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Quantitative Analysis of Intracellular Response Regulator Phosphatase Activity of Histidine Kinases

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Abstract

Quantitation of two-component protein activities is becoming increasingly important to understand the general design principles for this widely distributed prokaryotic signaling pathway. In many two-component systems (TCSs), phosphatase activity of the sensor histidine kinase (HK) plays a major role in controlling the system output and resetting the system to the prestimulus state. Quantitation of the phosphatase activity is often carried out *in vitro*, usually with truncated proteins that may not recapitulate the intact HK in the cellular environment. This chapter outlines a method for characterizing the intracellular phosphatase activity by investigating the TCS deactivation dynamics upon stimulus withdrawal. Two experimental approaches, the direct Phos-tag gel analysis and the indirect reporter assay, are described for measuring the TCS deactivation dynamics in cell. Combined with a mathematic model, the experimentally determined kinetics can lead to proper evaluation of the intracellular phosphatase activity.

1. Introduction

Two-component systems (TCSs) involving phosphotransfer between histidine protein kinases and response regulator proteins are a prevalent strategy for bacterial signal transduction. One important, although not universal, mechanism for regulating the phosphorylation level of the response regulator, and thus system output, is a response regulator phosphatase activity of the histidine protein kinase (Gao & Stock, 2009; Huynh & Stewart, 2011). Archetypical two-component systems consist of a transmembrane sensor histidine kinase (HK) and a response regulator (RR) that elicits the output response (Fig. 1a). The HK catalyzes autophosphorylation of a specific histidine residue, generating a high-energy phosphoryl group that is poised for subsequent transfer. Phosphotransfer from the histidine residue in the HK to an aspartate residue in the RR occurs within a complex, with both proteins contributing to catalysis. The phosphorylation level of the RR determines system output.

Two potential fates of the phosphoryl group of the RR are common to most all two-component systems. The phosphoaspartate of the RR can be hydrolyzed by an autodephosphorylation activity of the RR or alternatively, the phosphoryl group can be

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transferred back to the histidine residue of the HK and from there to ADP to regenerate ATP in a reversal of the phosphotransfer and autophosphorylation reactions. A third fate exists in the many systems that contain HKs with specific RR phosphatase activity. These HKs hydrolyze the phosphoaspartate of their cognate RRs without transfer to the histidine and regeneration of ATP. In these systems, the phosphatase activity of the HK plays an essential role in determining the level of phosphorylated response regulator and system output. Stimuli control the activities of the HK by stabilizing conformational states that favor either catalysis of autophosphorylation and phosphotransfer or the opposing RR dephosphorylation. The phosphatase activity of an HK is important not only for stimuli-mediated responses, but also for maintaining low basal levels of phosphorylated RR in the unstimulated state, counterbalancing phosphorylation of the RR via the cognate HK as well as cross-talk from non-cognate HKs and/or intracellular small molecule phosphodonors such as acetyl phosphate (Laub & Goulian, 2007; Siryaporn & Goulian, 2008; Wayne, Li, Kazmierczak, Tsui, & Winkler, 2012).

Quantitation of the phosphatase activity of HKs is challenging because of the intrinsic complexity of the integrated enzymatic reactions, the lability of the phosphoaspartate residue of RRs, and the technical difficulties of biochemical analyses of transmembrane proteins. To circumvent the latter, most quantitation of phosphatase activity of transmembrane HKs using *in vitro* biochemical analyses has been conducted with cytoplasmic fragments. Such studies have limitations. While the truncated, soluble constructs retain autophosphorylation, phosphotransfer and RR phosphatase activities, their levels relative to those of the intact proteins are unknown. The importance of HK sensing and transmembrane domains in regulating HK activities raises questions about the signaling state(s) represented by isolated domains. Perhaps due to the absence of the regulatory domains or the natural membrane environment that may be required to maintain the phosphatase signaling state, phosphatase activities measured *in vitro* using cytoplasmic fragments have been found to be substantially less than those estimated for intact HKs in cellular studies (Gao & Stock, 2017). Furthermore, cellular studies have the advantage of allowing comparison of enzymatic activities in the presence and absence of stimuli if environmental conditions that lead to activation are known, even for systems in which specific ligands or physical parameters have not been identified, or in which sensing is mediated by auxiliary proteins.

