FEBS Letters 583 (2009) 4013–4018

FEBS Letters

journal homepage: www.FEBSLetters.org

Review

Timing matters

Edda Klipp *

Theoretical Biophysics, Humboldt-Universität Berlin, Invalidenstr. 42, 10115 Berlin, Germany

ARTICLE INFO

Article history:
Received 1 September 2009
Revised 10 November 2009
Accepted 18 November 2009
Available online 24 November 2009

Edited by Måns Ehrenberg

Keywords:
Biochemical network
Time measure
Optimality

ABSTRACT

Cells are entities in space and time. Systems biology strives to understand their composition, structural organization as well as dynamic behavior under different conditions. Here, measures for dynamic properties such as characteristic times, time hierarchy and time-dependent response are reviewed. Using a number of examples from yeast and micro-organism systems biology, the importance of considering the timing in experimental and theoretical research is discussed.

1. Introduction

Systems biology is based on at least two different scientific strategies: on one hand collecting comprehensive information about various aspects of cell biology, e.g. cataloging or counting all proteins [1], all genes [2], all protein–protein interactions (e.g. [3,4]), all metabolic reactions [5], the expression of all genes under certain circumstances, and so on, and, on the other hand, combining experimental research with mathematical modeling. Model development and simulation becomes more and more important. The reason is that mechanisms and phenomena in biology are often very complex and that model formulation can help to bring different observations into one concept and to phrase stringent hypothesis. When observations cannot be understood by pure intuition, for example the occurrence of oscillations or the effect of negative or positive feedback loops, comparison with phenomena from physics and engineering may help to enlighten the underlying mechanism. Especially, if the observed phenomenon strongly depends on the specific parameter values, explanations profit a lot from sound and carefully parameterized mathematical models.

Cells are individuals in space and time. To decode their organizational principles, many general properties and details have to be measured. While levels of different compounds or changes thereof can be detected with increasing precision, it is often hard to quantify the impact of different processes on timing. In order to fully understand not only the composition of cells, but also regulation, development, adaptation, or reproduction it is necessary while not sufficient to accumulate static data. Instead, we will more and more observe the changes either inherent during development or induced by external stimuli. At least for small or simple systems we can even today use those observations to quantify the impact of regulatory processes on timing as will be demonstrated for a few selected examples below.

Time scales of biological processes vary. Molecular state transitions occur within femtoseconds or nanoseconds, protein–DNA binding in the order of microseconds, transcription and translation steps are in the order of milliseconds to seconds, cell cycle of *Escherichia coli* takes about 20 min, cell cycle of yeast about 2 h, circadian oscillations last about a day, evolutionary processes can take a few hundred generations (in case of microorganisms) or million to billion years considering life on earth.

It is common in biological model formulation (both verbally and with equations) to neglect or simplify processes that are much slower or faster than the processes under consideration. When, for example, studying transcription we usually neglect the details of molecular dynamics, since they are assumed to be fast enough. We also typically disregard cell growth and development, since we have chosen a time window small compared to the time necessary for the cell to considerably change in time. Metabolic research and modeling, at least traditionally, considered enzyme concentrations as constant despite changing metabolite levels (e.g. [6]). Global gene expression studies such as the analysis of expression changes of virtually all yeast genes during a metabolic shift from fermentation to respiration [7] have revealed a temporal program of gene expression and, thereby, most likely enzyme availability.

Neglecting temporal aspects in biology can lead to a number of problems that, in the best case, can be referred to as producing many false positive data. For example, reconstruction of biochemical networks is frequently based on detection of interaction

* Fax: +49 30 2093 8813.
E-mail address: edda.klipp@rz.hu-berlin.de

0014-5793/$36.00
doi:10.1016/j.febslet.2009.11.065

between proteins. The experimental procedures, such as yeast two-hybrid analysis, require artificial conditions. Thus, it remains an open question whether the potential interaction partners are expressed in vivo at the same time, such that they have the chance to meet at all.

