weight ~ 150–250 Da) organic molecules that bind weakly to the target. Two or more fragment hits can be advanced by linking them together to capture all of their binding interactions within a single, larger molecule that potentially binds much more strongly due to configurational cooperativity. (**b**) A successful example of fragment linking. Two weak-binding fragments (shown in orange, overlaid on the natural BH3 peptide ligand shown in green) were combined to form a molecule that bound to the target, Bcl-xL, ~10^4-fold more strongly than either fragment. Protein image reprinted with permission from ref. 18.

One plus one equals three

A very widespread form of cooperativity in biological systems involves the assembly of weakly binding components into stable multimolecular complexes. If two molecules form a complex that contains a high-affinity binding site for a third molecule, then the three components together can form a strong complex even if the binary interactions between any two components in isolation are very weak (**Fig. 3a**). This phenomenon represents a form of configurational cooperativity, in that pre-organization of two of the components enhances binding of the third.

An example of this effect is seen in the cooperative assembly of the so-called β-interferon enhanceosome[9], a set of proteins that together bind to the interferon-β (IFN-β) enhancer site on DNA to induce IFN-β expression in response to viral infection (**Fig. 3b**). At least six different proteins are involved in the complex, some of which interact to create a binding site for others and some of which are believed to bind to and distort the DNA to pre-organize it for binding. The high cooperativity of this system is evident from the fact that the absence of any one component greatly destabilizes the complex. The overall effect is to tightly control IFN-β expression by making it dependent on multiple factors, each of which is essential to the response. In systems where a complex contains multiple copies of the same protein, the effect can be to increase the dependence of the system's output on the concentration of that component, thereby producing a sharper dose-response threshold for function.

Cooperativity via the cell membrane

The large enhancement of binding that can be achieved by presenting multiple binding groups within a single ligand molecule, such as in the chelation of metal ions, has long been appreciated. Much less studied are the consequences of constraining interacting species to a membrane surface—a strategy that is widely used in nature. Colocalizing reversibly interacting species to a common membrane substantially reduces their relative freedom of motion, even if they retain the freedom to independently diffuse and rotate on the membrane surface, and thus partially pre-organizes them for binding (**Fig. 4**). The net effect of constraining the reactants to the membrane is to reduce the entropy of the unbound state, leading to an enhancement of binding compared to the same molecules interacting in solution.

Examples of membrane-derived cooperativity abound in biology. For example, a commonly invoked mechanism for the acti-

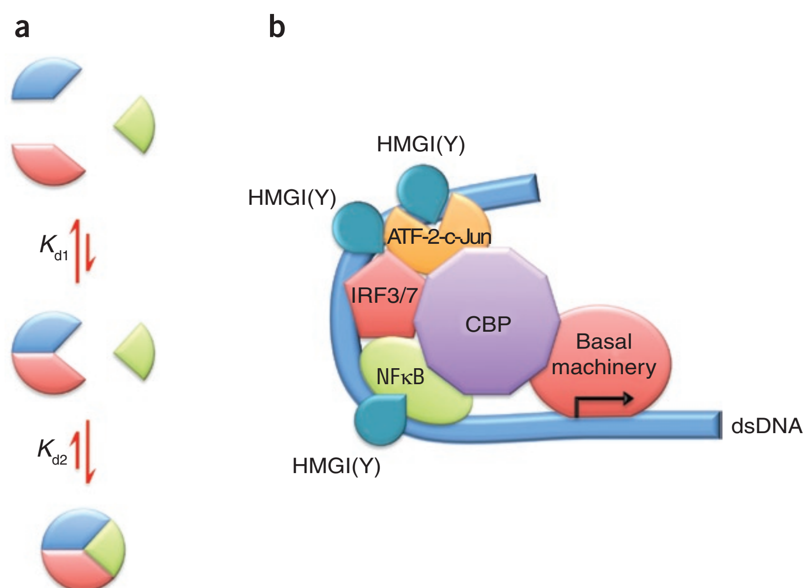

Figure 3 Cooperative assembly of a multimolecular complex. (**a**) Two components bind to form a complex that contains a high-affinity binding site for one or more additional components. The resulting multimolecular complex can be highly stable even if all binary interactions between any two components are weak. (**b**) The IFN-β 'enhanceosome' complex, which requires the cooperative assembly of multiple protein components to form a stable, functional complex on DNA. Diagram adapted from ref. 10.

