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Signal transduction in bacteria: phospho-neural network(s) in *Escherichia coli*?

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Abstract

The molecular basis of many forms of signal transfer in living organisms is provided via the transient phosphorylation of regulatory proteins by transfer of phosphoryl groups between these proteins. The dominant form of signal transduction in prokaryotic microorganisms proceeds via so-called two-component regulatory systems. These systems constitute phosphoryl transfer pathways, consisting of two or more components. Most of these pathways are linear, but some converge and some are divergent. The molecular properties of some of the well-characterised representatives of two-component systems comply with the requirements to be put upon the elements of a neural network: they function as logical operators and show the phenomenon of autoamplification. Because there are many phosphoryl transfer pathways in parallel and because there also appears to be cross-talk between these pathways, the total of all two-component regulatory systems in a single prokaryotic cell may show the typical characteristics of a 'phospho-neural network'. This may well lead to signal amplification, associative responses and memory effects, characteristics which are typical for neural networks. One of the main challenges in molecular microbial physiology is to determine the extent of the connectivity of the constituting elements of this presumed 'phospho-neural network', and to outline the extent of intelligence-like behaviour this network can generate. *Escherichia coli* is the organism of choice for this characterization.

Keywords: *Escherichia coli*; Two-component regulation system; Phosphoryl-transfer pathway; Neural network characteristics; Signal transfer; Cross-talk; Auto-amplification

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1. Introduction ..................................................... 310

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1. Introduction

To cope with the ever fluctuating environment, microbial cells have to adjust their cellular make-up to the extracellular conditions. This is particularly true for unicellular organisms, which have a very limited capacity to apply strategies in which the immediate environment around the cell is subject to a homeostatic regime. The molecular basis of many of the specific responses to changing environmental conditions have been described in the literature. Responses to stressful conditions, such as extremes of temperature and nutrient deprivation [1], have received most attention. To adjust smoothly to these stress conditions, many types of cells have developed signal transduction systems that report to the cytoplasm aspects of the changes in the physico-chemical conditions of the external world. In this manner, entrance of the sometimes distressing signal-carrying molecules into the cytoplasm is not necessary to evoke a response (for review see [2]).

Biological signal transduction systems were discovered via the characterization, at the molecular level, of the mechanism of action of hormones in higher organisms. Up until the sixties, it was generally assumed (see e.g. [3] and [4]) that signal transduction across biological membranes would require at least two proteins, each embedded — from opposite sides — into the hydrophobic interior of the membrane. Through protein–protein interactions, the two partner proteins were assumed to mediate signal transfer. Since that time, our views have changed drastically. It was argued on thermodynamic grounds that such a topology for membrane proteins is unlikely to exist [5]. Simultaneously, it was demonstrated that hormone-induced signal transfer across the cytoplasmic membrane in eukaryotic cells is brought about by single intrinsic membrane proteins. These proteins may have as few as only one transmembrane α-helix (e.g. the receptor tyrosine kinases) or many more, like seven in the best known class of the eukaryotic signal-transducing proteins from the cytoplasmic membrane, the so-called β-adrenergic receptor family (or: the 7 α-helical bundle family), of which eye-rhodopsin is the best characterized example. However, it has to be noted that receptor oligomerization in the plane of the membrane is nowadays considered to be an integral part of signal transduction through sensors with a single transmembrane α-helix ([6]; see also below).

Systems with a similar molecular design were subsequently described in unicellular eukaryotic microorganisms. Initially, the properties of these systems were considered to differ fundamentally from those operative in prokaryotic microorganisms, in their tactic responses to light and chemicals (i.e. in phototaxis and chemotaxis; see e.g. discussion in [7]). Again, this view has changed drastically. Signal
transfer in bacterial chemotaxis has turned out to be one special manifestation of a general and abundantly present mechanism of signal transfer in bacteria [2,8], catalysed by so-called 'two-component regulatory systems'. These bacterial systems are composed of pairs of proteins (i.e. sensors and regulators), each with a characteristic domain structure and with a very high degree of homology between corresponding domains. Representatives of these two-component systems play a role in signal transfer in such complex and important, yet ill-understood processes as sporulation, competence development, virulence, response to nitrogen, phosphate or carbon starvation, production of secondary metabolites, cell division, etc., in a large variety of prokaryotic organisms.