2. Characteristic times

An important feature of cell processes is the wide range of time scales in which changes may occur. Some modifications may happen within milliseconds, other processes take minutes, hours or even longer. Even on the level of enzymatic reactions we may find large differences in the time they need to respond to changes. In the frame of ordinary differential equation (ODE) systems, the time regime for the metabolic reactions is characterized by the kinetic constants. It has been a long-standing challenge in theoretical biology to correctly describe the timing of cellular or biochemical events. A major obstacle is the fact that many changes do not occur abruptly, but smoothly. So, what is the time to consider relevant? Below are reviewed different quantitative measures for the temporal characterization of dynamic processes in biochemical networks.

A time constant τ for a decay process, such as $S \xrightarrow{k}$ is given by $\tau = 1/k$, denoting the time when concentration S has reached the $1/e$-fold of its original value (Fig. 1).

A time constant τ for the isolated reversible first order reaction

$$S_1 \underset{K_-}{\overset{k_+}{\rightleftarrows}} S_2 \tag{1}$$

is given by

$$\frac{1}{\tau} = k_+ + k_-, \tag{2}$$

determining τ as the *relaxation time* for the decrease of perturbation x of the original equilibrium of S_1 and S_2 from its initial value to the $1/e$-fold value. This relaxation time refers to concentration changes. In general, one can distinguish between time constants for reaction rates and time constants for the change of concentrations.

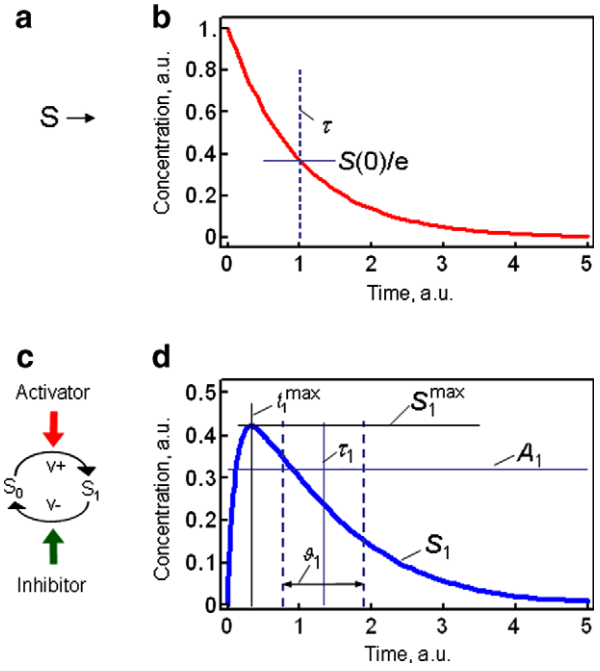

Fig. 1. Characteristic times. For different processes, temporal measures have been defined. (a) Decay of substance S, (b) temporal behavior of S with its characteristic time t denoting the time when S reached $1/e$-fold of its original value, (c) signaling pathway motif with activator (A), inhibitor (I) and two states of S (S_0 and S_1), (d) dynamics of S_1 with different temporal measures indicated (for notions see text). Used equations: $dA/dt = A,\ A(0) = 1,\ dI/dt = 0,\ I(0) = 1,\ dS_1/dt = -dS_0/dt = k_+S_0 - k_-S_1,\ S_0(0) = 1,\ S_1(0) = 0$. Parameters: $k_+ = k_- = 5$.

While these definitions are suited for isolated reactions, it is also interesting to consider what happens if a system of biochemical reactions is perturbed. If the system is described with a set of ordinary differential equations, $dS_i/dt = f_i(S_1, \ldots, S_n)$ with $i = 1, \ldots, r$, then close to steady state its dynamics can be approximated by the Jacobian $J = \{\partial f_i/\partial S_j\}$, which has the eigenvalues λ_i. If, moreover, the system is stable ($Re(\lambda_i) < 0$), then a sensible measure for the time necessary to respond to perturbations are the characteristic times

$$\tau_i = \frac{1}{|Re(\lambda_i)|}. \tag{3}$$

A general definition of a time constant for reactions was given by Higgins [8]. The *response time* is defined as

$$\tau_j = \left(\sum_i n_{ij} \frac{\partial v_j}{\partial S_i} \right)^{-1}, \tag{4}$$

where v_j is the rate of reaction j, S_i is the concentration of the ith compound and n_{ij} is the stoichiometric coefficient of compound i in reaction j. This definition can be applied to reactions with more than one substrate or product and even with non-linear rate expressions. For example the response time for the reaction