vation of cytokine and growth factor receptors involves ligand-induced dimerization or oligomerization of the receptor. According to this mechanism (**Fig. 4a**), receptor activation involves two distinct steps. First, the soluble cytokine or growth factor binds to a receptor protein on the cell membrane. The binary complex thus formed subsequently recruits an additional receptor protein, which may be identical to or different from the first, to form the activated receptor complex. A key step in this mechanism is thus the interaction between receptor components that occurs on the cell membrane after the initial binding of ligand. Some receptors comprise three or more independently diffusing membrane components, with activation involving multiple membrane-constrained association events[10]. Higher order interactions in which entire activated receptor complexes associate into ordered structures might also occur. Receptors that function by ligand-induced oligomerization use the cooperativity arising from colocalization to a common membrane to enhance the interaction between the receptor components, as part of the mechanism by which ligand binding is coupled to receptor activation.

Membrane-derived cooperativity is also important in many intracellular signaling processes in which proteins are dynamically recruited to the plasma membrane. For example, recruitment of signaling molecules to the membrane through lipidation or via adaptor proteins, with the reduction in dimensionality that results, is an important mechanism for enhancing and dynamically regulating the intermolecular interactions that govern intracellular signaling. One example is the Ras signaling pathway (**Fig. 4b**)[10]. However, the quantitative contributions of this strategy to enhancing the rates and affinities of interactions have not yet been fully elucidated.

Membrane-mediated cooperativity illustrates a common feature of cooperative processes in that its effect is highly contextual, such that the same system of molecular components can display different properties in different situations. This is because the equilibrium distribution between bound and unbound species on the membrane depends both on the magnitude of their mutual binding affinity and on the two-dimensional concentrations or densities of the interacting species on the membrane. It is therefore possible, for example, that a given cytokine receptor might display quantitatively different properties (and perhaps even qualitatively different activation

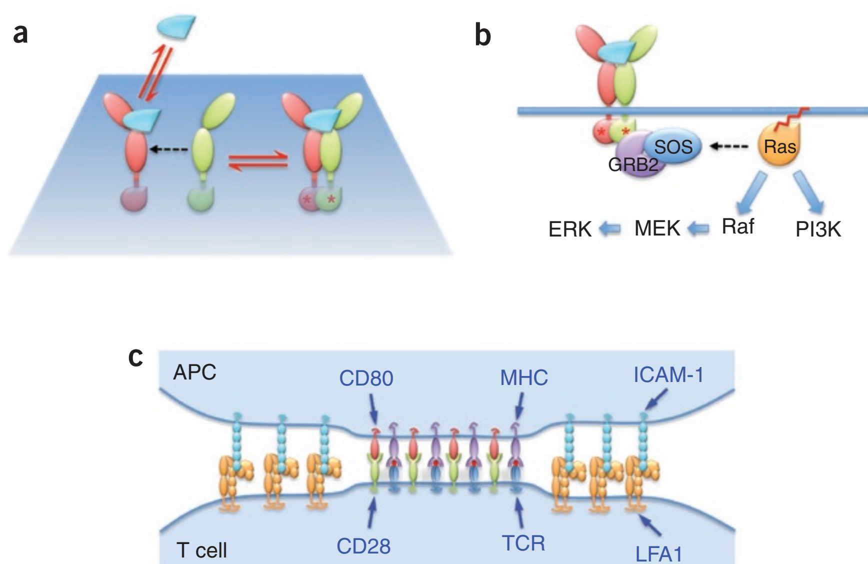

Figure 4 Cooperativity due to colocalization on the cell membrane. (**a**) Binding partners constrained to a common membrane are partially pre-organized for binding, relative to free solution, even when they retain free lateral mobility. For example, for receptors that are activated by ligand-induced dimerization, the affinity of the second, on-membrane step is enhanced through constraint to the two-dimensional membrane surface. (**b**) Intracellular signaling can also exploit cooperativity on the membrane. For example, the adaptor protein GRB2 recruits SOS to the membrane through binding to an activated growth factor receptor, where they are poised to interact with the Ras oncogene, which is tethered to the membrane via one or more covalently attached lipid groups (shown in red). Activation of Ras in turn activates downstream signaling pathways, including a cascade involving the kinases Raf, MEK and ERK and other events downstream of PI3 kinase. (**c**) The immunological synapse is a highly organized interaction between closely apposed cells—in this case a T cell and an antigen-presenting cell (APC)—that is mediated by the highly cooperative interactions of a complementary array of adhesion and co-stimulatory receptors on each cell.

