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Use of Two-Component Signal Transduction Systems in the Construction of Synthetic Genetic Networks

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Summary of Recent Advances

Two-component signal transduction systems are a common type of signaling system in prokaryotes; the typical cell has dozens of systems regulating aspects of physiology and controlling responses to environmental conditions. In this review, I consider how these systems may be useful for engineering novel cell functions. Examples of successful incorporation of two-component systems into engineered systems are noted, and features of the systems that favor or hinder potential future use of these signaling systems for synthetic biology applications are discussed. The focus will be on the engineering of novel couplings of sensory functions to signaling outputs. Recent successes in this area are noted, such as the development of light-sensitive transmitter proteins and chemotactic receptors responsive to nitrate.

Introduction and Background

Synthetic biology is a relatively new discipline focused on engineering novel cell activities (1-3). Many of the engineered systems built so far are fairly simple, and should be considered as demonstrations of the potential for engineering cell behavior, as opposed to applications suitable for commercial or medical applications. For example, “cameras” (4), oscillators (5-8), and bistable genetic systems (genetic toggle switches) have been developed (6, 9, 10). Bacteria have been developed that can form patterns on solid medium (11, 12), sense the presence of various compounds or conditions (13), detect edges of a light-dark interface (14), or regulate the turbidity of their own cultures (15). Some of these synthetic genetic devices contain components from two-component systems (4, 6, 7). These initial synthetic genetic devices demonstrated the feasibility of engineering cell activities, as well as the properties of the component parts and principles of genetic regulation. From the point of view of this review, a unifying theme of all of these initial devices is that the components used as parts were sufficiently well-understood at the outset to permit network engineering. Furthermore, the components allowed novel connections between sensory stimuli and output functions, such as the expression of target genes or pathways. A major challenge for synthetic biology is developing novel connections between stimuli and gene expression networks; to build increasingly complex systems, a large expansion of the “tool kit” will be required (2, 3). The potential benefits of synthetic biology approaches are illustrated by the use of engineered cells to make an important pharmaceutical compound

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(16). Creating novel connections between stimuli and responses should allow synthetic biologists to develop many different types of useful engineered cells.

The importance of functional modularity in engineering cannot be overstated. Modularity of function enables the engineer to predict the properties of composite structures from the known properties of the components. But, while biology provides numerous examples of modular functions, modularity is not a universal property. Here, I will try to note aspects of the two-component systems proteins that enable or discourage their use as modular components suitable for building larger structures with predictable properties. I will then note some successful examples and point out some possible uses of two-component systems in synthetic biology devices.

Two-component systems are genetically tractable, and provide a huge number of possible parts

Most bacterial cells and about 50% of Archaea contain dozens of two-component systems, working in parallel and functionally-isolated from one another, to connect specific stimulatory effectors to discrete output responses. Many two-component systems are not essential for survival, and many system regulate only a small number of target genes. Furthermore, systems can be introduced into host cells that have no analogous system. Thus, systems may be removed or introduced as necessary to allow development of well-isolated circuits. As a very large class of prokaryotic signalling systems, two component systems can provide thousands of components for synthetic biology.

Insulation from non-partner systems

Although the typical prokaryotic cells contains many two-component systems, these remain functionally isolated from one another. This is due to highly specific phosphatase activities associated with the transmitter protein itself (18-24), or with distinct phosphatases (25, 26) as well as a high degree of specificity in the protein-protein interactions between cognate transmitter and receiver proteins (27-29). It has long been known that non-physiological crosstalk between systems can be observed *in vitro* using elevated concentrations of proteins, or can be observed *in vivo* upon deletion of a cognate transmitter and with overexpression of a non-cognate transmitter (30). But, under natural circumstances, crosstalk between unrelated systems appears to be rare. Physiologically important networks exist that include multiple transmitter and receiver proteins, a phenomenon referred to as cross-regulation (31). Systems that participate in cross-regulation are not insulated from one another, but even these display insulation from other, non-cognate, systems. The natural mechanisms that result in high specificity of two-component signal transduction can be used to advantage for engineering well isolated systems.