One strategy for assessing phosphatase activity is the measurement of system output as an indirect report of RR phosphorylation. For the majority of two-component systems that regulate gene expression via RR transcription factors, this can be achieved using reporter gene assays. While this method allows real-time analyses in intact cells, some experimental precautions are necessary. Different operons in a single regulon can show substantially different expression profiles (Gao & Stock, 2015; Zwir, Yeo, Shin, Latifi, Huang, & Groisman, 2014), thus it is important that a reporter gene system be constructed with a promoter for which activity has been shown to correlate with levels of RR phosphorylation. It is also important to recognize that reporter output is delayed by the time required for transcription, translation, and maturation of the reporter protein and because of the long lifetime of the reporter protein, measurements of reporter output are cumulative. Furthermore, as for any cellular assay, measurement of reporter proteins is subject to interference by other cellular components.

A second, more direct strategy is the quantitation of RR phosphorylation in cell extracts using the phosphoryl chelator Phos-tag (Barbieri & Stock, 2008). This method relies on gentle lysis of cells to preserve the labile phosphoaspartate; separation of phosphorylated and unphosphorylated forms of the RR by electrophoresis in SDS Phos-tag acrylamide gels; and subsequent detection of the RR bands by immunoblotting. However, the rates of dephosphorylation of RRs catalyzed by HKs upon stimulus withdrawal are typically too rapid for kinetic characterization by this method. Nonetheless, this method can be used to set upper limits for the half-lives of RR dephosphorylation and validate the dephosphorylation kinetics measured by reporter assays. More accurate estimates of phosphatase activity can be obtained by combining such experimental data from wild-type and phosphatase-diminished mutant HKs with modeling to establish boundaries for the kinetic parameters of the phosphatase activity of a wild-type HK.

This chapter will focus on methods for analysis of the phosphatase activity of HKs in cells using Phos-tag analyses, reporter gene assays and a combined approach of experimental analysis of RR phosphorylation with modeling. These methods were developed to characterize phosphatase activity in the *Escherichia coli* PhoR-PhoB two-component system that regulates expression of genes involved in phosphorus assimilation in response to phosphate limitation. However, the approach is generalizable and should be broadly applicable to characterization of intracellular phosphatase activity in other bacterial two-component systems.

2. Phos-tag Gel Analyses of RR Dephosphorylation

Phosphatase activity of HKs plays a major role in resetting the system to prestimulus state once the stimulus is removed. Characterization of phosphatase activity in cells relies on measurements of RR dephosphorylation kinetics upon stimulus removal. At the heart of such studies on TCS deactivation dynamics is the experimental determination of intracellular RR phosphorylation (RR~P) levels. SDS PAGE gels containing the phosphoryl chelator Phos-tag (Wako Chemicals) covalently linked to acrylamide allows separation of phosphorylated and unphosphorylated forms of RR from cell lysates, and the subsequent direct quantitation of RR phosphorylation in complex mixtures using immunoblots (Fig. 1B and 1C). Detailed procedures of Phos-tag SDS-PAGE have been well documented (Kinoshita, Kinoshita-Kikuta, & Koike, 2009; Kinoshita, Kinoshita-Kikuta, Takiyama, & Koike, 2006) and customized for measuring RR phosphorylation (Barbieri et al., 2008; Boulanger, Chen, Hinton, & Stibitz, 2013; Gao & Stock, 2013; Wayne et al., 2012). Thus, the step-to-step protocol of Phos-tag gel analyses is not described in detail here. Instead, practical experimental considerations will be discussed to ensure reliable measurements of RR phosphorylation levels.

2.1 Experimental considerations

Acyl phosphate on the conserved aspartate residue of an RR is labile under many conditions. Base, strong acid and heat can lead to significant hydrolysis of phosphoaspartate. Preserving the labile acyl phosphate throughout an experiment is key to the success of measuring phosphorylated RR levels in cell lysates. Common lysis protocols involving sample heating

or sonication can cause substantial loss of RR phosphorylation (Barbieri et al., 2008). A mild formic acid treatment has been initially used for lysing cells and preparing RR~P samples (Barbieri et al., 2008; Boulanger et al., 2013) but it requires an additional sample neutralization step that can increase salt concentration and raise the possibility of electrophoretic distortion in Phos-tag gels.

Detergent-based methods using reagents specialized for bacterial lysis (Gao et al., 2013; Richards, Lawrence, Su, Yang, Yang, Dulebohn et al., 2015), such as BugBuster (EMD Millipore) or B-Per (ThermoFisher), provide a gentle and rapid protocol for preparing cell lysate samples. Nuclease or DNase can be added in the detergent-containing lysis buffer to reduce sample viscosity for optimal Phos-tag gel results. Trituration (pipetting up and down) for 15–30 s is usually sufficient for lysing 0.3–0.5 OD-mL ($\text{OD}_{600} \times \text{volume in ml}$) of *E. coli* cells. The entire lysis step including bacterial cell harvesting can be accomplished in 1–2 min, which sets a limit of sampling time intervals for kinetic studies. Alternative methods using lysing beads have also proved successful (Park & Groisman, 2014; Wayne et al., 2012). All these methods may need further optimization for individual TCSs in different bacteria species and the central principle for optimization is to minimize the exposure of the RR~P to various hydrolyzing conditions.

