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MODEL BASED ANALYSIS OF SIGNALING PATHWAYS

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The paper is concerned with application of mathematical modeling to the analysis of signaling pathways. Two issues, deterministic modeling of gene transcription and model-driven discovery of regulatory elements, are dealt with. First, the biological background is given and the importance of the stochastic nature of biological processes is addressed. The assumptions underlying deterministic modeling are presented. Special emphasis is put on describing gene transcription. A framework for including unknown processes activating gene transcription by means of first-order lag elements is introduced and discussed. Then, a particular interferon-β induced pathway is introduced, limited to early events that precede activation of gene transcription. It is shown how to simplify the system description based on the goals of modeling. Further, a computational analysis is presented, facilitating better understanding of the mechanisms underlying regulation of key components in the pathway. The analysis is illustrated by a comparison of simulation and experimental data.

Keywords: Signaling pathways, dynamical systems, systems biology, interferon-beta.

1. Introduction

The term *signaling pathways* (also called regulatory, or transduction pathways) relates to a cascade of processes, initiated by an external event (e.g., ligand binding to its specific receptor on a cell surface) or by an internal event (e.g., DNA damage). These processes involve creation or degradation of protein complexes, activation of enzymes, and usually lead to activation or repression of transcription of genes specific for a given pathway. This results in production of new proteins (or their disappearance, if the genes are suppressed) which may affect earlier stages of the cascade, thus creating positive or negative feedback loops.

Following rapid developments in new experimental techniques, mathematical modeling of regulatory pathways that control intracellular biological and chemical processes is gaining increasing interest in the biomedical research. Though the models are unavoidably much simplified, they can significantly contribute to the biological field (Tyson *et al.*, 2003). Knowledge about the dynamics of the processes involved in a given pathway facilitates better experiment planning. Mathematical models can help formulate or reject new hypotheses about unknown processes underlying results observed in experimental work. As a result, directions to be taken in experimental work may be suggested by mathematical models. Moreover, modeling can be used to analyze perturbed behavior even before experiments are undertaken, and answer the question if the desired effects are possible. Finally, an analysis of dynamics leads to determination of parameters defining system time responses (such as time constants, oscillation periods, damping coefficients) and thus helps us in experiment design, if only a limited number of measurements can be taken.

Though ordinary differential equations are not the only tool in mathematical modeling of signaling pathways, they undoubtedly are one of the most often used. In this approach variables describe molar concentrations of the molecules playing important roles in an analyzed pathway, and the processes taken into account include production of new molecules and their degradation, dissociation of complexes, nuclear shuttling and conformational changes in the form of molecules (e.g., caused by their posphorylation or dephosphorylation) leading to their activation or inactivation.

2. Applicability of deterministic modeling

While stochastic effects play a great role in any signaling pathway, deterministic modeling seems to be justifiable, at least in some cases. In this approach the model descri-

bes the behavior of an average cell in a population (even though such a cell does not exist), and the dynamics obtained reflects the cellular behavior seen at the population level. It corresponds to data collected by means of Western Blots, EMSA blots, Real-Time PCR, etc., where actually the average levels of molecules of interest over population are measured. In real life, however, the heterogeneity of cells contributes to the stochastic distribution of initial conditions, which, in turn, results in different dynamics of intracellular processes. Moreover, if there are large oscillations of state variables (molecular levels) in single cells, data gathered during experiments do not reflect real dynamics at all (Lipniacki *et al.*, 2006).

Taking into account that in the pathway analyzed in this paper there are no oscillations in single cells and that very clear trends in experimental data (in terms of increasing or decreasing levels of essential pathway components) are observed, it is reasonable to adopt a deterministic approach. Moreover, if the goal of the modeling is to link intracellular processes with population responses, it is the average response, not that of an individual cell, that should be taken into account.

The modeling of complex formation is based on the law of mass action (Segel, 1991). Though it does not apply when the number of molecules is small, the number of interacting proteins reaches a high level very quickly in the analyzed pathway, and therefore it can be assumed that the influence of this simplification on the results is negligible.