$$S_1 + S_2 \rightleftarrows S_3 + S_4 \tag{5}$$

is given by

$$\tau = (k_+(S_1 + S_2) + k_-(S_3 + S_4))^{-1}, \tag{6}$$

when we consider mass action kinetics. Time constants for metabolite concentrations have been defined in different ways. The so-called *turnover time* was introduced by Reich and Sel'kov [9], describing the time necessary to convert a metabolite pool once:

$$\tau_i^{turn} = \frac{S_i}{\sum_{j=1}^{r} (n_{ij}^- v_j^+ + n_{ij}^+ v_j^-)}, \tag{7}$$

v_j^+ and v_j^- are the forward and backward part of every reaction rate with $v_j = v_j^+ - v_j^-$. Accordingly, n_{ij}^+, n_{ij}^- are the stoichiometric coefficients of substance S_i in the individual reaction directions.

Easterby [10,11] has defined a *transition time*. Considering that an empty pathway, i.e. a pathway defined by a set of enzymes but without metabolites available, is supplied with the initial substrate, the transition time describes the time necessary to build up the intermediate pools. Steady state is reached only asymptotically and the transition time is a handy temporal measure. For each intermediate holds

$$\tau_i = \frac{S_i^{SS}}{J}, \tag{8}$$

where S_i^{SS} and J denote intermediate concentration and flux in the final steady state. The transition time of the complete pathway is the sum of the transition time of all intermediates,

$$\tau = \sum_{i=1}^{n} \tau_i. \tag{9}$$

Another measure for the time necessary to return to a steady state after a small perturbation is the transition time introduced by Heinrich and Rapoport [12]. If $\delta(t) = S(t) - \bar{S}$ denotes the deviation from the steady state concentrations, then the transition time is defined as

$$\tau = \frac{\int_0^\infty t \cdot \delta(t) dt}{\int_0^\infty \delta(t) dt}. \tag{10}$$

This definition is applicable, if $\delta(t)$ vanishes asymptotically for large t. A generalization of this measure was introduced by Llorens et al. [13]. Be f a function such as flux or a concentration subject to perturbation. The characteristic time can be calculated in analogy to center of mass as

$$T = \frac{\int_0^\infty t \cdot |\frac{df}{dt}| dt}{\int_0^\infty |\frac{df}{dt}| dt}. \quad (11)$$

This definition may be applied even for oscillating response to the perturbation.

Specific quantitative measures have been introduced for signaling pathways [14]. Let $S_i(t)$ be the time-dependent concentration of compound i, e.g. an active kinase. The quantity

$$I_i = \int_0^\infty S_i(t) dt \quad (12)$$

is the time integrated concentration of S_i, which can be considered as a combined measure for the amount and life time of active kinase i generated during the signaling period, i.e. of the compound able to transmit the signal further down the cascade. The signaling time

$$\tau_i = T_i / I_i \quad \text{with } T_i = \int_0^\infty t \cdot S_i(t) dt, \quad (13)$$

describes the average time to activate the kinase i, similar to the transition time introduced above. The signal duration defined as

$$\vartheta_i = \sqrt{Q_i / I_i - \tau_i^2} \quad \text{with } Q_i = \int_0^\infty t^2 \cdot S_i(t) dt, \quad (14)$$

gives the average time during which the kinase i remains activated, which also corresponds to a standard deviation of a statistical distribution. The signal amplitude $A_i = I_i/(2\vartheta_i)$ is a measure for the average concentration of activated kinase i. Note that the amplitude expressed this way might be different from the maximal value $S_i^{\max}$ that $S_i(t)$ assumes during the time course (Fig. 1).

Discussing measures for the timing of events, leads to another notion, the time hierarchy in cellular systems [15,16]. The following measure for time hierarchy was introduced

$$H_\tau = \frac{r}{r-1} \frac{\sum_{i=1}^r (\tau_s - \tau_i)}{\sum_{i=1}^r \tau_i}, \quad (15)$$

where τ_s is the maximum characteristic time among all τ_i. H_τ becomes maximum when one reaction is slow and all other reactions are as fast as possible.