Phosphorylated RRs may inevitably lose some of their phosphoryl groups due to the intrinsic lability of acyl phosphate, particularly during electrophoresis when heat is generated. RR~P standards with different fractions of phosphorylated RR are often run along with lysate samples on the same gel (Fig. 1B) to calibrate for the loss of phosphorylation. Because the chemical stability of phosphoaspartate should be similar in all denatured RRs, it is not essential that the RR~P used for standards be the same RR being experimentally analyzed, however the use of different RRs complicates detection on a single immunoblot. To generate RR~P standards, purified RR proteins are first phosphorylated, ideally to completion, or at least to a known extent, by excess small molecule phosphodonors, such as phosphoramidate or acetyl phosphate, and subsequently mixed with known amounts of unphosphorylated RR. The resulting standards are often added to lysates of the bacterial cells whose RR gene is deleted to maintain gel-running conditions identical to those of experimental samples. Quantification of these standards on immunoblots are calibrated with the known RR~P fractions to generate a standard curve that ensures accurate quantitation of RR phosphorylation levels.

2.2 Limitations

Direct measurement of RR~P levels by Phos-tag gel analyses relies on harvesting of sufficient bacterial cells and generation of cell lysates, both of which take time and could be laborious if a large number of samples needs to be collected. As discussed above, sample preparation takes at least a couple of minutes even with a rapid lysis protocol. However, some HKs have strong phosphatase activity and dephosphorylation of RR~P occurs extremely quickly (Gao et al., 2017). For example, upon stimulus removal, PhoB~P is completely dephosphorylated within two minutes, even before completion of processing of the first sample (Fig. 1B). Rapid RR dephosphorylation was still observed in phosphatase-diminished mutants (Fig. 1C). Such rapid dephosphorylation leaves little time to collect

enough samples for kinetic analyses but dephosphorylation half-lives can be estimated for these proteins, WT (1 min), Mut1 (1 min) and Mut2 (2.5–4 min). Moreover, the Phos-tag gel method is inherently low throughput. The number of sample wells in each Phos-tag gel limits the total number of samples that can be analyzed simultaneously. The necessity of including RR~P standards further reduces the sample numbers for kinetic characterization.

3. Transcription Reporter Assays

Transcription reporters have been routinely used to infer RR phosphorylation levels in cells. A wide variety of genes encoding different reporter proteins, such as beta-galactosidase, luciferase and fluorescent proteins (FPs), can be fused with RR-regulated promoters to report the system output. Green fluorescent protein (GFP) and its color variants represent one of the most versatile reporters that allow high-throughput, real-time, continuous and even single-cell measurement of gene expression. Thus, we will use FP reporter as an example to describe necessary considerations for characterization of TCS deactivation dynamics. Upon stimulus removal, temporal dynamics of fluorescence output can be tracked with any fluorescence detector, such as a fluorometer or microplate reader, to report the kinetics of RR dephosphorylation. As an indirect reporter of RR~P, FP reporters have some limitations associated with the complexity of transcription regulation as well as physicochemical properties of FPs. Some of these issues may be specific to FP reporters but common principles are shared with other reporter systems for establishing proper controls and improving the experimental design.

3.1 Time derivatives of reporter dynamics

TCS activity or RR phosphorylation status is frequently indicated by overall fluorescence output. However, the overall fluorescence value at a particular time is the result of all fluorescent proteins expressed prior to that time point and determined by the cumulative output of promoter activity. In contrast, the first derivative of fluorescence with respect to time, i.e. the rate of fluorescence change, reflects the real-time promoter activity. The time derivative of fluorescence is also influenced by the FP decay rate. Fluorescent proteins are usually very stable and the half-life of GFP is longer than one day in *E. coli* (Andersen, Sternberg, Poulsen, Bjorn, Givskov, & Molin, 1998). At the timescale of activation or deactivation of the exemplified PhoR-PhoB system (1–2 h), FP decay is negligible, thus the time derivative of fluorescence is directly used to represent promoter activity and track RR~P dynamics (Fig. 2A and 2B). For TCSs functioning at a longer time scale (days) or in different organisms, the FP degradation rate may require re-evaluation and need to be considered when calculating promoter activity from fluorescence derivatives.