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mechanisms) on different cell types, on different cells within a single population, on the same cell at different times or even on different surface regions of a single cell[11]. Cooperativity on the cell membrane is thus very much a local affair. Nature exploits this property by engineering heterogeneity in the structure of the cell membrane in the form of cholesterol-rich lipid rafts[12], leading certain receptors and other signaling proteins to accumulate at high local densities. Other ways in which nature might exploit the contextual nature of membrane cooperativity to dynamically regulate cellular responses remain largely unexplored.

Surprisingly for such a biologically important phenomenon, the magnitude of the enhancement in binding that might be expected from constraining an interaction to a cell membrane is unknown. This significant gap in our knowledge is partly due to the slow pace of development of experimental and theoretical tools for the quantitative investigation of interactions on membrane surfaces. Moreover, there seems to be no accessible theoretical model to guide us as to what advantage might be expected even for a simplified, ideal system. Progress on both the experimental and theoretical fronts is needed to elucidate the quantitative consequences of membrane colocalization and thus to fully understand the role that this aspect of cooperativity plays in the many biological processes to which it contributes. An improved understanding of the quantitative aspects of membrane cooperativity would also aid in the design of new biopharmaceutical drugs by increasing our ability to exploit this phenomenon in predictable ways.

Cooperative interactions between cells

Membrane cooperativity is not always restricted to molecules on the same cell. The close approach of two cells allows arrays of membrane proteins on the two cell surfaces to come into contact at the interface. The resulting interactions, which are important mediators of cell-cell adhesion and communication, can involve a high level of organization that is intricately linked to biological function. A particularly elegant and well-characterized manifestation of this effect is the 'immunological synapse'[13]. This term describes the interaction formed between antigen-presenting cells and T lymphocytes during T-cell activation, or between cytotoxic T lymphocytes or natural killer cells and their target cells during cellular cytolysis. The contact between the cells is intimate, involving a substantial area of membrane surface, and is mediated by an organized array of protein-protein interactions involving adhesion molecules and co-stimulatory receptors present on the surface of each cell (**Fig. 4c**). The interacting molecules at the interface segregate into concentric zones, the arrangement of which evolves as the interface matures from initial cell-cell contact to the final, stable immunological synapse, providing the interaction with temporal as well as spatial organization[14]. The interactions at the interface have been studied in considerable detail; cooperativity effects arising from the constraint of the binding partners to the two-dimensional membrane surfaces at the contact region in conjunction with active transport processes involving interactions of membrane proteins with the cytoskeleton together promote and regulate the formation and function of this complex and highly organized interaction[15]. The extent to which other cell-cell interactions involve similarly high levels of molecular organization remains unclear. Cooperative interactions between cells, such as those seen in the immunological synapse, have been exploited by some biopharmaceuticals that incorporate an antibody Fc fusion partner to mediate bridging with natural killer cells to bring about selective killing of the target cell[16,17]. The tools and approaches that have been developed to study the immunological synapse have thus opened up an important new area of study involving cooperative processes at the multicellular level.

Conclusion

Cooperativity is a key organizing principle in chemistry and biology without which the complex molecular systems required for life could not function. Higher level biological systems are characterized by a complex network of interdependencies. Cooperativity represents a primary and highly versatile mechanism for achieving such interdependency at the molecular and cellular levels. Our increasing ability to explore the details of such processes, in the context of single molecules, multimolecular networks and whole cells, is opening up new levels of understanding of the critical role that cooperativity plays in the function of complex biological systems. There are, however, some very basic questions about the thermodynamic and kinetic consequences of cooperativity that must be answered if we are to achieve a truly quantitative understanding of this phenomenon and thus of the systems in which it functions.

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