Potential for building complex sensory devices that integrate multiple signals

In general, engineers desire modular unit functions. Two component signal transduction systems provide the possibility of engineering simple connections between stimulus and output (e.g. 4). However, in some cases, the natural function of these systems seems to be to provide the integration of distinct signals, a power that could conceivably be harnessed in future engineering of complex devices. Integration of signals can occur at the single-protein level and at the system level. As an example of integration at the single protein level, the FixL transmitter of the FixLJ system of *S. meliloti* appears to be controlled by two distinct mechanisms: oxygen binding to a heme cofactor within a PAS domain controls the balance between kinase and phosphatase activities, while the cytoplasmic FixT protein appears to act as a receiver analogue that acts as a competitor to reduce FixJ phosphorylation (32-34). Similarly, the VirA transmitter is regulated by phenolic compounds and by a periplasmic sugar-binding protein (35); the KinA transmitter is regulated by a stimulus that acts through

a PAS domain, as well as by proteins that inhibit its autophosphorylation (36). In a recent review, it is noted that in many transmitters, apparent sensory domains are present for which the stimulus is unknown, and in some cases the domain arrangement of transmitters suggests multiple sensory inputs (37). CheA presents a special case, as it functions in a large array with receptors and additional components (e.g. 38). Thus, transmitters appear to have the capacity for signal integration at the level of the single protein. This is a double-edged sword for engineering, as the capacity to integrate signals at the single protein level is a powerful benefit, but not understanding all the stimuli that can affect the output is an obvious drawback.

Alternatively, integration of signals can occur at the system level. A striking example comes from sporulation control in *B. subtilis*, where distinct cross-regulating transmitter proteins permit the integration of the signals sensed by the transmitters and controlling the expression of the transmitters, multiple accessory proteins regulate transmitter activity allowing additional input signals, and multiple phosphatase proteins acting upon the phosphorelay allows yet additional signals to control flux through the phosphorelay. Here, the obvious modularity of sensory perception in the natural system suggests an engineering approach for integrating multiple signals as well as for tuning system output.

Re-wiring of sensation

As already noted, a major bottleneck is in engineering novel linkages of stimuli to regulatory outputs (target genes). In this review, I consider the two-component systems as forming four classes based on general mechanisms of signal perception (Fig 1). The stimulating signal may impinge upon the transmitter either directly or indirectly, and either at the cell surface or within the cytoplasm. Thus: (a) direct sensation by a periplasmic domain of the transmitter, with transmembrane signaling, (b) sensation by a distinct integral membrane or periplasmic protein, that controls transmitter activities by tight interaction with the membrane-localized transmitter protein, (c) sensation by specialized cytoplasmic sensory domains that may include one or more copies of the PAS domain and/or sensory cofactors, (d) sensation by cytoplasmic proteins that interact with cytoplasmic domains of the transmitter protein. A much more detailed discussion of signal perception has been provided (37). Here, I will briefly discuss status and prospects for engineering each type of sensation.

The first class of mechanisms (Fig 1A), namely, the direct sensation by a periplasmic domain of the transmitter protein, with transmembrane signaling, is a family of mechanisms by different types of integral membrane proteins. One large family of transmitter proteins contains a HAMP domain immediately inside the membrane, and a similar domain occurs in chemotaxis receptor proteins. This suggested a common mechanism of transmission of signals through the membrane by this family of proteins. M. Inouye and colleagues showed that a fusion between the Tar chemoreceptor protein and the EnvZ transmitter protein resulted in a hybrid protein that was able to sense a normal stimulatory effector of Tar (aspartate), and use this information to regulate a gene under the control of EnvZ (37). This remarkable result demonstrated a generality in the functional mechanism of transmembrane signaling by proteins containing the HAMP domain. Similarly, a chimera of the NarX and CpxA transmitters could use the stimulating signal for the Nar system (nitrate) to control CpxA activity (40). The NarX sensory domain has also been used to form chimeric proteins with chemotaxis receptors such as Tar, DifA, and FrzCD (41-43), resulting in cellular chemotactic responses to nitrate and nitrite. Finally, a light-sensing phytochrome could be used to graft light-sensing capability onto EnvZ (4), enabling development of a bacterial camera and edge-detection program (4, 14). These examples demonstrate that a family of related signaling proteins can be used to form functional chimeras. Not unexpectedly, the sensory capacity of the Tar chemoreceptor could not be grafted onto an unrelated transmitter (44). Prospects for future engineering of the HAMP-containing proteins seem favorable, and