Reporter dynamics constitute discrete data measured at different time points. The first derivative needs to be computed. Considering that the data may contain unequal time intervals, the following equation can be used to calculate the fluorescence derivative by differentiating the 2nd order Lagrange interpolating polynomial,

$$f'(t_i) = \frac{t_i - t_{i+1}}{(t_{i-1} - t_i)(t_{i-1} - t_{i+1})} f(t_{i-1}) + \frac{2t_i - t_{i-1} - t_{i+1}}{(t_i - t_{i-1})(t_i - t_{i+1})} f(t_i) + \frac{t_i - t_{i-1}}{(t_{i+1} - t_i)(t_{i+1} - t_i)} f(t_{i+1}).$$

$f(t_i)$ represents fluorescence of the entire culture at the t^{th} time point. The first derivatives are normalized to the culture optical density (OD) for calculation of the promoter activity, $(d\text{Fluo.}/dt)/\text{OD}$. The numerical differentiation process will amplify any noise in the data. When fluorescence readings are particularly noisy, it may be necessary to smooth the time series data by adjacent-averaging of the fluorescence reading with measurements at the preceding and subsequent time points, or by a more sophisticated smoothing algorithm. To boost the signal-to-noise ratio, the FP reporter assay is best performed in multi-well microplates with a large number of replicate samples. Mean fluorescence derivatives from these samples illustrate the temporal profiles of promoter activity, which can be used to evaluate different reporters and define RR~P kinetics.

3.2 Evaluation of the reporter promoter

Not every RR-regulated promoter is a suitable reporter for RR phosphorylation kinetics. Transcription of specific promoters may be subject to additional regulation by other transcription factors, small RNAs or global physiological effects. Temporal profiles of promoter activity can be compared with RR~P dynamics to evaluate whether a specific reporter can recapitulate intracellular RR phosphorylation kinetics. This is best demonstrated by the example shown in Fig. 2A and 2B. Both *phoA* and *phoB* promoters are activated by the PhoR-PhoB system in response to Pi-depleted conditions. Fluorescence of the two YFP reporters increased monotonically after stimulation (Fig. 2A), but with different temporal patterns in fluorescence derivatives (Fig. 2B). Promoter activity of the *phoB-yfp* reporter peaked at ~20 min and gradually decreased afterward. The *phoA-yfp* reporter reached a steady level of promoter activity after the initial increase, recapitulating the RR phosphorylation kinetics measured by Phos-tag gels. The *phoA* promoter is clearly more suitable to report RR~P levels than the *phoB* promoter. In addition, the steady phase of promoter activity also marks the suitable timing for removing stimulus and initiation of TCS deactivation. A steady promoter activity of *phoA-yfp* ensures minimal interference from additional inhibitory mechanisms, such as that apparent in the *phoB* reporter, which would complicate assessment of the decrease in reporter activity expected after stimulus removal.

3.3 Correction of reporter fluorescence

Cellular fluorescence signals can be affected by factors not associated with FPs, such as the autofluorescence emitted by other cellular metabolites, which require appropriate corrections before further differentiation analyses. Various methods have been used to correct for bacterial autofluorescence (de Jong, Ranquet, Ropers, Pinel, & Geiselmann, 2010; Zaslaver, Bren, Ronen, Itzkovitz, Kikoin, Shavit et al., 2006). Briefly, cells without the corresponding reporter are assayed as the control. Autofluorescence of the control cultures is either directly subtracted or growth-calibrated and then subtracted from fluorescence of bacteria carrying the reporter.

One often-overlooked factor in FP reporter analyses is the absorbance or scattering of the excitation and/or emission light by bacterial suspensions. When the reporter assay involves significant growth of bacterial cultures, the increased bacterial turbidity will reduce the intensity of excitation and/or emission light by light-scattering, leading to reduced fluorescence values. This light-scattering effect may be corrected with a calibration curve

that establishes the relation between fluorescence and culture turbidity (Fig. 2C). Identical amounts of either pure FP or fluorescent cells can be mixed with different amounts of non-fluorescent cells to generate an empirical correlation between measured OD and fluorescence. The empirical curve is then used to calibrate the fluorescence reading of the sample of interest.

