Point of VIEW

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How the "Melting" and "Freezing" of Protein Molecules May Be Used in Cell Signaling

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ABSTRACT The motile response of *Escherichia coli* bacteria to attractants and repellents is one of the best-understood examples of a signal transduction pathway. A number of recent studies suggest that the receptors in this system undergo major changes in both their degree of structural order and their state of aggregation in the membrane. We discuss the thermodynamic basis for this effect and argue that the "freezing" or "melting" of protein structure may be the language of signaling.

The binding together of molecules through weak noncovalent bonds is, not to put too fine a point on it, the basis of life. And yet despite its importance, most of us have an astonishingly one-dimensional view of this process. Biologists, at least, usually have in mind the powerful lock-and-key metaphor proposed in 1895 by the German chemist Emil Fischer. This depicts the interaction between a large molecule such as a protein and a smaller molecule such as a sugar as a fit between two complementary surfaces. If the larger molecule has a cavity on its surface that matches the smaller one, then the two can come together. Given suitable chemistry, neighboring atoms will interact, especially through hydrogen bonds, and the two molecules will stick. If we now measure the binding affinity or dissociation constant of the interaction, we will then know all we need to about its strength, specificity, and potency. End of story.

Well, not quite. Proteins are large, complex, dynamic structures that have evolved over billions of years to perform very sophisticated tasks. When they associate with a specific binding partner, most proteins respond actively in a far from simple manner. You can find proteins that snap shut like a gin trap, open like a jack knife, or assemble into long filaments. There are proteins that respond to binding events by splitting into pieces or by emitting light. In doing all these things, and much more, proteins are manipulating aspects of thermodynamics to enable the processes of life.

When two molecules in solution bind to each other, there is in one respect an increase in local order. The molecules become more constrained in their translation and rotations. The problem is, as we know from the second law of thermodynamics, that any spontaneous process must involve a net increase in disorder in the universe. So, if binding is to occur, there must be a compensatory increase in disorder, and the question arises about where this comes from. The usual answer is that it arises from a change in the thermodynamic quantity called enthalpy, or heat content, of the system. Heat produced by the binding event is released to the surroundings (*i.e.*, the universe), where it causes other molecules to become more disordered. However, there is a second strategy that is less familiar but just as effective. Here, the binding event causes disorder in the system itself, in regions of the two molecules other than the actual binding site. In this case, the system of the two interacting molecules actually increases its overall disorder, measured by the thermodynamic variable entropy. If this increase of disorder is sufficiently large, it can even be associated with an overall absorption of heat from the surroundings. So, binding interactions can be driven by a reduction in enthalpy (heat release), an increase in entropy, or some combination of the two (*1*).

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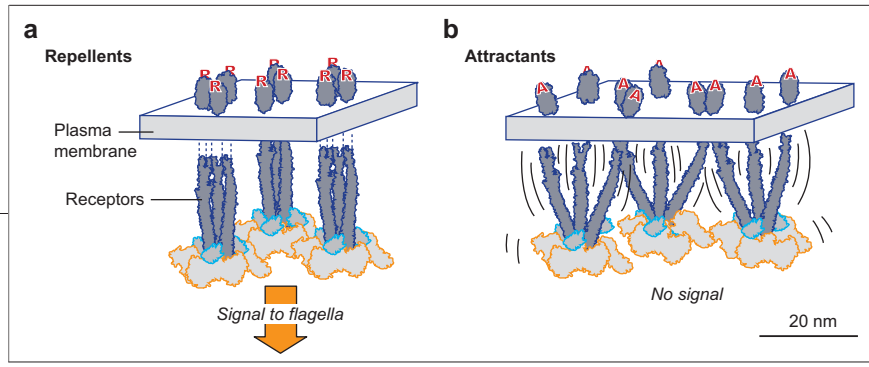

Figure 1. Freezing and melting of chemotaxis receptors. *E. coli* chemotaxis receptors are dimeric transmembrane proteins with a globular ligand-binding extracellular domain and a long α-helical coiled-coil cytoplasmic domain. They are shown here associated into trimers, linked at their base by signaling proteins. Here, we suggest that the receptors exist in two states that differ in rigidity or order. a) In the active state, the receptors are more highly ordered and associate into a relatively rigid lattice able to generate a downstream phosphorylation signal. This "frozen" state is promoted by the (enthalpy-driven) binding of repellents. b) By contrast, receptors in their inactive state possess a significant degree of disorder and are less highly organized into a lattice. This "molten" state is promoted by the binding of attractants and driven by an increase in entropy, as described in the text.

A good example of an enthalpy-driven binding interaction may be seen in the binding of biotin to the bacterial protein streptavidin. The remarkably high affinity of this interaction ($\sim 10^{14}$ M^{-1}) is strongly exothermic and accompanied by the formation of 24 new hydrogen bonds (*2*). Most bonds are made not with biotin, which in any case is too small to form so many bonds, but within the protein itself. A flexible loop in the free protein changes from an "open" to a "closed" conformation as it binds to biotin, thereby amplifying the strength of the interaction. This process is analogous to that of freezing, in which molecules of a substance such as water come together in a more highly ordered and restricted arrangement with the release of heat.

At the other extreme, an example of an interaction driven by entropy may be found in the binding of an oxygen molecule to hemoglobin. The first oxygen molecule binds with essentially no change in enthalpy even though, in isolation, the combination of oxygen with an iron atom would produce an appreciable amount of heat. What happens instead is that the strength of this binding is used to distort the protein, with a net increase in entropy. Indeed, it is well-known that hemoglobin becomes less well-ordered when it binds to oxygen, a change classically described as a change from a "tense" to a "relaxed" state (*3*). In this case, the analogy is with the process of melting, in which molecules in a highly ordered solid state become dispersed.