a variety of possible graftable sensory capabilities remain to be examined. Furthermore, structure of sensory domains are beginning to appear (eg. 45-48), which should aid their engineering to new specificities.

Additional sensory capabilities could potentially be obtained by further manipulation of the Tar-EnvZ chimera and other already-existing chimeras. For example, rationally designed or selected mutations that allow sensation of additional small molecules may be sought. Chemotaxis receptor chimeras containing the sensory domain of the receptors also sense the presence of periplasmic binding proteins bound to regulatory effectors (49); altered versions of the binding proteins able to sense additional small molecules may provide expansion of sensory capabilities. An appealing aspect of the latter system is that the binding protein functions as a sensory module that can be expressed separately to change the sensory capabilities of networks.

A second class of mechanisms (Fig 1b) employs sensation by an integral membrane or periplasmic protein, that controls transmitter activities allosterically. The paradigms for this class are VirA, which responds to the periplasmic ChvE sugar-binding protein, and the UhpBA system responsible for the uptake of hexose phosphates, where the sensory function is provided by the UhpC protein (50). As noted above for the periplasmic sugar-binding proteins, an appealing feature of such systems is the modularity of sensation; it ought to be possible to develop altered versions of the sensory protein responsive to different molecules. Alternatively, it may be possible to identify altered versions of the sensory protein that “constitutively” activate or inhibit the transmitter protein, without regard to the presence or absence of the stimulatory effector. If such a protein were available, genetic regulation of its expression under the control of different stimuli would control the two-component system output.

The third class of mechanisms (Fig 1c) employs sensation of environmental conditions by specialized cytoplasmic sensory domains, often consisting of one or more PAS domains. In some cases, these domains contain regulatory cofactors, such as heme or flavin. A large number of two-component systems appear to use this sensory mechanism, but so far detailed biochemical information is limited. In the case of the FixL protein of *S. meliloti*, binding of oxygen by a heme cofactor allows a PAS-containing sensory domain to regulate transmitter activities (32). Notably, many of the proteins utilizing PAS-mediated sensory mechanisms are transmembrane proteins, but lack significant periplasmic domains. Apparently, in these proteins a membrane location is required to allow the PAS-containing sensory domain to function. Although little is known concerning the biochemical details of sensation and signaling by the PAS-containing sensory domains, the simple observation of conservation of structure suggests that grafting of sensory capabilities may be possible. This was shown to be true by Moffat and colleagues, who formed light-regulated transmitters by fusing the light-sensitive, flavin-containing PAS domain (known as a LOV domain) of YtvA to the DHP and CA domains of FixL (51). This is highly significant for two reasons. Light-sensitive transmitter proteins are likely to be very useful for development of efficient interfaces for bacteria and machine, and between different bacteria. Even more importantly, PAS domains are found in numerous proteins not involved in two-component signal transduction, raising the obvious possibility of grafting sensory functions from those proteins onto members of the two-component systems. However, much remains to be learned; other efforts to graft PAS-mediated sensation have failed for no obvious reason (52).

