THEORETICAL AND EXPERIMENTAL DEVELOPMENTS OF PHOSPHORYLATION ROBUSTNESS STUDY IN BACTERIAL TWO-COMPONENT SYSTEMS

Abstract

Every cell sense environmental fluctuation (input signal) and accordingly generates a response (output). Since the constituents vary from cell to cell, different cells will behave in a different manner to the same stimulus. But in case of stress signals every bacterium activates the stringent responsive pathways uniformly irrespective of the concentration of their constituent proteins. This kind of cellular response is said to be robust if the output is directly proportional to the input signal strength as well as independent of variations in the concentrations of the components that make up the system. The robustness theories in bacteria were founded by Silhavy and Leibler. Using Michaelis-Menten rate kinetic theories (1992-93), Silhavy proposed that bacterial signalling systems can achieve robustness if the sensor kinase (SK) is involved in phosphorylation and dephosphorylation reaction simultaneously. Later in 2003, Batchelor and Goulian came up with a mathematical model and showed that when the response regulator (RR) is much more abundant than the SK, then the phosphorylated response regulator (RR-P) is insensitive to fluctuations of the SK. Innoye (2002) using quantitative Western Blot assay showed that the SK to RR ratio is 1:35, supporting the assumption of Goulian. However, Goulian's model is limited only to Escherichia coli as in other organisms, the amount of SK and RR are comparable with each other. Shinar and Alon in 2007, and then, Shinar and Feinberg in 2010, came up with theoretical propositions that successfully provided robustness initially within the input-output circuit and then in concordant chemical reaction networks. Later on in 2013, Gao and Stock did phosphorylation profiling of the PhoB/PhoR two-component system (TCS)in E. coli, and observed that one of the major

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condition of robustness is that within the saturating range of the phosphatase activity of the SK, the total RR concentration is minimally effected by the level of RR-P. Recently in 2020, using MprAB TCS and promoter region of ppk1 gene as an experimental model, Dutta and Mukhopadhyay have showed that robustness exists in the input-output relations of bacterial signalling systems, both in vitro and in vivo, and have validated two distinctly significant aspects of robustness: (i) the output (RR-P and its downstream gene regulations) is insensitive to fluctuations of the SK, and (ii) the output is dependent on the total RR concentration up to a certain threshold value above which, it becomes insensitive to RR variations. In this book chapter, we summarise the chronological development, both theoretical as well as experimental, highlighting the history of science that led to the current understandings of the input-output robustness theory in bacterial two-component signalling systems.

Keywords: Theoretical Experimental, Phosphorylation., Robustness, Bacterial Component.

I. INTRODUCTION

Cell is an integral part of any living organism. A cell must monitor the environmental conditions and, accordingly, changes its responses for survival. It also must regulate the number of proteins within it as well as the need for activation or repression of the production of the proteins depending on the environmental signals called the input signals. The complex network of proteins and other metabolites in the cell that processes the input signals and generates an output in response to it is called a signalling network of the cell (1). One of the most important functions of this information processing signalling unit is largely carried out by transcription networks. In a well-studied microorganism like Escherichia coli, several network motifs have been characterized which serves as the building blocks of the whole transcription network. One of them is the two-step signalling cascade (2), especially known for executing rapid responses. They are composed of interactions between signalling proteins which functions to sense information from the environment, process this information and, accordingly, regulate the activity of transcription factors or other effectors proteins. One of the best examples of the two-step signalling cascade is the two-component system found extensively in bacterial signalling networks.

Two-component system is a gene regulatory network that allows bacterium to sense changes in the external environment and to generate necessary response for survival. The name, two-component system, refers to two different proteins, one is a sensor kinase (SK) and the other one is a response regulator (RR). A sensor kinase is usually a histidine kinase, which has a transmembrane domain and a cytosolic domain. Any change outside the cell is sensed by the transmembrane domain of the SK and subsequently, causes phosphorylation at the conserved histidine residue in its own cytosolic domain. This process is called autophosphorylation and requires ATP from the cell. The phosphorylated SK (SK-P), then transfers the phosphate group to the cytosolic response regulator (RR)at a conserved Aspartate residue. Phosphorylated response regulator (RR-P) the nacts as a transcription factor, causing downstream gene regulation to generate a response (output) against the environmental stimuli (input signal).