The stochastics of the binding of molecules activating the pathway to their respective receptors has been neglected. The model have been built for very high extracellular ligand concentration, for which all receptors can be assumed to be used. This is particularly relevant if the ligands are active drug components, further justifying the approach describing the average dynamics of a cell in a given population.

3. Induced gene transcription

In the deterministic approach the induced gene transcription is usually modeled by a single ODE:

$$\frac{\mathrm{d}(mRNA)}{\mathrm{d}t} = f[(TF)] - k_{\deg}(mRNA), \qquad (1)$$

where (TF) and $(mRNA)$ denote the concentrations of an active transcription factor (TF) and $mRNA$ produced, respectively, and $f[(TF)]$ is a transcription rate. The function $f[(TF)]$ is given by

$$f[(TF)] = \frac{v_{\max} \cdot (TF)}{k + (TF)}, \qquad (2)$$

with a Michaelis-Menten nonlinearity resulting from physical constraints of the speed of polymerase movement

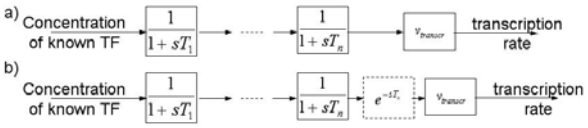

Fig. 1. Block diagram of the transcription initiation in the case when several processes are involved in transcription activation (a) for long genes, (b) for short genes, where the delay in finishing the first polymerase run can be neglected.

along the transcribed DNA. However, if the (TF) is relatively small ($(TF) \ll k$), which is often the case in the analyzed systems, this can be reduced to the following:

$$f[(TF)] = v \cdot (TF), \qquad (3)$$

which will be used further in this section.

Since a single event of binding an active TF to the promoter region can result in a burst of the $mRNA$ level and, consequently, newly synthesized proteins, transcription is a process where stochastic effects are most apparent (Paszek *et al.*, 2005). However, taking into account that at least some TF proteins bind to their respective regulatory elements for time ranging from seconds up to maximum a few minutes and the transcription induction can be observed for over one hour or more, it can be safely assumed that in such cases the transcription rate is proportional to the nuclear concentration of the TF. This is the case in the analyzed pathway (Vinkemeier *et al.*, 1996), and hence the deterministic modeling of early gene transcription is justified. By contrast, when in addition to the binding of the known TFs other processes must take place in order to initiate transcription, another approach is required. It has been shown that, if these processes include the binding of the molecules of the transcriptional machinery, which is necessary for chromatin remodeling and attracting sub-

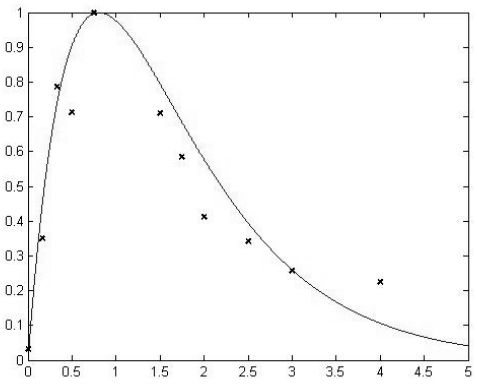

Fig. 2. Typical time evolution of an active TF concentration (crosses) and its approximation by a sum of two exponents (solid line).

sequent elements of polymerase complex, each of them can be represented by a first-order time-lag element, as illustrated in Fig. 1 (Śmieja *et al.*, 2006). A typical time evolution of the TF level can be represented by a sum of two exponential functions (see Fig. 2):

$$(TF) = k_0 \left(\exp(-t/T_0) - \exp(-t/T_1)\right), \qquad (4)$$

with T_1 and T_2 being time constants to be identified from experimental data. If such a signal is applied to the input of serially connected time-lag elements, as in Fig. 1(a), the output of the last element, determining the transcription rate, is given by