Recently, the relevance of two-component regulatory systems also for sensing and signal transfer in eukaryotic organisms was demonstrated explicitly. First, it was reported that proteins, homologous to the two basic components of a two-component regulatory system, do occur in eukaryotes [9–11] and subsequently, the phosphoryl transfer underlying signal transfer through such components was demonstrated to be (part of) the basis of osmosensing in yeast [12].

The generality of the mechanism of signal transfer, as it is mediated through the sensors of two-component regulatory systems, has perhaps been demonstrated most convincingly with the chimeric protein, containing the sensing domain of a prokaryotic aspartate sensor (see below) and the cytosolic portion of the human insulin receptor. This chimeric receptor activated the insulin pathway of eukaryotic cells in response to aspartate [13]. Indeed, signal transfer in the prokaryotic two-component regulatory systems and eukaryotic signal transfer show significant similarity [14].

2. Two-component regulatory systems

Initial results of sequence analyses of regulatory proteins from bacteria revealed a surprising relation-

Figure 1. Schematic representation of a canonical two-component regulatory system. S and R refer to the sensor and regulator component, respectively. The numbers indicate the sequential steps in the (linear) phosphorylation pathway, referred to in the text.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Sensor</th>
<th>Regulator</th>
<th>Signal</th>
<th>Elicited response</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Ntr</td>
<td>NtrB</td>
<td>NtrC</td>
<td>Gln/α KG&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Expression of glutamine synthetase</td>
<td>[29]</td>
</tr>
<tr>
<td>Omp</td>
<td>EnvZ</td>
<td>OmpR</td>
<td>Osmolarity</td>
<td>Expression of outer membrane proteins</td>
<td>[28]</td>
</tr>
<tr>
<td>Pho</td>
<td>PhoR</td>
<td>PhoB</td>
<td>P&lt;sub&gt;b&lt;/sub&gt;</td>
<td>Expression of high-affinity uptake system</td>
<td>[30]</td>
</tr>
<tr>
<td>Che</td>
<td>CheA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>CheY, CheB</td>
<td>Attractants and repellants</td>
<td>Swimming behaviour</td>
<td>[31]</td>
</tr>
</tbody>
</table>

<sup>a</sup> Gln/α KG, ratio of the glutamine and α-ketoglutarate concentrations in the cytoplasm.

<sup>b</sup> An as yet unknown signal is generated by limitation in the availability of inorganic phosphate.

<sup>c</sup> Actually a hetero-trimeric complex between a methyl-accepting chemotaxis protein, CheW and CheA forms the regulated histidine kinase.
ship between proteins that had never been shown, nor thought, to be related beforehand (c.f. refs. [8,15]). A striking example is formed by sets of proteins involved in the regulation of nitrogen metabolism in enterobacteria, in chemotaxis in *Escherichia coli* and in sporulation in *Bacillus subtilis*. These proteins can be classified into two protein families: the sensors and the regulators; each corresponding pair then forms the basic unit of a two-component regulatory system (see also Table 1). More than 70 members of each family have been identified until now (for recent reviews see [2,14,16,17]).

The overall structure of these two components is as follows (see Fig. 1): most sensors are intrinsic membrane proteins with two or more transmembrane α-helices in their N-terminal part. Their C-terminal part forms an independently folded domain that extends into the cytoplasm, and can bind ATP and phosphorylate itself (i.e. it shows autokinase activity). This C-terminal domain is approximately 250 amino acid residues in size and shows significant similarity between all members of the sensor family. In this domain, two signature sequences for nucleotide-binding can be discerned, as well as a conserved histidine residue, that becomes phosphorylated through autokinase activity. This autokinase activity of the sensor is modulated by the presence of the cognate signal (molecule), which is often sensed by its extracytoplasmic N-terminal domain. Sensors thus have histidine kinase activity; most likely, autophosphorylation occurs through phosphoryl transfer between the two halves of a dimer of sensor molecules [18-20].