What happens when the environmental stimulus is gone? When the external input dies down, the autophosphorylation activity of the SK stops and a pool of unphosphorylated SK is generated. The unphosphorylated SKs then acts on phosphorylated response regulators to dephosphorylate them and restores the original pool of RRs within the cell. However, some of the two-component systems (TCSs) utilize a third protein to carry out the last dephosphorylation step of the RR. In those TCSs, the SKs are monofunctional and are not involved in the dephosphorylation of the RR-P to RR. But in most cases, the SKs are bifunctional sensors that are antagonistic in nature. Bifunctional means the protein can perform two different function and antagonistic means the same protein will act as a kinase as well as a phosphatase. In presence of ATP, the sensor phosphorylate the regulator, whereas unphosphorylated sensor carries out dephosphorylation of the RR-P(3). Interestingly, ATP is also required in the last dephosphorylation step but as a co-factor. The theory of robustness is only applicable in case of bifunctional antagonistic network motif.

Robustness in a biological system may be defined as the persistence or prolonged existence of a certain traits in a system under perturbations or when there are conditions of uncertainty. The input-output relation in any logical circuit is said to be robust if the output is

directly proportional to the input signal strength and is insensitive to fluctuations of the system components. Robustness can be applied in a two-component system if changes in the environmental conditions (input signal) cause direct effect in phosphorylated protein concentration and subsequent downstream gene regulation (output), and this relation is independent of variations in the components of the cell. Since the protein and metabolite concentrations, such as ATP, vary from cell to cell, different cells may behave in a different manner to the same stimulus. Such heterogeneity in the response of different cells (output) is inevitable. But in case of stress signals every bacterium activates the stringent responsive pathways uniformly irrespective of the concentration of their constituent proteins. It has been observed that the level of phosphorylated protein does not vary from cell to cell in response to a stress signal such that the downstream gene regulation is carried out unequivocally and homogeneously within the population of the bacterium. Thus, the principle of robustness may be applied in case of bacterial two-component systems(2,4).

Existence of paradoxical components led to the emergence of robustness in biological systems. Interacting components in a biological circuit that have antagonistic bifunctional features, that is, having two opposing effects on the same target or biological process simultaneously, exhibits robustness. In case of a two-component system, sensor kinase acts as a kinase as well as a phosphatase. With the onset of stress signals or any other stimulus, histidine kinases get autophosphorylated andshifts the phosphate moiety to the regulator acting as kinaseas well as generating a pool of phosphorylated response regulators. However, during resuscitation, histidine kinases act as a phosphatase and dephosphorylate response regulators for establishing physiological conditions. Now the concentrations of histidine kinases and response regulator proteins may vary from cell to cell including the metabolites like ATP. But it has been observed thatirrespective of the fluctuations in the system constituents, all cells respond in a similar manner to similar stress signals. This property of precise functioning and providing an accurate output to the specific input signal despite the naturally occurring variations in a biological system is called robustness. Two-component systems exhibit robustness only when the sensor kinase protein is antagonistically pleotropic or bifunctional, example: EnvZ-OmpR family proteins in E. coli. Another set of two-component systems exists where the dephosphorylation of the response regulator is carried out by a third protein and not by the cognate histidine kinase. In this case, the histidine kinase acts only as the phosphate donor and does not take part in the phosphatase reaction, that is, the sensor kinase is monofunctional in nature, example: CheX-CheY family of proteins in E.coli, where the third protein is CheZ(1). Robustness is absent in monofunctional two-component systems. Thus, the necessary and sufficient condition for robustness is the existence of bifunctionality in biological systems. A part from two-component systems, robustness principles can be extended to other systems having antagonistically bifunctional enzymes. For example, (a) in the carbon-fixation pathways of C4 plants there is an enzyme (Regulatory Protein) catalysed by pyruvate orthophosphate dikinase (PPDK) that shows robustness (5); (b) in the bacterial TCA cycle, isocitrate dehydrogenase (IDH) enzyme regulates the glyoxylate bypass, which in turn, gets regulated by a bifunctional IDHKP enzyme and exhibits robustness (6-8); (c) cytokines, small diffusible messenger proteins, shows antagonistic pleiotropy causing both cell growth and cell death (9-12).