The possibility that the "freezing" and "melting" of proteins might have a functional significance in cell signaling was raised in an examination of membrane receptors. A pioneering 1994 study by Borea and colleagues (*4*) reviewed thermodynamic data on a wide range of drug–receptor interactions. They found that the 136 interactions examined ranged between those driven almost entirely by enthalpy and those driven almost entirely by entropy, with many intermediate cases. Intriguingly, it appeared that agonists for a particular receptor, such as the nicotinic acetylcholine receptor or the β-adrenergic receptor, almost always acted in the opposite manner as that of antagonists. That is, if a receptor is activated by an enthalpic reaction, then chances are that it will be inactivated by an entropic reaction, and *vice versa*. It could be, therefore, that discrimination is achieved not by the atomic minutiae of conformational changes but by broader features of the protein such as its rigidity or flexibility—whether it is too a large degree frozen or melted (*5*).

There is resonance here with a series of studies of the cluster of receptors responsible for chemotaxis in bacteria, the most recent of which by Borrock *et al.* appears on page 101 of this issue (*6*). Many bacteria move in response to their chemical environment by the mechanism of chemotaxis, which involves a two-component signal transduction system. Like other sensory receptors, bacterial chemotaxis receptors respond to changes in the chemical environment with a high sensitivity and a broad dynamic range. The high sensitivity is manifested by an observation that the binding of attractants to <1% of the receptors can induce increased swimming motion of *Escherichia coli* (*7*). This high sensitivity is achieved at the beginning of the signal transduction pathway and is thought to arise through the clustering of the receptors (*8, 9*). Various models have proposed cooperative interactions between elements of the receptor cluster, but their structural basis is still unclear (*10, 11*).

The chemotaxis receptors are long dimeric molecules with a globular ligand-binding domain on the outside of the cell and a four-helix, coiled-coil bundle ~30 nm long on the cytoplasmic side (Figure 1). Molecular modeling, together with more recent biochemical and electron microscopic evidence, suggests that the receptors are arranged in sets of three, tethered at their cytoplasmic ends and held in a lattice through their interactions with the kinase CheA and other molecules. In some fashion, binding of attractants and repellents to the outer globular domains sends a message to the CheA kinase attached to the bottom of the receptor, which in turn sends signals to the flagellar motors. The current view is that the receptor acts as a two-state, on–off switch in which different inputs shift the equilibrium between two signaling states by triggering conformational changes (*12*). Evidence from X-ray diffraction studies, cysteine cross-linking, and comparative sequence analysis points to a piston-like sliding of one of the membrane helices toward the cytoplasm and bending of the four-helix bundle at a glycine-rich region midway along its length. Attractants binding to the receptor are seen as causing a downward piston displacement that in turn increases flexibility at the glycine hinge and in some manner turns off the kinase. Repellents have the opposite effect.

The notion of "protein machines" in which molecules adopt rigidly defined struc-

tures and interact with their neighbors through sterically precise interactions is an underlying tenet of contemporary biology. However, it is now known that very high proportions of proteins in a cell have long regions of disorder, lacking any discernible structure (*13*). Recent findings also indicate that a significant amount of structural disorder or polymorphism can be preserved in protein complexes (*14*). Moreover, this could apply to the bacterial chemotaxis system, as first suggested by Kim and colleagues in 2002 (*15*). These authors observed that that the crystallographic temperature factor, a measure of the flexibility or disorder, of the cytoplasmic domains was much higher in a wild-type receptor than in a mutant receptor locked in an "on" state. This led them to suggest that a receptor exposed to repellents is in a more "frozen" state compared with one exposed to attractants. Moreover, they hypothesized that communication between receptors in a cluster could be through propagation of a highly ordered state, so that "changes in dynamic property of the receptors on ligand binding or methylation may be the language of the signaling by the chemotaxis receptors." Another possibly related observation was made on living cells by measuring changes in fluorescence anisotropy. Vaknin and Berg (*16*) traced these changes to relative movements of receptor dimers, which appear to move further apart in the presence of attractants, with the opposite happening in repellents.

The Article in this issue from Kiessling's group (*6*) is the latest in a series of reports describing their use of chemical probes to analyze bacteria chemotaxis signaling. Their strategy is to synthesize multivalent ligands carrying multiple copies of attractant or repellent groups and to examine their effects on bacterial swimming and the distribution of receptors. In this case, they used polymers bearing leucine pendant groups, because leucine is known to act as a repellent in this system. They found, unexpectedly, that although leucine itself and the smaller polymers do indeed act as repellents, the largest polymers provoked an attractant response and, moreover, seemed to disrupt the receptor clusters. Although the interpretation of these results is far from simple, they do seem congruent with a previous study in which cells were exposed to high concentrations of attractant. Once again, the chemotaxis receptors were dispersed (*17*).

The broad message appears to be that signaling in the chemotaxis system entails changes in both the dynamic state of the receptors and their state of aggregation. Repellents reduce the dynamic motions of the receptors and increase their lateral association into clusters. From a thermodynamic standpoint, we surmise that repellent binding is driven mainly by enthalpy and, if such measurements could be made, would be accompanied by a release of heat. Conversely, we think it likely that attractant binding will have a much larger entropic content. Binding in this case will promote disorder in individual receptors and disrupt their interactions with neighboring proteins in the membrane. This should be accompanied by a much smaller release of heat to the surroundings (or even be endothermic). Seen from this perspective, signaling in the receptor cluster seems analogous to a phase transition, with local regions of disorder spreading, like the melting of ice cause by small quantities of salt.

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