The measures introduced above provide information about how fast or slow a system responds to perturbations. In the frame of metabolic control analysis [17–19], a type of sensitivity analysis which is also applicable to other regulatory networks, it has been shown that fast sub-systems hardly contribute to the control of the full system, since they adapt immediately to changes, while slow sub-systems severely determine the impact of a perturbation on the system's behavior [20].

Time-dependent response coefficients are a specific type of sensitivity coefficients, which have been introduced by Ingalls and Sauro [21] and defined as

$$R_{p_k}^{S_i(t)} = \frac{p_k}{S_i(t)} \frac{\partial S_i(t)}{\partial p_k}, \quad (16)$$

where $S_i(t)$ are time-dependent concentrations and p_k are parameter values. The calculation requires information about the network stoichiometry, about the functional dependence of the individual reaction rates on substrate concentrations and parameters, and about parameter values. Given this information, they express the sensitivity of concentrations or rates to parameter values over time. They are global quantities, meaning that not only the sensitivity to parameters directly affecting a substrate or reaction are considered, but the sensitivity with respect to all, even very remote, parameters in system are determined. As for all quantitative measures that employ derivatives, all conclusions hold only strictly for infinitesimally small perturbations. Still, one gets inside into the impact of certain parameters on certain concentrations at different periods of time. An example for the yeast osmostress response is discussed below.

Example 1. Different time scales of metabolic changes and gene expression effects in metabolic regulation.

Why are we interested in the timing of a process? Does it matter whether transcription and translation are slower or faster than enzyme modifications or changes in metabolite concentrations due to changing substrate levels? What is the impact of such different regulation mechanisms on the temporal behavior of a metabolic network?

To tackle such questions, Bruggeman and collegues have introduced the time-dependent hierarchical regulation coefficients. For defining hierarchical regulation coefficients, the property of most enzymatic rate laws is used that they can be split into two factors: one factor is dependent on the enzyme concentration, the other factor on substrate concentrations and parameter values:

$$v_i = v_i(E_i, S_1, \ldots, S_n) = f_i(E_i) \cdot g(S_1, \ldots, S_n). \quad (17)$$

Taking the difference of the logarithmic values leads after a few steps to the following expression:

$$1 = \frac{\Delta \ln f_i}{\Delta \ln v_i} + \frac{\Delta \ln g_i}{\Delta \ln v_i} = \rho_h + \rho_m, \quad (18)$$

where ρ_h and ρ_m are denoted as hierarchical and metabolic regulation, respectively. The time-dependent version

$$1 = \frac{\partial \ln v_i}{\partial \ln E_i} \frac{d \ln E_i/dt}{d \ln v_i/dt} + \sum_{j=1}^n \frac{\partial \ln v_i}{\partial \ln S_j} \frac{d \ln S_j/dt}{d \ln v_i/dt} = \pi_h(t) + \pi_m(t), \quad (19)$$

then quantifies the relative contribution of enzyme concentration changes – through gene expression or protein degradation – and direct metabolic changes. This concept has been applied to regulation of yeast metabolism under different conditions [22,23].

Example 2. Regulation of glycerol accumulation in osmostress response – fast and long-term contributions.

With yeast osmoresponse we denote all processes contributing to the adaptation of the budding yeast *Saccharomyces cerevisiae* to osmotic stress, usually induced in experimental settings by adding an osmotically active substance such as NaCl, KCl, or sorbitol to the medium. The identified regulatory network comprises the high osmolarity glycerol (HOG) signaling pathway with membrane-bound receptors, interacting proteins, and a highly conserved stress-activated (or mitogen-activated) protein kinase (SAPK or MAPK) cascade as well as a number of genes regulated depending on the activity of the most down-stream kinase (Hog1), the production and accumulation of glycerol in metabolic pathways and, finally, the membrane-bound glycerol channel, Fps1. It is important to note that among the osmostress-regulated genes are several which code for enzymes in glycolysis and the glycerol production pathway. For a long time, research on osmoresponse focused on the HOG pathway and the gene expression changes induced by osmostress via Hog1 phosphorylation and nuclear accumulation. Only careful quantitative studies combined with mathematical modeling [24] have revealed that glycerol accumulation – a short-term response which is mainly characterized by metabolic adaptations including the closure of the glycerol channel Fps1, which prevents export of produced glycerol, and a long-term adaptation process which requires the production of new enzymes for metabolic pathways.