II. THEORETICAL BACKGROUND OF INPUT-OUTPUT ROBUSTNESS

Over the past thirty years scientists around the globe have tried to establish and extend robustness principles in biological systems. In 1993, Russo and Silhavy observed that bacterial signalling systems maintain a balance between two opposed reactions: phosphorylation and dephosphorylation, in order to achieve downstream gene regulation (13,14). They worked in the E. coliEnvZ-OmpR TCS, where EnvZ is the SK and OmpR is the RR and are actively involved in the ompF and ompC porin genes osmoregulation. By isolating as well as generating various mutant alleles of envZ and ompR, Silhavy showed that by controlling the OmpR-P concentration, the osmoregulation phenomenon is mediated by EnvZ(14). Although the role of EnvZ as a kinase is clear, to generate OmpR-P species, its role as a phosphatase remained blurry as a phosphatase deficient envZ mutant showed constitutive high osmolarity phenotype (15). If the role of phosphatase activity of EnvZ is to restore the original conditions after the external stimulus is withdrawn, then loss of function mutant must get affected only by the signal persistence irrespective of the strength of signal. However, it has been observed that the loss of phosphatase activity is affecting the kinase activity and then, it may be argued that an intrinsic balance is maintained between the kinase and the phosphatase activities in such a manner that the phosphorylated pool of OmpR remains constant. Further, it has been observed that the porin regulation remain unaffected even when there is overproduction of EnvZ and OmpR(16). As the OmpR-P pool is generated by the kinase reactions, the phosphatase activity shifts the equilibrium back to OmpR. At steady state, rate of phosphorylation and dephosphorylation are equal, which means that the OmpR-P species gets accumulated unless the rate of phosphatase becomes equal to that of the kinase reaction. Thus, the level of OmpR-P can be kept constant even if there is variation in the concentration of OmpR.To explain the dependence of OmpR-P on EnvZ concentration, Silhavy proposed rate kinetics model applying Michaelis-Menten equations and showed that the OmpR-P concentration is dependent only on two parameters, K_m (p) and Q_{kp}, and both are independent of the actual EnvZ and OmpR concentrations. However, it has been assumed that (a) the phosphorylation and dephosphorylation reactions must be carried out by the same protein, in this case EnvZ, and (b) the phosphatase reaction gets saturated later than the kinase reaction and the two reactions are independent processes, so that a constant level of OmpR-P is already present. Thus, two component systems can generate output signals that are stable to large number of varying conditions, a hallmark of a well-designed regulatory system.

III. GOULIAN'S THEORETICAL MODEL AND ITS DRAWBACKS

Silhavy proposed two important conditions for the application of robustness in EnvZ/OmpR two-component system. Further experiments related to the structure function characterisation of EnvZ-OmpR system remained consistent with the first condition that a bifunctional enzyme (that can catalyse both phosphorylation and dephosphorylation reaction) is required. But the second condition that the two processes are independent of each other is the major drawback of Silhavy's model as later on it has been shown that the autophosphorylation domain, transmitter domain and the phosphatase domain of EnvZ interacts with each other (17-21). In 2003, Batchelor and Goulian came up with a mathematical model to analyse the cycle of EnvZ mediated phosphorylation and dephosphorylation of OmpR. Goulian's model is based on the assumptions that (a) the SK concentration (EnvZ) is much less than that of the RR (OmpR) and (b) the response regulator

concentration (OmpR) is sufficiently high. Considering these assumptions, the model predicted that the concentration of OmpR-P is independent of fluctuations in the total EnvZ and OmpR concentrations (22). Goulian's mathematical model is more effective than Silhavy's model because it considers total amount of EnvZ to be divided among its different intermediate forms, which includes phosphorylated EnvZ interacting with OmpR, EnvZ interacting with phosphorylated OmpR, unphosphorylated EnvZ and phosphorylated EnvZ taking part only in specific reactions. Thus, under the boundary conditions that total OmpR concentration is very much greater than the total EnvZ concentration and the total OmpR concentration is sufficiently high, Goulian's model have successfully showed that the output is independent of total amount of proteins (SK and RR) present in a cell (22).