As a rule, the regulators are cytosolic proteins, composed of two independently folded domains. Their N-terminal domain (approx. 125 amino acid residues) exhibits the mutual similarity among the regulators, while the C-terminal domain only shows significant homology amongst certain subclasses of regulators (for instance, those that function together with a specific minor sigma factor). The N-terminal domain of a regulator can be phosphorylated by phosphoryl transfer from the autokinase domain of the corresponding sensor. However, the actual enzyme activity, required for this phosphoryl transfer and determining its specificity, appears to be located in the regulator itself [21], rather than in the sensor. In turn, this phosphorylation activates (the C-terminal domain of) the regulator, which then may result in either (i) stimulation of transcription of specific genes (for which there are many examples), (ii) regulation of the direction of rotation of flagellae (like CheY ~ P) or (iii) modulation of enzyme activity (like stimulated esterase activity of CheB ~ P).

Little is known about the conformational transitions that underlie these signalling events. Current thinking about the structure of sensors is guided by what is known of the methyl-accepting chemotaxis protein(s) from *E. coli*. The structure of the periplasmic domain of Tar, the methyl-accepting chemotaxis receptor protein, that functions in the detection of aspartate in chemotaxis in *E. coli*, has been determined by X-ray crystallography [22]. On the basis of subsequent molecular modelling, a hypothetical structure of the intra-membrane and periplasmic part of this sensor has been proposed [23]. The conformational transitions in this structure during transmembrane signalling will be discussed below.

Thinking about regulator structure is guided by the information available for CheY. The structure of the unphosphorylated form of this protein was resolved through X-ray crystallography [24]. CheY, which essentially corresponds with only the N-terminal domain of the average regulator, has an α/β-barrel structure with 5 α-helices surrounding a 5-stranded parallel β-sheet. In the other regulators, phosphorylation of this N-terminal domain must activate the independently folded C-terminal domain. Recently, the structure of CheY and CheY ~ P were compared through the use of 1H-NMR spectroscopy. These studies revealed that, upon phosphorylation, nearly half of all the resonances in the spectrum of CheY were shifted to a different position [25], indicating that a major conformational transition is induced by the phosphoryl transfer. This large conformational change was anticipated, based upon the involvement of a phosphorylated aspartate residue in the regulators [2]. The so-called γ-turn loop of the regulator domains may form a hinge for this conformational transition, which moves regulators into the signalling state, as was already predicted by Volz [26], on the basis of sequence comparisons. Recent NMR work has shown that the overall structure of SpoOF, a *B. subtilis* regulator involved in sporulation, is similar to the structure of CheY [27].

Sequence comparisons have been pivotal in the
identification of many of the members of the sensor and regulator families and the elucidation of their role in signal transfer. Simon and colleagues have discussed the limits of this approach [16]. Once identified, straightforward predictions can be made as to the critical amino acid residues for functioning of a particular sensor or regulator, and as targets for site-directed mutagenesis. The biochemistry and physiology of many of these (linear) phosphoryl-transfer pathways has been described in considerable detail, in particular of the Omp [28], Ntr [29], Pho [30] and Che system [31]. Only a single example (i.e. NifL of Azotobacter vinelandii) has been reported until now in which such a predicted phosphorylation site could not be confirmed in subsequent biochemical experiments [32].

For most of the two-component systems, we know very little about the actual signal molecule (like for EnvZ, which senses medium osmolarity somehow; see also Table 1) that is/are perceived by the sensors. Of course, in this respect, too, there are exceptions, like the methyl-accepting chemotaxis proteins, UhpBC (which detects glucose 6-phosphate [33]) and FixL (which detects molecular oxygen [34]).

Variations on the basic theme outlined in Fig. 1 do occur: the sensor may be a soluble cytoplasmic protein (like in the Ntr system [35]), and even when the sensor is an intrinsic membrane protein, the signal sensing domain may be located on the cytoplasmic rather than on the periplasmic side of the membrane [36]. Also, the regulator may essentially be a single domain protein (like CheY and SpoOF; [2,37]) and a sensor and a regulator domain may be combined into a single protein (like in FrzE [38]). Particularly this latter class of sensors has gained considerable importance.

During the last 2 years, it has been demonstrated that components with homology to sensors and regulators also occur in eukaryotic cells, like in yeast [9] and Arabidopsis [10] and maybe even in mammals [16] (see also above). Amongst the two-component sensors from eukaryotes, particularly the type with a combined sensor and a regulator domain, within a single protein, abounds.