The fourth class of sensory mechanisms (Fig 1d) employs sensation by sensory components that are cytoplasmic proteins, that interact with the cytoplasmic portions of the transmitter protein to regulate its activities. As already noted, KinA is controlled in this fashion (36, 53),

FixL is controlled by FixT (33, 34), and CheA forms part of a large sensory apparatus (eg. 38). In the *E. coli* Ntr system, the transmitter protein is a soluble, dimeric, cytoplasmic protein, NRII (also called NtrB). Although NRII contains an N-terminal domain containing a PAS motif, it is not known whether this is involved in sensation. Signals of nitrogen status are communicated to NRII by PII, which binds to the ATP-binding and catalytic (CA) domain of NRII (54). Binding of PII to the NRII CA domain results in the inhibition of NRII autophosphorylation and the activation of the NRII phosphatase activity. Detailed biochemical analysis of altered proteins and heterodimeric forms of NRII containing two different altered proteins (55), as well as structural information for the CA domain of NRII (56), has allowed a detailed hypothesis for the mechanism of regulation of NRII activities by PII (55, 56). PII is one of the most widely-distributed signal transduction proteins in nature, and serves as sensor of α -ketoglutarate and adenylate energy charge (57). Grafting regulation by PII onto distinct transmitter proteins may prove difficult, as the PII-NRII interaction surface may be extensive and distributed broadly across the CA domain. Whether it is possible to use the NRII CA domain in hybrid transmitters along with non-partner dimerization and histidine phosphorylation (DHP) domains to expand regulation by PII to other systems is unknown. Furthermore, extensive studies of PII in a variety of organisms has shown that signaling is complex and not a modular function of any small portion of PII. Finally, PII plays a key role in metabolism; it is not suitable for engineering an isolated circuit.

Returning to the example of the FixT protein of *S. meliloti*, it is thought that this protein behaves as a competitive inhibitor of the FixJ receiver (34). If this proves to be true, it would provide a useful paradigm for tuning the output of a transmitter protein in engineered systems. Competitive inhibitors may be developed from altered versions of the cognate receiver domain that lack output domains and/or the site of phosphorylation. Recent studies have provided an idea as to how the binding affinity of cognate DHP domain and receiver proteins may be regulated, and these studies could guide future approaches to rationally design competitors of desired affinity for the transmitter (27-29).

Acknowledgments

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BOX1

- The author envisions a future where synthetic biological genetic networks are routinely used both as probes of the significance of natural network topologies (58) and as part of sophisticated commercial and medical applications.
- The two-component signal transduction systems, are likely to provide many of the component parts for the assembly of synthetic genetic networks.
- Promising areas for future research include grafting of sensory capabilities and development of synthetic phosphorelay systems, where genetic expression of the phosphorelay components as well as auxiliary factors (phosphatases, inhibitors) might be used for integration of distinct signals.
- Challenges include increasing our understanding of the biochemical details of the mechanisms for regulation of the autophosphorylation and phosphatase activities of the transmitter proteins. Understanding of the mechanisms of regulation will greatly advance our ability to engineer novel regulatory properties into synthetic systems.

Figure 1.

Four different mechanisms of sensation by two-component signal transduction systems. In all cases, the stimulatory effector is depicted as a yellow arrow, and the output (kinase and phosphatase activities) are depicted with an orange arrow emanating from the DHp domain. The membrane is depicted as two horizontal lines in panels A-C. Transmembrane regions of transmitter proteins are depicted in light blue, the DHp domains are depicted with dark blue, the CA domains are depicted with green, and PAS domains, where present, are depicted in cyan. A. Signals impinge upon a periplasmic sensory domain and control the activities of the DHp domain by transmembrane signalling. Numerous examples of this class are available, including EnvZ. B. An auxiliary protein receives the signal and communicates the signal to the transmitter protein. An example of this class is the UhpBA system, where UhpC is the auxiliary sensory protein. C. Sensation by a cytoplasmic sensory domain. In the transmitter depicted, a periplasmic sensory domain is absent. An example of this mechanism is provided by the FixL protein, which senses oxygen by its cytoplasmic, PAS-bound, heme. D. A cytoplasmic protein (grey) senses the stimulus and communicates this to the transmitter by direct interaction with the CA domain. The example depicted is the NRII (NtrB) transmitter protein. Binding of the PII signal transduction protein to the CA domain of NRII regulates the NRII activities.