Fig. 2D and 2E show how the scattering correction affects interpretation of fluorescence reporter results measured for deactivation of the PhoR-PhoB system. Once the stimulus was removed and Pi became replete, cells resumed fast growth and raw fluorescence of the entire culture decreased gradually along with the increase in OD. If no correction were made, it would lead to a negative fluorescence derivative and suggest the loss or degradation of FPs. This hypothetical “loss” will potentially interfere with interpretation of the deactivation kinetics. When the scattering effect is corrected with the calibration curve shown in Fig. 2C, fluorescence of the entire culture appeared constant during the majority of the time course, suggesting a rapid shut-off of the promoter activity and a constant total level of the stable FP. FP per cell, or the OD-normalized fluorescence, decreased only due to the growth dilution. The deactivation dynamics were monitored with high temporal resolution in Fig. 2E. Upon stimulus removal, corrected fluorescence continues to rise for ~10 min and the first derivative of fluorescence rapidly decreased with a half-life of ~4 min. The corrected fluorescence allows precise evaluation of the fluorescence derivative. The light-scattering effect is not unique to fluorescent reporters. Any reporter involving optical measurements of light signals, such as luciferase, may also require similar calibration controls to evaluate the influence of bacterial turbidity on reporter output.

3.4 Measuring the maturation time of fluorescent protein

The time needed for the maturation of FP, i.e. formation of the fluorophor, may be of concern for analyzing reporter kinetics. The time required for FP maturation can be ignored only when it is much more rapid than the examined signaling event, otherwise, it needs to be accounted for. Unfortunately, there is not a standard value for FP maturation to assess its impact on the observed fluorescence dynamics. FP maturation time depends on the specific FP variant, the culture conditions, the organism and even the strain in which FP is expressed (Balleza, Kim, & Cluzel, 2018; Heibisch, Knebel, Landsberg, Frey, & Leisner, 2013). It is necessary to experimentally measure the FP maturation time for the particular reporter used.

FP maturation time in bacteria cells can be determined by measuring the fluorescence increase after addition of a translation inhibitor (Fig. 3A). The FP reporter is activated until the fluorescence derivative reaches a steady state. Protein synthesis is then inhibited by translation inhibitors, such as the antibiotics chloramphenicol (Cm) or erythromycin (Ery). To ensure complete inhibition, the inhibitor concentration is usually much higher than the minimal inhibitory concentration documented for these antibiotics. It is assumed that the inhibition occurs instantaneously and the subsequent increase in fluorescence results from maturation of FPs that are expressed prior to translation inhibition. The increase in fluorescence can be fitted with a first-order kinetic model to derive the maturation half-time. The maturation half-life of cyan fluorescent protein (CFP) is calculated to be 8.0 ± 0.9 min for the data shown in Fig. 3A, very close to the time scale of PhoR-PhoB deactivation. When

reporter dynamics of FP maturation and TCS deactivation are measured in parallel (Fig. 3B), the almost identical temporal profiles of the two suggest that deactivation of the PhoR-PhoB system is extremely fast and the relatively slow FP maturation kinetics dominate the promoter activity dynamics. Effects of FP maturation on reporter dynamics must be considered for understanding the deactivation kinetics and characterization of the PhoR phosphatase activity.

4. Modeling the RR Dephosphorylation Kinetics

As discussed above, RR dephosphorylation kinetics can be either directly measured by Phos-tag analyses or indirectly inferred from reporter dynamics. Both methods have limitations that prevent direct quantitation of the phosphatase activity using traditional enzyme kinetic analyses. Due to the sample preparation time, the number of kinetic samples that can be collected for Phos-tag analyses is often limited, especially for HKs with fast dephosphorylation kinetics. Unlike *in vitro* biochemical assays in which the enzyme concentration can be lowered to slow down the reaction for sufficient sampling, measurement of RR~P dynamics in cells is usually restricted by inherent expression levels of HK and RR. Thus, complete kinetic characterization is not always achievable with limited numbers of data points but the Phos-tag method can provide an estimation of half-lives of RR dephosphorylation. On the other hand, the indirect FP reporter is influenced by transcription and FP maturation rates, thus the measured promoter activity dynamics cannot be directly used for enzyme kinetics. Understanding the reporter dynamics requires a kinetic model that accounts for these non-phosphorylation reactions.

4.1 Kinetic model of reporter dynamics

Phosphatase activity is believed to be the main effector for RR dephosphorylation upon stimulus withdrawal. Therefore, other activities, such as autodephosphorylation of RR~P, reverse phosphotransfer from RR~P to HK and phosphorylation of RR by HK or non-specific sources are not considered in order to minimize the number of parameters that must be estimated. Three reactions are modeled to derive the reporter dynamics (Fig. 4A). Dephosphorylation by phosphatase is modeled with a reversible binding equilibrium between RR~P and HK with the on rate k_1 and the dissociation constant K_d , followed by the subsequent dephosphorylation with the catalytic rate constant k_p . Reaction rates are as follows:

$$-d[RR\sim P]/dt = k_1[RR\sim P][HK] - k_1K_d[HK \cdot RR\sim P] \quad (1)$$

$$-d[HK \cdot RR\sim P]/dt = k_p[HK \cdot RR\sim P] + k_1K_d[HK \cdot RR\sim P] \quad (2)$$