The covalent coupling of a sensor and a regulator domain may play an important role in regulating the rate of phosphoryl transfer from a sensor to the corresponding, but separate, regulator. Phosphorylation of the sensor-linked regulator domain may open up the site containing the histidine-linked phosphoryl group for access by the regulator. Also, ArcB from E. coli conforms to this type of multiple-domain protein [39]. For at least one prokaryotic representative of this atypical type of sensor (FrzE), a separate regulator appears to be absent, which implies that for this system, regulation of gene expression by FrzE must take place at the membrane surface (just like for instance for ToxR; see [40]).

In two-component systems like Spo, Pho and Deg, the situation is even more complex. Some of these phosphoryl transfer pathways diverge, because more than one regulator is phosphorylated by a single kinase (e.g. the Spo system in B. subtilis [41], which is involved in the regulation of sporulation and competence development). Others converge, when more than one kinase phosphorylates a single response regulator (like for instance PhoB [42], NarL [43], RegA [44], LuxN from Vibrio [45], etc.). In Myxococcus, the latest variant of a part of a two-component system has been discovered: a protein containing only the transmitter domain of a sensor and, at its N-terminus, a receiver domain of a regulator [46]. This protein may play a role in the proposed phospho-relay system that leads to differentiation of this organism [46].

In many organisms additional phosphoryl transfer pathways play an essential role. The phosphoenolpyruvate-dependent carbohydrate phosphotransferase system (PTS) is a well known example (for a review, see [47]). In this four-step phosphoryl transfer pathway a phosphoryl group from phosphoenolpyruvate is invested in the uptake and concomitant phosphorylation of carbohydrates. Some of the PTS components show a weak similarity with sensors and regulators [15].

3. Monomers versus dimers and the mechanism of transmembrane signalling

An important aspect of the functioning of sensors is the mechanism of signal transfer across the membrane. The dominant secondary structure element of sensors, intrinsic to the cytoplasmic membrane, is the α-helix. Because of the rigidity of this latter structure, it is very unlikely that signals can be
transferred through a single transmembrane $\alpha$-helix. In agreement with this notion, in eukaryotic signal-transducing receptors, which contain only a single transmembrane helix (like the epidermal growth factor receptor), association/dissociation equilibria, laterally in the plane of the membrane, are at the basis of signal transfer through the membrane [6]. In the prokaryotic sensors, monomer-multimer transitions do not appear to be involved in signal transfer [49,50], be it that sensors do appear to function as dimers, as it has been demonstrated for instance for the methyl-accepting chemotaxis proteins (which function together with pairs of CheA, mutually linked through a pair of CheW; see e.g. [51]), as well as for VirA [19]. Surprisingly, a dimeric structure does not appear to be required for transmembrane signal transfer through the sensors [52,53]. As the periplasmic and cytoplasmic domain of individual sensors are often linked by two trans-membrane helices, it is generally assumed that the molecular basis of signal transfer across the membrane must be contained in some form of mutual movement of these helices, with respect to each other (for a recent discussion see [48]). This can for instance be a piston-like, a scissor-like or a rotational movement.

Binding of a signal molecule (e.g. aspartate) to the Tar sensor induces a small rearrangement in its periplasmic domain, as has been revealed through crystallization of the periplasmic domain of Tar, with and without aspartate, by D.E. Koshland's group [23,54,55]. Binding of aspartate to Tar is negatively cooperative, in E. coli even to such an extent that crystals can be analysed with only a single aspartate bound per dimer of the periplasmic domain of Tar [55]. The extent of the transition, initially blurred by binding of a sulfate anion to the aspartate-binding site in an unoccupied periplasmic domain of Tar [23], is very small. In Salmonella Tar, the average displacement of amino acids around the two binding sites in a dimer is about 1–2 Å, which also explains the negative cooperativity. Nevertheless, this transition induces a rotational movement of the two periplasmic domains, relative to each other [48]. Since signals can also be relayed through sensor monomers [53], most likely also the two helices, mutually, of a single sensor within the membrane rotate (see Fig. 2) [48,55]. Based on these results, it is speculated that a rearrangement of the autokinase domain results from this rotational transition, with subsequent modulation of its activity. Cross-linking experiments, too, have made it unlikely that a piston model can explain the activation of the sensors [56] and evidence has been presented (through helix-swop experiments between methyl-accepting chemotaxis proteins [57]) that the transmembrane helices indeed may function as quite non-specific and inert rods.