The effect of different parameters on the temporal profile of glycerol accumulation can be visualized using the time-dependent control coefficients (Eq. (16)) as shown in Fig. 2. A slightly simpli-

fied model based on the model presented in [24] has been used to calculate the coefficients. This model is formulated as set of ordinary differential equations. It considers the effect of the external osmotic pressure changes on the receptor Sln1 and the glycerol transporter Fps1. This effect is mediated via turgor changes, where turgor is the pressure that cell membrane or cell wall subtends cell swelling due to differences in internal and external osmotic pressure. Dephosphorylation of Sln1 induces the signaling cascade including transitory dephosphorylation of Ssk1 and phosphorylation of Hog1. Active Hog1 enters the nucleus to activate transcription, among others of genes coding for metabolic enzymes. The enzymes catalyze reactions leading to glycerol production. Without stress, glycerol may leak out of the cell through the Fps1 channel, which closes upon stress. Hyper-osmotic stress induces water outflow and volume shrinkage. Glycerol is osmotically active and its accumulation balances the osmotic pressure differences, thus leading to cell re-swelling.

Fig. 2b shows the time course for glycerol and selected time-dependent response coefficients with respect to glycerol. It can be seen that the parameter for Fps1 closure has the highest positive response, at least one order higher than mRNA synthesis or protein synthesis (for enzymes in the glycerol production pathway), but only for the first about 20 min. Hog1 nuclear accumulation and Hog1 regulated transcription and translation become important only at later stages. Some parameters have also negative response coefficients, such as Hog1 nuclear export or mRNA degradation, indicating that their increase would slow down glycerol accumulation in the respective periods.

The general finding that yeast osmoresponse operates on at least two times scales has been further supported and elaborated. Mettetal et al. [25] stimulated yeast with different frequencies of osmotic stress. Using a simpler model in the frame of linear systems-theory, they found a fast-acting negative feedback through the Hog1 kinase and a slower negative feedback through gene expression. An interpretation of the slow mode is that cells can prepare to respond faster to future stimuli.

Quantitative single cell measurements[26] revealed that osmotic stress induces cellular processes at different time scales: loss of turgor pressure (and volume) within 20 s, Hog1 nuclear transition between 20 s and 5 min, turgor (and volume) recovery between 5 and 25 min and resuming of cell growth after 25 min.

Example 3. Optimality of regulation – cellular response to changing nutrients.

Theoretical biology has often formulated the question whether biological structure or function can be explained with optimization principles. Are enzymes designed to allow for a maximal reaction rate? Are steady state fluxes in metabolic networks maximized or minimized [27,28]? Are regulatory systems designed to be fragile, to be robust, to use the least resources, or to respond as quickly as possible to perturbations?

Assuming that cells have experienced various pressures during evolution, we can learn from applying optimization principles to models of biological processes, whether the actual design comes close to an optimized design.

An example case is the response of cells to nutrient changes. For micro-organisms, levels of nutrients may vary and, in some cases, desired but non-essential nutrients might become available suddenly.

Bacterial amino acid production pathways are interesting example cases for such a scenario: Zaslaver et al. [29] studied experimentally the biosynthesis of several amino acids in *E. coli* and identified the expression pattern in time. The promoter activities of about 100 genes were monitored in parallel using appropriate reporter libraries. The time course of pathway activation was analyzed by diluting cells in a defined medium in which all amino acids except one (e.g. arginine, methionine, or serine) were present. The different promoters were found to become activated successively with delays of the order of 10 min. This temporal order is in accordance with the enzyme sequence in the various unbranched reaction chains of amino acid biosynthesis. Thus, a hierarchy of expression was observed that matched the enzyme order predicted earlier for unbranched pathways [29]. The phenomenon was termed "just-in time expression" because the enzymatic genes investigated were expressed just in time when needed.