$$\frac{\mathrm{d}(mRNA)}{\mathrm{d}t} = v_{\max} \cdot \sum_{k=0}^{n} \frac{\prod_{i=1}^{n} 1/T_i}{\prod_{i=0, i\neq k}^{n} (1/T_i - 1/T_k)} \exp(-t/T_k) - k_{\deg} \cdot (mRNA), \qquad (5)$$

where T_is ($i > 1$) are time constants of successive time-lag elements. This result corresponds directly to the expected value of the $mRNA$ level derived in (Paszek *et al.*, 2005) for a stochastic model. This approach allows including unknown processes in the model. Moreover, only a couple of such elements need to be included, regardless of the number of the processes that take place in the transcription initiation. If the time constants significantly differ and only the final output of the system is of interest, faster processes can be neglected, reducing the model order. Alternatively, if they are similar, introducing new elements beyond the first couple of them does not affect the output significantly, at least for the type of input that is possible in the analyzed system (meaning no high frequency oscillations). In practice, three to four elements are sufficient to reproduce system responses.

4. Early stages of an interferon- β activated pathway

Interferons (IFNs) are very important components of the immunodefense system (Janeway, 2001). Their role and elements of interferon induced signaling pathways are subjects of ongoing research (see, e.g., the reviews (Levy and Darnell, 2002; Sen, 2001; Pestka *et al.*, 2004)) . However, there are very few attempts to model the processes involved in the signaling pathways activated by IFN, and they are mostly concerned with IFN-γ that is produced only by activated lymphocytes (Yamada *et al.*, 2003; Zi *et al.*, 2005).

In this section, the mathematical model description of early processes activated by IFN-β, a cytokine produced by most cell types following viral infection, will be briefly introduced. The analysis presented in this paper is constrained only to the early processes, and therefore no induced transcription is actually modeled. The full model is based on experimental results, which have not been published yet.

The following notation is used:

- Variables names correspond to proteins, complexes and transcripts they represent; for greater clarity, they are put in brackets.
- Variables denote cytoplasmic molar concentrations if no subscripts are present, while the nuclear concentration is represented by the subscript n.
- The phosphorylated form of proteins is indicated by the subscript p.
- Finally, k_v is the ratio of cytoplasmic and nuclear volumes.

The other symbols not mentioned above are model parameters.

The following description of an IFN activated pathway is based on the reviews (Kalvakolanu, 2003; Shuai and Liu, 2003). The graphical representation is shown in Fig. 3.

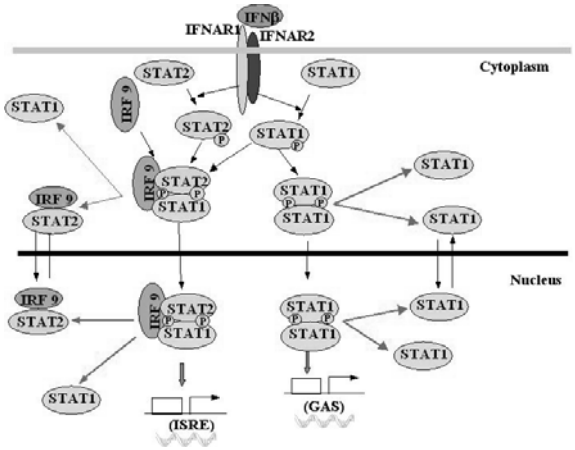

Fig. 3. Early processes in the interferon-β activated pathway.