One should keep in mind, however, that many of the inferences about conformational transitions in transmembrane signalling actually are based only on measurements of (Tar) methylation, instead of on measurements of the rate of phosphoryl transfer to a histidine kinase domain of a true sensor.

An as yet unexplored area of research is the putative modulation of the intensity of signal transfer through sensors by the size of the electrochemical potential gradient of protons across the cytoplasmic membrane. Observations on the functioning of sensory rhodopsin I from Halobacterium salinarium [58] make it likely that indeed such modulation can exist. This photoactive signal receptor protein is an
intrinsic membrane protein, but it does not translocate charge across the membrane. Nevertheless, its turnover is modulated by the electrical potential gradient across the membrane, most likely because the conformational changes during signalling are accompanied by rearrangements of charged or dipolar groups in the electric field across the membrane. Similar rearrangements may underly the transmembrane signal transfer through sensors.

Dimer formation is of utmost importance for the functioning of regulators, too. This is true for transcriptional activators in general, which often are functional in dimeric form (see e.g. [59,60]). Dimer formation of activators is reflected in their target promoter structures. For example, the phospho box, which is the binding-site for PhoB of phosphate limitation inducible promoters, contains a two-fold repeat of a 7-bp element [30]. Recently, it was observed that a mutant form of OmpR, when forced to form dimers through a mutation that introduces a cysteine and which leads to disulfide bridge formation, is simultaneously activated for transcription of ompC [61].

4. Dephosphorylation of response regulators

The pathway of phosphoryl-transfer within a two-component system ends with a phosphorylated response regulator. The in vitro stability of this component, though quite variable amongst the regulators of two-component systems (ranging from seconds to hours), is typical for acyl-phosphates [62]. Also quite variable is the nature of the components that are involved in the release of the phosphate group from regulators. In the first place, response regulators themselves may be involved in the catalysis of phosphate release. However, in many two-component systems, the sensor, too, stimulates phosphate release from the corresponding response regulator (like in Ntr, Omp, Uhp, etc.), particularly when the former is in the unstimulated form [14]. This type of nonlinear regulation allows an exquisite fine-tuning of the intensity of signalling, detected by the sensor, to the degree of phosphorylation of the response regulator. In the Nar system, in which two sensors govern a single regulator, both sensors also have phosphatase activity in addition to kinase activity. Interestingly, the ratio of kinase over phosphatase activity of these two sensors is characteristically different [43]. In a limited number of systems, specifically dedicated proteins function directly or indirectly in the de-activation of the phosphorylated response regulator. Examples are the P-II protein, which stimulates the phosphatase activity of the sensor NtrB, and CheZ in the Che system, both in enterobacteria.

Through these mechanisms, the in vivo stability of phosphorylated response regulators may vary from a few seconds (for CheB and CheY [63,64]) to several hours, i.e. as long as several generation times of the organism (for OmpR and VirG [65,66]; for review see [14]). In combination with the amplification of the signalling machinery, which is observable in some signal transfer pathways (see below), the extremely long lifetime of a number of the phosphorylated response regulators provides the cell with some sort of memory. As a consequence, the response of a cell upon excitation with a particular stimulus will depend on the history of that particular cell for that type of stimulus.

5. Auto-amplification in signal transduction

In some of the signal transfer systems, signalling itself gives rise to an amplification of the amount of the two components of that particular signalling system in the cell, via transcriptional activation. The best characterized example in this respect is NtrB/C [67]. Phosphorylation of NtrC, upon nitrogen deprivation, induces the synthesis, not only of glutamine synthetase, but also of the signal-transduction components themselves, i.e. NtrB and NtrC. Consequently, upon maximal stimulation of signal transfer, the amount of the signal transduction components in the cell may increase up to ten-fold. A similar mechanism of amplification of the amount of signalling components is present in the VirA/G system [68] and in PhoR/B of E. coli [69,70]. As will be discussed below, it is of utmost importance to determine in future work how widespread this phenomenon of autoamplification is amongst the ensemble of two-component systems and to determine for each the dynamic range of this autoamplification.
6. Cross-talk between two-component systems