Using mathematical modeling and optimization, the experimentally observed behavior had been predicted previously through

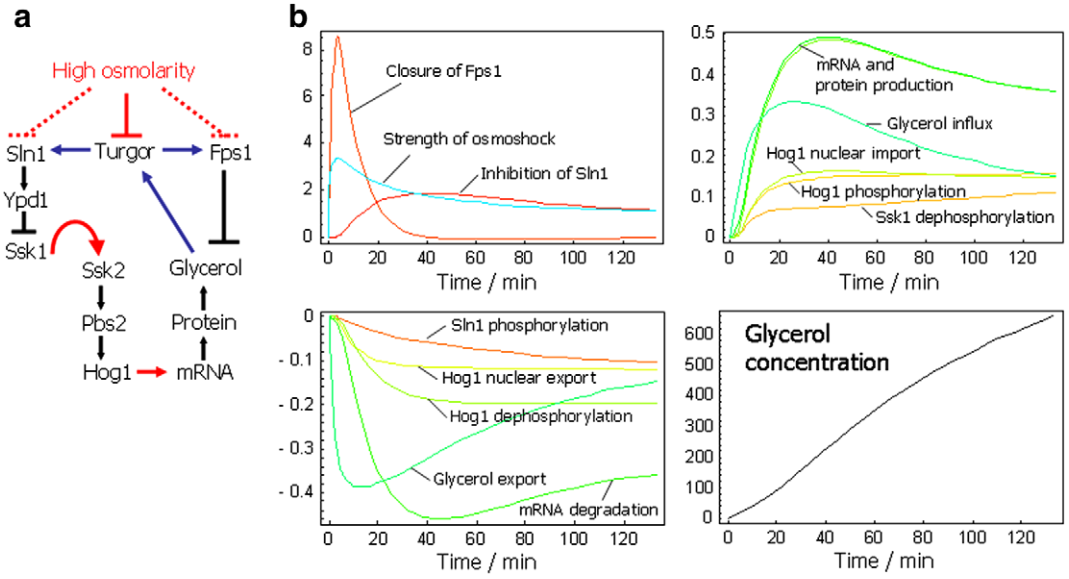

Fig. 2. Time-dependent response in yeast osmoresponse. Using an established model describing yeast response to osmotic stress including volume and turgor dynamics, signaling, gene expression and metabolism, the time-dependent response coefficients for glycerol with respect to various parameters have been calculated. (a) Simplified schema of the network relevant for yeast osmoresponse. (b) Time courses of glycerol accumulation and time-dependent response coefficients. Strikingly, the response coefficient for Fps1 closure is by far the largest, but only for the first 20 min. Processes relevant for gene expression regulation show later and smaller rise of response coefficients.

the study of the dynamics of the enzyme levels in a metabolic pathway after immediate supply of substrate S_0, specifically asking for the timing of presence of the enzymes [30]. The model reveals interesting results under a set of assumptions: (i) when no substrate is available, the pathway is at rest, i.e. no enzymes are synthesized for sake of economy, (ii) when S_0 becomes available, it will be converted to an end product S_n with maximal rate. This is motivated by considering that S_n be important, but not essential for the cell's survival and reproduction and that the faster S_n is formed from S_0, the faster the cell can reproduce itself and out-compete other organisms. The simple set of system equations describing the pathway dynamics is

$$\frac{dS_0}{dt} = -k_1 \cdot E_1 \cdot S_0, \tag{20a}$$

$$\frac{dS_i}{dt} = k_i \cdot E_i \cdot S_{i-1} - k_{i+1} \cdot E_{i+1} \cdot S_i \quad (i = 1, \ldots, n-1), \tag{20b}$$

$$\frac{dS_n}{dt} = k_n \cdot E_n \cdot S_{n-1}. \tag{20c}$$

The modeling concerned the question of how the cell can convert S_0 into S_n as fast as possible after S_0 has become available to the cell. To this end, conditions for the enzymes must be specified: it was assumed (i) that the cell can produce the enzymes immediately when needed, thus neglecting the time span necessary for transcription and translation, and (ii) that the total amount of enzyme protein is bounded due to limited capacity of producing and storing proteins in a cell. Otherwise, one could achieve a mathematical solution with infinite rates by infinite enzyme concentrations. The time necessary to convert S_0 into S_n was quantified by the transition time