The most important molecules mediating cell responses after IFN (both of type I and II) stimulation are STAT (Signal Transducer and Activator of Transcription) proteins. In particular, two members of this family of proteins, STAT1 and STAT2, mediate the responses taken into account in the analyzed pathway. Binding IFN-β to a cell receptor results in phosphorylation of STAT proteins. The intermediate stages of this process are not directly modeled. Instead, the process is assumed to follow simple first-order dynamics. Subsequently, phosphorylated STATs form hetero- and homodimers. In cytoplasm, STAT1|STAT2 heterodimers form a complex with

an IRF9 protein, called ISGF3. Both STAT1 dimers and the ISGF3 complex are rapidly transported into the nucleus, where they serve as active transcription factors. In the presented model, the STAT2 protein exists in cells in dimmer with IRF9, and therefore directly after phosphorylation the ISGF3 complex can be formed. STATs are dephosphorylated by phosphatases both in the nucleus and in cytoplasm. Dephosphorylation results in dissociation of complexes leading to nuclear export of STATs and making them available to subsequent phosphorylation/dephosphorylation cycles.

It is assumed that the total amount of proteins is constant within the chosen time horizon. Therefore there are neither production nor degradation terms in the model. This assumption is justified, since the STAT proteins are stable, with half-life times of approximately 15 hours (Heinrich *et al.*, 2003), and they are constitutively present in cells. The shuttling of molecules between the nucleus and cytoplasm is not modeled by any kind of diffusion equations. Since it is not diffusion driven, for the sake of simplicity it was assumed that the rate of nuclear import and export is proportional to cytoplasmic and nuclear concentration of molecules, correspondingly. This stems directly from treating the nucleus and cytoplasm as two different compartments and the transport as the flow in the compartmental model (Godfrey, 1983).

Summarizing all that was discussed above, the dynamics of free STAT proteins in phosphorylated and unphosphorylated forms in cytoplasm and in the nucleus are described by the following ODEs:

$$\begin{aligned}\frac{\mathrm{d}(STAT1)}{\mathrm{d}t} &= -i_{s1}\cdot(STAT1)+e_{s1}\cdot(STAT1)_n\\ &\quad -k_{s1\_\mathrm{phos}}\cdot(STAT1)+k_{s1\_\mathrm{deph}}\cdot(STAT1_p)\\ &\quad +2k_{\mathrm{inv\_s1s1}}\cdot(STAT1_p|STAT1_p)\\ &\quad +k_{\mathrm{inv}\_ISGF3}\cdot(ISGF3),\end{aligned} \tag{6}$$

$$\begin{aligned}\frac{\mathrm{d}(STAT2)}{\mathrm{d}t} &= -i_{s2}\cdot(STAT2)+e_{s2}\cdot(STAT2)_n\\ &\quad -k_{s2\_\mathrm{phos}}\cdot(STAT2)+k_{s2\_\mathrm{deph}}\cdot(STAT2_p)\\ &\quad +k_{\mathrm{inv}\_ISGF3}\cdot(ISGF3),\end{aligned} \tag{7}$$

$$\begin{aligned}\frac{\mathrm{d}(STAT1)_n}{\mathrm{d}t} &= k_v i_{s1}\cdot(STAT1)-k_v e_{s1}\cdot(STAT1)_n\\ &\quad +2k_{\mathrm{inv\_s1s1\_}n}\cdot(STAT1_p|STAT1_p)_n\\ &\quad +k_{\mathrm{inv}\_ISGF3\_n}\cdot(ISGF3)_n,\end{aligned} \tag{8}$$

$$\begin{aligned}\frac{\mathrm{d}(STAT2)_n}{\mathrm{d}t} &= k_v i_{s2}\cdot(STAT2)-k_v e_{s2}\cdot(STAT2)_n\\ &\quad +k_{\mathrm{inv}\_ISGF3}\cdot(ISGF3),\end{aligned} \tag{9}$$

$$\begin{aligned}\frac{\mathrm{d}(STAT1_p)}{\mathrm{d}t} &= k_{s1\_\mathrm{phos}}\cdot(STAT1)-k_{s1\_\mathrm{deph}}\cdot(STAT1_p)\\ &\quad -2k_{s1s1}\cdot(STAT1_p)\cdot(STAT1_p)\\ &\quad -k_{ISGF3}\cdot(STAT1_p)\cdot(STAT2_p),\end{aligned} \tag{10}$$

$$\begin{aligned}\frac{\mathrm{d}(STAT2_p)}{\mathrm{d}t} &= k_{s2\_\mathrm{phos}}\cdot(STAT2)-k_{s2\_\mathrm{deph}}\cdot(STAT2_p)\\ &\quad -k_{ISGF3}\cdot(STAT1_p)\cdot(STAT2_p).\end{aligned} \tag{11}$$