In view of the large degree of homology among sensors and among regulators, it is not very surprising that phosphoryl-transfer pathways converge and diverge. In in vitro experiments, it was observed that a sensor from one particular system rapidly phosphorylated several different response regulators [71]. This has led to the notion that in vivo there may be significant cross-talk in the signal transfer between various systems that otherwise operate in parallel (for review see [42]; note, however, that the term ‘cross-talk’ is used in the literature also to refer to completely unrelated processes [72]). The notion of the occurrence of cross-talk has recently been strengthened by the observation that some sensors (like ArcB) contain a second domain with a histidine residue that can be phosphorylated [73]. In particular this second histidine residue is active in non-cognate phosphoryl transfer. Stock et al. [74] estimated that on average any sensor-linked histidine kinase can phosphorylate any regulator with a relative rate of at least 1% of the rate of phosphorylation of its cognate sensor. The total flux of phosphoryl groups will of course also strongly be affected by the relative concentration of the components involved.

One may question the extrapolation of the in vitro data on cross-talk to the in vivo situation. In many cases, a putative low background rate of phosphorylation of a given regulator, by an unrelated sensor, may be counteracted by the phosphatase activity of the cognate sensor. For instance, cross-regulation of the Pho regulon by the sensor CreC was only observed in a mutant strain, lacking PhoR [42]. However, it is conceivable that under the appropriate growth conditions such cross-talk does have a physiological meaning. In this respect, it is relevant to note that additional evidence has been obtained that supports this assumption. Several observations (e.g. in the Ntr, Omp and Nar systems [75–77]) indicate that the remaining activation of a particular response regulator, after elimination of the cognate sensor through mutation, is still meaningful for the cell within the physiological context of the regulator’s signalling system (i.e. nitrogen starvation for Ntr and osmotic stress for Omp). It may be more than just coincidence that these examples of cross-talk were reported for systems like Ntr, Nar and Pho, which function in the center of intermediary metabolism. It is therefore particularly relevant to further investigate the cross-talk between systems regulating the response to carbon, nitrogen and phosphorus limitation. Each of these three limitations is known to lead to the induction of not only a set of specific but also of general stress proteins [1].

In addition to this cross-talk sensu strictu, sensor and/or regulator proteins can also be phosphorylated by metabolites from intermediary metabolism (acetyl-phosphate, carbamoyl-phosphate, etc. [78] and possibly γ-glutamyl phosphate [79]). But in the rate of phosphorylation by intermediary metabolites, the different regulators show specificity, too. For instance, in contrast to most regulators, CheB is hardly phosphorylated by acetyl-phosphate [74]. As a third source, regulators might be phosphorylated by proteins involved in other phosphoryl transfer networks in prokaryotes, such as the phosphoenolpyruvate phosphotransferase system [80]. Such a transfer has already been suggested to occur between the PTS and chemotaxis (Che) proteins [81].

7. Neural networks

A network classifies as a neural network when it conforms to a number of criteria [82]. These criteria imply the presence of (i) more than one signal transduction route, (ii) cross-talk between those routes, (iii) logical operators at the cross-talk points (i.e. AND, OR, NOR, etc., ports that give an output that depends on the value of more than a single signal), and (iv) amplification of signal transduction elements in response to signal transduction through those elements. The molecular basis of all these properties differs between the various materializations of neural networks, of which the most accurately described examples are: (i) certain computer programmes, (ii) the immune system of higher eukaryotes and (iii) the human brain.

Neural networks [83] are characterized by their ability to generate a pattern of output signals (a ‘response’) as a consequence (and function) of a pattern of input signals (a ‘signal’), such that the relation between the former and the latter pattern depends on the signal transduction history of the network. The latter dependence is such that a re-
response corresponding to a signal is achieved more readily or extensively when similar signals have been received in the past; i.e. the network is able to 'learn' (note the difference with 'memory'). This learning may even take the form of association: a network can be provided with a training set of signals. After processing of these signals, the network subsequently will associate a similar response to both these initial signals and to incomplete variants of the signals it was trained with. For instance, if A and B are 'proper' responses, corresponding to signals a and b, respectively, one may train a network by always giving the signals a and b in combination. After such training, a neural network will produce both A and B, even if only signal a is offered.