The rate change due to growth dilution is not considered for the PhoR-PhoB system because the growth rate is low with a doubling time near 100 min, much greater than the time scale of dephosphorylation and FP maturation. For TCSs with slow dephosphorylation kinetics or organisms with high growth rates, growth dilution may not be neglected. A Hill function

with the DNA binding affinity K_{DNA} and the maximal expression rate V_m is used to describe RR-activated expression of the nascent FP that has yet to mature to a fluorescent form. Maturation of FP to the fluorescent form FP* follows first-order kinetics with the rate constant k_{mat} while the scaling factor α denotes the relation between the measured fluorescence values and FP* concentrations.

$$d[FP]/dt = V_m[RR\sim P]^n/([RR\sim P]^n + K_{DNA}^n) - k_{mat}[FP] \quad (3)$$

$$d[FP^*]/dt = k_{mat}[FP] \quad (4)$$

$$d[fluor.]/dt = \alpha * d[FP^*]/dt \quad (5)$$

The ultimate goal of modeling reporter dynamics is to quantitate the phosphatase constant k_p and K_d . Other kinetic parameters and initial concentrations of each species need to be properly estimated to fit the observed fluorescence dynamics. Total concentrations of HK and RR can be measured with quantitative western blots using purified proteins as standards (Gao et al., 2013). The initial concentration of RR~P before stimulus withdrawal can be calculated from the total RR level and the RR~P fraction determined by Phos-tag analyses. A high value close to the diffusion rate limit can be initially assigned to k_I because the binding equilibrium is usually fast and the fast rate k_I has little effect on dephosphorylation. For reporter transcription and maturation, the initial concentration of the immature form of FP protein is not readily available, however a relative value can be derived based on the steady-state value prior to TCS deactivation. As described in section 3.2, stimulus is preferably removed during a steady phase of fluorescence derivative, suggesting a steady value for immature FP and a value of zero for $d[FP]/dt$. Based on equations (3–5), the following equations can be derived to assign the initial values:

$$[FP]_0 = \frac{d[fluor.]/dt|_0}{\alpha \cdot k_{mat}}$$

$$V_m = \frac{d[fluor.]/dt|_0}{\alpha} \cdot \frac{[RR\sim P]_0^n + K_{DNA}^n}{[RR\sim P]_0^n}$$

in which $d[fluor.]/dt|_0$ indicates the initial value of the fluorescence derivative. The maturation constant k_{mat} can be measured as described in section 3.4 while the DNA-binding affinity K_{DNA} and the Hill coefficient n can be approximated by values determined *in vitro*.

With all the initial conditions and corresponding parameters defined, the model can be simulated with the Simbiology tool of Matlab and the phosphatase parameters can be estimated to fit the observed fluorescence dynamics. However, different pairs of K_d and k_p values can yield very similar time courses of fluorescence derivatives (Fig. 4B). This is likely due to the much higher value of RR~P concentration than the DNA binding constant K_{DNA} for the modeled PhoB protein, which saturates the binding site, maximizes the transcription rate and masks any early-stage kinetic difference in RR~P concentration caused by different K_d and k_p values. Accurate estimation of parameters may not be possible with a single set of kinetic data. Global fitting with multiple data sets at different TCS expression levels or RR~P levels is likely to allow more robust fitting and better estimation of the phosphatase activity.

Alternatively, the dephosphorylation kinetics can be approximated by an exponential decay with a single parameter, the half-life $t_{1/2}$. The fluorescence reporter dynamics can be fit to determine the value of $t_{1/2}$. As shown in Fig. 4C, PhoB~P dephosphorylation half-lives derived from FP reporter assays compare well with the half-lives measured by Phos-tag analyses. When combined with *in vitro* characterization of the phosphatase activity, half-lives of a few phosphatase mutants can be used to define the boundaries for the kinetic parameters of the phosphatase activity in cell.

4.2 Quantitative estimation of the phosphatase activity

In order to use half-lives to estimate the phosphatase activity, half-lives of RR dephosphorylation need to be calculated for different values of K_d and k_p . The same phosphatase model and same initial concentrations as those discussed in section 4.1 can be used to derive the half-lives of RR dephosphorylation (Fig. 4D). For HKs with strong phosphatase activity, it is typical that only an upper limit of half-life can be experimentally estimated because Phos-tag analyses cannot capture the fast dephosphorylation kinetics and FP reporter dynamics are dominated by FP maturation kinetics. For the PhoR-PhoB system, both the WT and a phosphatase-diminished mutant Mut1, which exhibits lower phosphatase activity than WT, showed fast dephosphorylation kinetics with an estimated upper limit of half-life at ~1 min. A large parameter space (shaded area in Fig. 4D) can give half-lives shorter than 1 min.