Goulian's mathematical model is based on two major approximations that the total SK concentration is very less than the RR total concentration and the total response regulator concentration has to be sufficiently high. Now, the number of EnvZ molecules in a singleE. coli cells has been estimated by Inouye, in 2002, to be 100, where as that of OmpR is found to be nearly 3500 using quantitative Western Blot Assay (23) which, in turn, favoured the first approximation of the theoretical model. But later on it has been found that the model is true only in case of E. coli, cause in other species the sensor kinase and the response regulator concentrations are usually comparable. For example, in case of the two-component system MprAB of M. tuberculosis, the response regulator MprA is only 5 fold higher than the sensor kinase MprB(24). Secondly, no specific limit or threshold value of the response regulator concentration is mentioned in the model except that it has to be sufficiently high. Thirdly, the model does not acknowledge the situation where response regulator concentration becomes comparable or even lower than that of the sensor kinase. Lastly, the model is sensitive to variations in the ATP levels. During phosphate deficiency or nutrient starvation or any other form of stress that can lead to the depletion of ATP or delay its production, will the robustness principles still be valid is one of the major questions that Goulian's model failed to answer.

IV. THEORETICAL FORMALISM FOR STUDYING INPUT-OUTPUT RELATIONS

In 2007, Guy Shinar and Uri Alon introduced a theoretical formalism providing an analytical solution to define the input-output relation using the framework of two-component systems. Considering the influx of the external stimulus (input) and the consequent out flux via the formation of phosphorylated response regulator (output), where the sensor kinase is capable of three different functions: autophosphorylation, transfer of the phosphate and dephosphorylation, Shinar have successfully defined the output as a function of the input signal strength and showed that the phosphorylated response regulator concentration (output) is independent of the total concentration of components of the cell, including sensor kinase, response regulator and ATP, without any boundary conditions imposed upon sensor kinase with respect to response regulator,except the fact that robustness principle can only be applied to bifunctional enzymes.

Regarding the condition that response regulator concentration has to be sufficiently high, Shinar's model specifies that there is a limiting value or a threshold value of the total response regulator concentration above which ATP is consumed at steady state and the input-output relation of the system is robust. If the total response regulator concentration is below the threshold value, then ATP is not consumed at steady state and the output, that is,

phosphorylated response regulator is dependent on the total response regulator concentration. ATP is the source of phosphate donor in most of the two-component systems. During the phosphorylation reaction ATP binds with the sensor kinase and actively takes part in the reaction by carrying out the autophosphorylation of the sensor kinase releasing ADP. However, in the phosphatase reaction ATP acts as a cofactor and binds at a different site of the sensor kinase to carry out the dephosphorylation reaction. Shinar's model also predicted that the output is insensitive to variations in the ATP levels of the system.

V. EXPERIMENTAL VERIFICATION OF ROBUSTNESS IN BACTERIAL TWO-COMPONENT SIGNALLING SYSTEMS

Batchelor and Goulian have not only prescribed the mathematical model but also performed reporter assays to experimentally validate their theory. They have prepared a two-colour fluorescent reporter strain in E. coli and by varying the concentrations of EnvZ and OmpR proteins, monitored the effect of the perturbation on the transcription of ompF and ompC genes corresponding to low and high osmolarity respectively. The experimental results validated the predictions of their theoretical model and have clearly shown that the expression of porin genes ompF and ompC are insensitive to wide range of variations in the concentrations of EnvZ and OmpR. However, despite of capturing basic features of the two-component signalling systems, Goulian's reporter assays suffer from some minor experimental issues. (1) In their assay system, the output is a ratio of two OmpR dependent promoter expressions. The ratio may not vary with the concentration of each component in the same way as the individual promoter unless the dependence of expression of each promoter varies with the OmpR-P concentration in the same way. (2) Over-expression of EnvZ and OmpR by IPTG may lead to the partial formation of inclusion bodies. In the event, the concentration measured as the total concentration could include an insoluble fraction that did not take part in the reaction. These two factors could influence the data on the dependence of two-component system's output on the component concentrations. In addition, there could be an indirect effect on the output at very high concentration of EnvZ, not controlled by the OmpR-P. However, it is needless of saying that Goulian's path breaking concepts have opened newer avenues for sophisticated theoretical modelling and experimental verifications for establishing robustness principles in bacterial signalling systems.