Signal transduction in a single living cell has not yet been identified as an example of a neural network, in part perhaps because it falls far short of the highly sophisticated behaviour of the human brain. Yet, essentially all the fundamental mechanistic requirements which classify a system as a neural network are present in prokaryotic signal transduction: (i) a significant number of transduction routes operate in parallel, (ii) these routes are interconnected through branching and cross-talk, and (iii) some of their elements are present at concentrations that depend on previous signalling, i.e. they show autoamplification. All these properties are present in the phosphoryl transfer through two-component regulatory systems. Future work on the physiological levels of cross-talk and auto-amplification may reveal the extent to which these properties bestow prokaryotic signal transduction with (perhaps primitive) aspects of neural network behaviour, such as memory and learning.

8. Multiple phospho-neural networks?

A single *E. coli* cell may contain as many as 50 different two-component systems [2]. Through a specific regulation of expression, and because of their high biochemical specificity, these systems primarily form linear phosphoryl-transfer routes. Nevertheless, additionally, these pathways mutually interact and they interact with other phosphoryl transfer cascades, such as the PTS (c.f. [47]) and phosphorylated intermediary metabolites. Furthermore, because the sensor/regulator combinations of individual phosphoryl transfer pathways comply with all the prerequisites for neural network elements, the picture emerges of a 'phospho-neural network' in each individual bacterial cell. Upon signalling from the environment, input of phosphoryl groups into this network, primarily via sensors, is followed by transfer to various output systems, involved in the regulation of gene expression, of enzyme activity, of flagellar rotation and/or of cell division (see Fig. 3). This network has neural network characteristics since e.g. the concentration of its components is linked to the intensity of signal transfer through the network. Qualitative (i.e. mechanistically) and quantitative resolution of such a 'phospho-neural network' is of utmost importance for understanding the very essence of the performance of a prokaryotic cell, and it is extremely valuable as a model system to study regulation and signal transfer in more complex systems.

An additional challenge is the determination of the extent of connectivity of the constituting elements of this presumed 'phospho-neural network'. This should reveal whether or not it is appropriate to describe the sum of the phosphoryl transfer pathways...
in an individual prokaryotic cell as a single network, or whether it is more relevant to distinguish more or less 'isolated' subdomains in this network. *E. coli* is the organism of choice for this characterization, because it has been well-characterized, both genetically and physiologically [84], and because a large array of mutants of this organism is available for detailed molecular biological studies.

9. Outlook

A major challenge for molecular microbial physiology in the coming years will be to derive equations that quantitatively characterize the response of an intact phospho-neural network to extracellular stimuli. Signalling via these phosphorylation cascades often leads to changes in the level of gene expression. Such a description is therefore necessarily of a hierarchical nature, which generates additional problems in modelling [85]. Expertise to derive such a hierarchical model is emerging [86]. The quantitative description of this network will strongly benefit from these recent developments in the theory of control analysis of regulatory networks. Of particular interest in the phospho-neural network of a prokaryotic cell is the low copy number of some of the components involved, so that stochastic phenomena may come into play [17]. Most studies performed up to now on the biochemical and genetic aspects of signal transduction in prokaryotes have mainly concentrated on signal transfer within individual two-component systems. The same applies to modelling studies of signal transduction (see e.g. [87,88]). An approach in which the interactions between signalling pathways are characterized may reveal unexpected properties [73].

For a quantitative basis of these modelling studies, it is of utmost importance to develop assays, with proper accuracy and time resolution, for the determination of the in vivo phosphorylation level of a particular sensor or regulator, under physiological conditions. This problem can be tackled via attempts to separate the phosphorylated and non-phosphorylated forms of a sensor and/or regulator on a non-denaturing gel, in combination with antibody detection. Another approach may be to stabilize the linkage between the phosphate group and the regulator via chemical reduction. Independently, the phosphorylation level of a specific two-component system may be 'read out' by a reporter enzyme, under the control of the regulator of the system under study.

Through such studies, in the end, also our understanding of signal transduction in eukaryotic cells may strongly benefit. In those cells, too, the problem of a quantitative understanding of multiple, parallel, overlapping, and interacting signal transduction pathways has to be solved [89,90].

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