The relative difference between kinetic parameters of a WT protein and a phosphatase mutant can be used to establish boundaries for the WT parameter space. Mut1 has been shown to have a K_d value eight-fold higher than that of WT by *in vitro* kinetic analyses (Gao et al., 2013). Even though half-lives measured *in vitro* differ significantly from those determined in cells, the same eight-fold difference is assumed for intracellular K_d values between Mut1 and WT. Mut1 has an upper limit of half-life of ~1 min. Lowering the K_d by eight fold establishes a limit for the WT phosphatase parameters (dotted area in Fig. 4D and 4E). Characterization of a second mutant can further refine the quantitative estimation. Mut2 has a k_p value 4.5-fold lower than that of WT. The intracellular half-life between 2 and 4 min sets the boundaries for WT parameters given a 4.5-fold difference in k_p (Fig. 4E, dashed area shaded in gray). The cross-section of the two areas defined by the two mutants (Fig. 4E, dotted area in dark gray) represents the possible parameter values for WT, suggesting a

strong phosphatase activity with a much larger k_p and a smaller K_d than those measured *in vitro*. Caution should be exercised in such analyses because a single mutation may have complex effects on multiple aspects of TCS activities. The assumption that *in vitro* differences in parameter values can be readily translated into intracellular parameter differences may not be true for every protein.

5. Summary

Quantitation of HK activities with *in vitro* methods is often limited by the reconstituted system or the biochemical environment, neither of which mirror the native system under cellular conditions. Analyzing TCS deactivation kinetics in cells using a combined approach provides a quantitative assessment of intracellular phosphatase activity of HKs. For the exemplified PhoR-PhoB system, this approach reveals a strong intracellular phosphatase activity that is necessary to suppress non-specific phosphorylation in the absence of stimulus (Gao et al., 2017). Underestimation of the phosphatase activity by *in vitro* analyses may be attributed to the lack of regulation from the sensing and transmembrane domains that are truncated. Due to the wide usage of truncated proteins for *in vitro* characterization of HKs, such underestimation may be common in TCS studies. We hope the approach outlined in this chapter can be extended to other systems for evaluation of the phosphatase activity in the cellular environment. Because TCSs exhibit great diversity in regulatory details, such as the signaling time scale, cellular expression levels and cell growth rates, various assumptions and considerations discussed above need to be thoroughly examined to customize for an individual system.

Abbreviations

CFP	cyan fluorescent protein
Cm	chloramphenicol
Ery	erythromycin
Fluo.	fluorescence
FP	fluorescent protein
GFP	green fluorescent protein
HK	histidine kinase
OD	optical density
PAGE	polyacrylamide gel electrophoresis
RR	response regulator
RR~P	phosphorylated response regulator
SDS	sodium dodecyl sulfate
TCS	two-component system

WT wild type

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Fig. 1.

Measurement of intracellular RR dephosphorylation kinetics. (A) Multiple enzyme activities of two-component proteins. The RR~P level is determined by the autokinase, phosphotransferase and phosphatase activities of the HK. Promoters of RR-regulated genes can be fused with a fluorescent protein (FP) gene to report RR phosphorylation levels. (B and C) RR dephosphorylation kinetics analyzed by measuring RR~P levels with Phos-tag gels. After removing the stimulus by adding 1 mM Pi, PhoB dephosphorylation kinetics were measured in wild type (WT) shown in (B), and phosphatase-diminished mutants PhoB^{F20D} (Mut1) and PhoR^{T217M} (Mut2) shown in (C). Approximately 0.3 OD-ml of cells were harvested at indicated times and cell pellets were lysed by trituration in 55 μ l 1 $\times$ BugBuster reagent. After addition of 18 μ l 4 $\times$ SDS loading buffer for denaturation, all samples were immediately flash frozen and later analyzed using Phos-tag gels (10% acrylamide, 25 μ M Phos-tag acrylamide) and western blots. To prepare RR~P standards, pure PhoB protein (5 μ M) was phosphorylated in the presence of 50 mM phosphoramidate for 1.5 h in 50 mM Tris, pH 7.4, 100 mM NaCl, 5 mM MgCl₂, followed by denaturation in SDS sample loading buffer and mixing with known amounts of unphosphorylated PhoB.

Fig. 2.