There are no free phosphorylated forms of STATs in the nucleus, since these enter it only in the form of complexes and the dissociation of complexes in the nucleus is caused by dephosphorylation. STAT1 homodimers dynamics are given by

$$\begin{aligned}\frac{\mathrm{d}(STAT1_p|STAT1_p)}{\mathrm{d}t} &= -i_{s1s1}\cdot(STAT1_p|STAT1_p)\\ &\quad +2k_{s1s1}\cdot(STAT1_p)\cdot(STAT1_p)\\ &\quad -k_{\mathrm{inv\_}s1s1}\cdot(STAT1_p|STAT1_p),\end{aligned} \tag{12}$$

$$\begin{aligned}\frac{\mathrm{d}(STAT1_p|STAT1_p)_n}{\mathrm{d}t} &= -k_{\mathrm{inv\_}s1s1}\cdot(STAT1_p|STAT1_p)_n\\ &\quad +k_v i_{s1s1}\cdot(STAT1_p|STAT1_p),\end{aligned} \tag{13}$$

Since a large amount of phosphorylated STAT1 is carried by SGF3 complexes, their dynamics also must be included in the model:

$$\begin{aligned}\frac{\mathrm{d}(ISGF3)}{\mathrm{d}t} &= k_{s1s2}\cdot(STAT1_p)\cdot(STAT2_p)\\ &\quad -k_{\mathrm{inv}\_ISGF3}\cdot(ISGF3)\\ &\quad -i_{ISGF3}\cdot(ISGF3),\end{aligned} \tag{14}$$

$$\begin{aligned}\frac{\mathrm{d}(ISGF3)_n}{\mathrm{d}t} &= -k_{\mathrm{inv}\_ISGF3}\cdot(ISGF3)_n\\ &\quad +k_v i_{ISGF3}\cdot(ISGF3).\end{aligned} \tag{15}$$

It should be noted that the nuclear import of complexes is mediated by other molecules, present in cytoplasm only. Therefore, once in the nucleus, these complexes cannot be transported back into cytoplasm. Hence, in (12)–(15) the transport is only one-way.

The initial conditions for all phosphorylated forms of molecules, including their complexes, are zero, and for nuclear and cytoplasmic STAT1 and STAT2 they are assumed to be known values.

Once in the nucleus, STAT1 homodimers and ISGF3 activate transcriptions of the so-called *early genes*. Eventually, this leads to production of new STAT1 proteins, thus creating positive feedback. However, since it requires several intermediate steps, its effects start to influence

the pathway after several hours. This paper concentrates on the first three to four hours, and therefore such feedback is neglected to simplify the analysis.

There is a known mechanism of negative feedback in this pathway, based on the activity of the Suppressors of Cytokine Signaling (SOCS) family of proteins (Wormald and Hilton, 2004; Alexander and Hilton, 2004). However, in the analyzed HeLa cell line, this feedback is not present and therefore is omitted in the model.

5. Need for a model based analysis

The most important contribution of mathematical modeling to investigation of transduction pathways seems to be in the ability to test various hypotheses about unknown processes allowing us to choose the most promising ones for experimental testing. The model introduced in the preceding section is based on a widely accepted description of the signaling pathway. However, it fails to explain the mechanism behind the negative feedback whose effects are apparent in the time course of the STAT1 homodimer (see Fig. 4). Despite the availability of the phosphorylated STAT1 proteins, the homodimer level decays after reaching its peak value.