$$\tau = \frac{1}{S_0(0)} \int_{t=0}^{\infty} (S_0(0) - S_n(t))dt. \tag{21}$$

The optimization problem under study can be written as

$$\text{Minimize } \tau \text{ subject to } E_{tot} = \sum_{i=1}^{n} E_i(t) = const. \tag{22}$$

where the time-dependent enzyme levels, $E_i(t)$, are to be determined The optimal enzyme profiles can be derived by variational calculus or Pontryagin's maximum principle (Oyarzún et al., 2007). The optimal time course of enzymes is shown in Fig. 3 indicating that, within successive time periods and except from the last period, only a single enzyme is operative whereas all others in the pathway are shut off. At the beginning, the whole amount of available protein is allocated exclusively to the first enzyme in the pathway, since the intermediates S_1, S_2,... are still not available for the later enzymes. In the following phases, the total amount of protein is shifted to the enzyme catalyzing the following reaction. The last switch allocates a certain fraction of protein to all enzymes according to a series that is monotonic increasing from the first enzyme to the last. This scenario guarantees that each enzyme is active when its respective substrate is present.

3. Discussion

While the composition of cellular networks, their stoichiometry, interaction motifs are progressively deciphered, quantification of the dynamic properties of cellular processes becomes increasingly important. Here, a number of examples have been discussed, where the temporal organization of cell processes has been studied.

Why do we care? Are components and structure not sufficient? – The example osmostress shows, that pure network reconstruction according to classical molecular biology would have overlooked the different time scales of immediate adaptation and gene expression of metabolic enzymes. When considering the interplay of signaling pathways with cell cycle progression it occurs that a signal has different effects if hitting the cell at different stages, therefore with different concentrations of relevant players. Moreover, the coordination of different regulatory and developmental processes in time is extremely challenging.

Careful study of the timing of processes requires careful quantification. While many basic principle of regulation can be studied with simplified models and with methods from classical engineering, analysis of time-dependent processes is only possible with detailed determination of kinetics.

Further in-depth analysis requires precise time-resolved quantitative data. It has been discussed frequently that measurements with cell populations – although in many cases hard to avoid or replace – hide many dynamic aspects since they average out: for example, if cells respond to a stimulus in a step-wise fashion, but some earlier, some later, then data will often show a gradual change for the whole population. One way to deal with this type of problem is to synchronize cells. The drawback of synchronization is an artificial interference with the system before the actual experiment.

New approaches as quantitative time-lapsed fluorescence microscopy in single cells (for a recent review see [31]) can provide data that allow careful studies of the timing of individual events and thereby allow dissecting the contribution of different processes to cell cycle, cellular development, or stress response.

The development of mathematical models describing the dynamics of cell processes accurately can crucially improve the understanding of regulation in time and highlight interrelations that are not immediately obvious from experimental data. The further development of theoretical concepts to extract and quantify dynamic properties from data is therefore a future challenge. This way of research will also go together with the development of

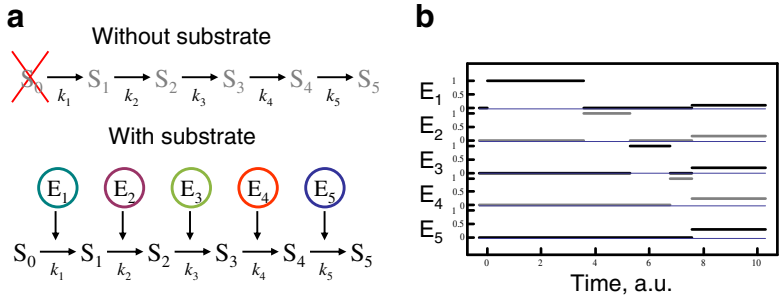

Fig. 3. Optimal temporal enzyme distribution for a linear metabolic pathway with sudden substrate supply. (a) The experimental scenario: when no substrate is present, the enzymes are not produced for sake of resources. When the first substrate becomes available, enzymes are expressed to convert it into the final product. (b) Optimal temporal profile of enzyme concentrations: each enzyme is present at a limited period to convert its substrate. Only in the final phase all enzymes are at hand to convert the remaining metabolite amounts.

experimental scenarios that allow tracing temporal changes of cellular components in the appropriate time scale.

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