Data analyses of reporter fluorescence dynamics. (A and B) Evaluation of reporters with time derivatives of fluorescence. Activation dynamics of the *phoA-yfp* and *phoB-yfp* reporters were measured in a fluorescence microplate reader with constant shaking. Eleven replicates for each reporter were assayed simultaneously. Upon stimulation by Pi-depleted conditions, cellular fluorescence (A) increased with distinct dynamics for two reporters. Solid and open circles represent data for the *phoA* and *phoB* YFP reporters. Normalized first derivatives of fluorescence (B), (dFluo./dt)/OD, reflect the real-time promoter activities. Dynamics of *phoB* promoter activity suggest an additional transcription inhibition

mechanism and are not consistent with RR phosphorylation kinetics measured with Phos-tag gels (open triangles). (C) Calibration of fluorescence scattering by bacterial turbidity. Pure yellow fluorescent protein (YFP) solution was diluted to give similar fluorescence readings as the activated *phoA-yfp* reporter. Dark triangles represent fluorescence of identical amounts of pure YFP mixed with different amounts (OD) of non-fluorescent bacteria. An empirically-fitted linear correlation (solid line) was used to correct for the fluorescence of tested samples (gray dots). (D and E) Correction of the fluorescence scattering. WT cells carrying the *phoA-yfp* reporter were stimulated in a Pi-depleted condition for ~90 min. For the deactivation dynamics shown in (D), stimulus was removed by resuspension of bacteria in Pi replete media. Raw fluorescence (triangles) decreased along with the increase in OD (solid line). After the scattering correction, corrected fluorescence (filled circles) appears constant while the OD-normalized fluorescence (open circles) decreases due to growth-dilution. High temporal resolution upon stimulus removal is achieved by direct addition of Pi to stimulated cultures (E). Time zero is defined as the time of Pi addition. OD-normalized fluorescence derivatives (grey circles) depict the deactivation dynamics.

Fig. 3. Measurement of FP maturation time. (A) CFP maturation kinetics. The *phoA-cfp* reporter was activated by Pi-starvation and protein synthesis was inhibited by 250 $\mu\text{g/ml}$ of erythromycin, as indicated by the arrow. Fluorescence and fluorescence derivatives are shown in diamonds and circles, respectively. Solid lines indicate the exponentially fitted line for fluorescence and the corresponding derivatives. (B) Comparison of the deactivation dynamics (solid circles) with CFP maturation kinetics (open circles).

Fig. 4.

Modeling the RR dephosphorylation kinetics. (A) Schematic diagram of reactions used for modeling. (B) Simulation of reporter dynamics with different values of K_d and k_p . Derivatives of total CFP fluorescence (open squares) are calculated from deactivation dynamics of the *phoA-cfp* reporter in the phosphatase-diminished mutant Mut2 (PhoR^{T217M}). Error bars indicate standard deviation from 11 individual wells. Dashed and solid lines represent the simulated curves with indicated values of K_d and k_p . Values for the rest of the parameters and initial species concentrations are as following: $[\text{RR}^{\text{P}}]_0$, 6.3 μM ; $[\text{HK}]$, 0.8 μM ; k_{mat} , 0.00144 s^{-1} ; K_{DNA} , 0.6 μM ; k_1 , 0.5 $\mu\text{M}^{-1}\text{s}^{-1}$; V_m , 0.0019 $\mu\text{M/s}$; α , 1; $[\text{FP}]_0$, 1.28 μM . The first four parameters are experimentally determined as described in section 4.1 while k_1 is assigned a high value for a fast binding equilibrium. The value of α does not affect the fluorescence derivative because both the initial value $[\text{FP}]_0$ and the changing rate can be converted to values relative to $1/\alpha$ and it cancels out the scaling factor α when $d[\text{fluo.}]/dt$ is computed with equation 5. The value α is arbitrarily set at 1 and the corresponding V_m and $[\text{FP}]_0$ are calculated as described in section 4.1. (C) Simulation of reporter dynamics with first-order kinetics of dephosphorylation. Exponential decay, instead of the typical Michaelis-menten kinetics, is used to approximate the RR dephosphorylation kinetics. Dynamics of fluorescence derivatives (symbols) were fitted (lines) to determine the dephosphorylation half-lives. (D and E) Contour plots of dephosphorylation half-lives derived from different values of K_d and k_p . Symbols indicate parameter values derived from

in vitro characterization of truncated HK proteins. Shaded areas represent parameter spaces for individual mutants based on their dephosphorylation half-lives in cells. Dotted and dashed areas are derived from the relative difference of K_d and k_p between the WT and mutants. Cross-section of the two (dotted areas in dark gray) highlights the boundaries for WT phosphatase activity.