In the year of 2012, Gao and Stock developed a novel method to estimate the level of phosphorylated response regulator in vivo and showed that the phosphatase activity is essential for maintaining the robustness of a two-component system (25). Using a PhoB/PhoR two-component system in E. coli, they demonstrated that the level of phosphorylated response regulator has little effect on the total concentration of the protein if it remains within a saturating range. Further, they observed that an unphosphorylated response regulator population exists throughout the transduction of the input signal which fits in accordance with the Goulian's model identifying a flaw in the Shinar's model, which states that below the threshold value total response regulator population is completely phosphorylated. Now, the input-output relation of any system is said to be robust if, the output is independent of variations in the concentrations of the system's components. In case of any two-component system, sensor kinase and response regulator are the component of the system and phosphorylated response regulator is the output. Previous experiments were successful in demonstrating that the output is insensitive to variations in the concentration of

the sensor kinase. However, regarding the dependency of the output on the response regulator concentration, theoretical model predicts that at steady state level there can be two scenarios. Either when total concentration of response regulator is above a certain threshold value, then phosphorylated response regulator becomes independent of the total concentration of response regulator or when total concentration of response regulator becomes much higher than that of sensor kinase concentration, then phosphorylated response regulator becomes independent of the total concentration of response regulator.

In 2020, Dutta and Mukhopadhyay came up with a four-plasmid synthetic circuit in Escherichia coli using a representative two-component system, MprAB of Mycobacterium tuberculosis, and monitored the in vivo output signal by systematically varying the concentration of either one component or both. Further, using Western Blot analysis they have quantified the amount of each protein and have successfully shown that the output is independent of variations in the sensor kinase concentrations. Moreover, they have also nullified the theoretical prediction that the output is linear unless sensor kinase and response regulator concentrations were comparable and saturated as the response regulator concentration became sufficiently high, rather they have shown that the output is independent of the variations in the response regulator concentrations above a certain threshold value. However, the in vivo reporter assay suffered from one limitation that even at higher inducer concentrations, the amount of sensor kinase remained lower than the response regulator (2 to 5 fold), which is in accordance with the MprB/MprA ratio in M. tuberculosis(24). So they performed in vitro assays and have estimated the phosphorylated MprA pool or MprA dependent transcription yield by varying either of the components of the two-component system and have shown that even when the sensor kinase concentration is higher than that of the response regulator concentration, the output is insensitive to fluctuations in the response regulator concentration above a specific threshold value and have successfully verified phosphorylation robustness for protein concentrations even below the saturation level. Considering the amount of ATP in a cell to be sufficiently high, it has been shown that the output is independent of variations in the sensor kinase concentrations and is independent of the response regulator concentrations above a certain threshold value.

VI. CONCLUSION

Active bio molecules in a chemical reaction network are liable to fluctuations within the cell. These fluctuations move forward in a downstream direction and bring about desirable changes in cellular and sub-cellular levels. However, excessive variations in certain bioactive molecules may bring about drastic changes undesirable for the cell and sometimes could be deleterious for its survival. Cellular mechanisms are designed in such manner that the absolute concentration of these bioactive molecules is maintained within restricted boundaries. Thus, significant nodal proteins, that is, proteins which are employed in the up regulation and down regulation of several chemical reaction networks, need to maintain their functions despite being subjected to environmental or structural variations. Hence, robustness theory, enriched with more sophisticated theoretical models and experimental verifications, must be employed as a design principle attributed to these signalling networks to have a holistic understanding of these biochemical systems.

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