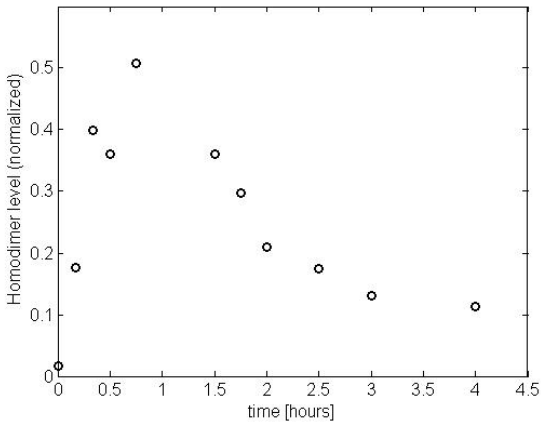

Fig. 4. Time evolution of homodimers of phosphorylated STAT1. Experimental data were normalized to the area under the plot.

On the basis of the experimental results (not published yet), three possible explanations have been rejected: (i) a SOCS based mechanism of phosphorylation inhibition; (ii) there is no reason to assume that in less than one hour phosphorylated STAT1 proteins loose their ability to form homodimers, while still forming heterodimers; and (iii) the control mechanism cannot work by blocking the nuclear import of the homodimers. Therefore, some other mechanism must be involved in controlling the level of homodimers. The type of dynamics they exhibit can be explained basically by one of the following: either the homodimer is rapidly degraded, or dephosphorylation and subsequent dissociation of the homodimer are the only mechanisms involved.

If the induced degradation is postulated to be behind the decrease in molecule levels, then the only modification needed in the system description given by (6)–(15) is the introduction of the degradation term to (13):

$$\begin{aligned}\frac{\mathrm{d}(STAT1_p|STAT1_p)_n}{\mathrm{d}t} &= -k_{\mathrm{inv\_s1s1}} \cdot (STAT1_p|STAT1_p)_n \\ &\quad + k_v i_{s1s1} \cdot (STAT1_p|STAT1_p) \\ &\quad - k_{\mathrm{deg\_s1s1\_n}} \cdot (STAT1_p|STAT1_p)_n.\end{aligned} \tag{16}$$

It is clear that homodimer degradation would result in a decrease in the total STAT1 level in cells, as illustrated in Fig. 5. However, measurements of the total STAT1 level (results not shown here) did not confirm that. Therefore, simulation results combined with experimental data disprove this hypothesis, and there is no reason for an experimental search for the molecule responsible for the induced degradation of homodimers.

Testing the other hypothesis is more challenging, since, in addition to indicating the cause of dissociation, another question must be answered, namely, if this effect can be caused by molecules that are constitutively active or requires, activation within the signaling pathway. It should be stressed that there are at least several known phosphatases that are responsible for STAT dephosphorylation (Hoeve *et al.*, 2002), and several others are postulated (Yamada *et al.*, 2003). It would be extremely difficult, if possible at all, to conduct experiments in which specific phosphatases would be blocked. Therefore, the computational analysis is a convenient tool to check this hypothesis.

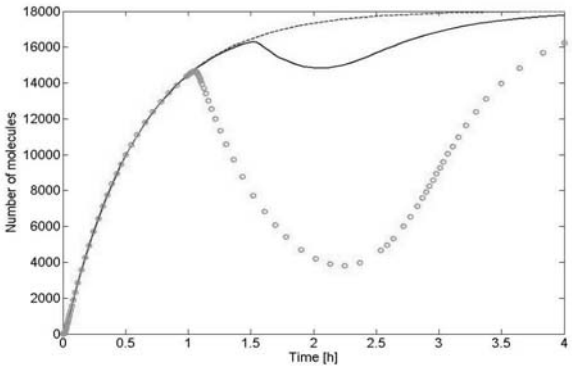

Fig. 6. $STAT1$ homodimers for various dephosphorylation rates (constitutively active phosphatases).

First, the behavior of a model with constitutively present and active phosphatases, described by (6)–(15), was analyzed numerically. Different rates of dephosphorylation yield results shown in Fig. 6. If dephosphorylation is

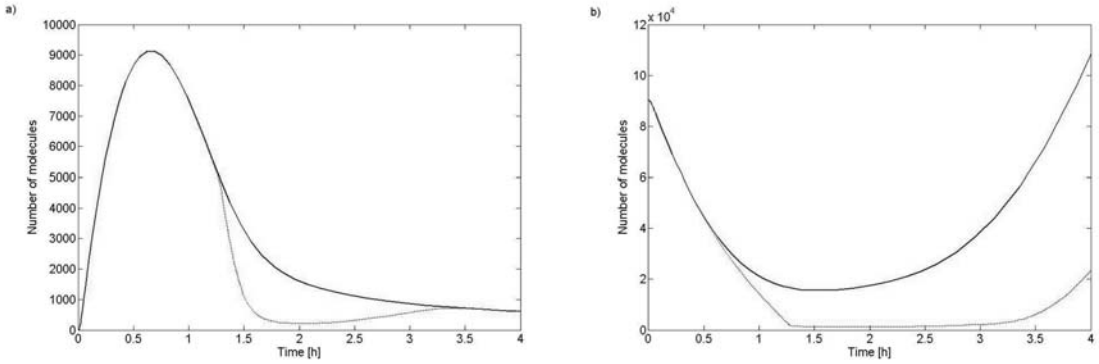

Fig. 5. Degradation (lower line) and dephosphorylation (upper line): (a) $STAT1$ homodimers, (b) total cytoplasmic $STAT1$.

very fast, then homodimer levels reach their steady state without any overshot, while for a slower rate of the process only the initial system behavior reflects experimental data, since after some time the homodimer level starts increasing again. Such dynamics would be characteristic also for processes other than dephosphorylation, if they were mediated only by constitutively active molecules. Therefore, based on simulation results one must assume that there is a nuclear phosphatase, denoted by PHX, that is activated in the pathway. It seems reasonable to assume that the rate of this activation is proportional to the nuclear concentration of ISGF3, which is the main signaling molecule for this pathway. Therefore we obtain

$$\frac{\mathrm{d}(PHX_{\mathrm{inactive}})_n}{\mathrm{d}t} = -k_{\mathrm{activ}} \cdot (PHX_{\mathrm{inactive}})_n \cdot (ISGF3)_n \quad (17)$$

$$\frac{\mathrm{d}(PHX_{\mathrm{active}})_n}{\mathrm{d}t} = k_{\mathrm{activ}} \cdot (PHX_{\mathrm{inactive}})_n \cdot (ISGF3)_n, \quad (18)$$

with initial conditions for the active form of PHX assumed to be zero. In place of (8) and (13) we now have, respectively,

$$\begin{aligned}\frac{\mathrm{d}(STAT1)_n}{\mathrm{d}t} &= k_v i_{s1} \cdot (STAT1) - k_v e_{s1} \cdot (STAT1)_n \\ &\quad + 2k_{\mathrm{inv\_}s1s1\_n} \cdot (STAT1_p|STAT1_p)_n \\ &\quad + k_{\mathrm{inv\_}ISGF3\_n} \cdot (ISGF3)_n \\ &\quad + 2k_{\mathrm{inv\_}xs1s1\_n} \\ &\quad \cdot (PHX_{\mathrm{active}})_n \cdot (STAT1_p|STAT1_p)_n,\end{aligned} \quad (19)$$

$$\begin{aligned}\frac{\mathrm{d}(STAT1_p|STAT1_p)_n}{\mathrm{d}t} &= -k_{\mathrm{inv\_}s1s1} \cdot (STAT1_p|STAT1_p)_n \\ &\quad + k_v i_{s1s1} \cdot (STAT1_p|STAT1_p) \\ &\quad - 2k_{\mathrm{inv\_}xs1s1\_n} \cdot (PHX_{\mathrm{active}})_n \\ &\quad \cdot (STAT1_p|STAT1_p)_n.\end{aligned} \quad (20)$$

A comparison of simulation results and experimental data is made in Fig. 7.

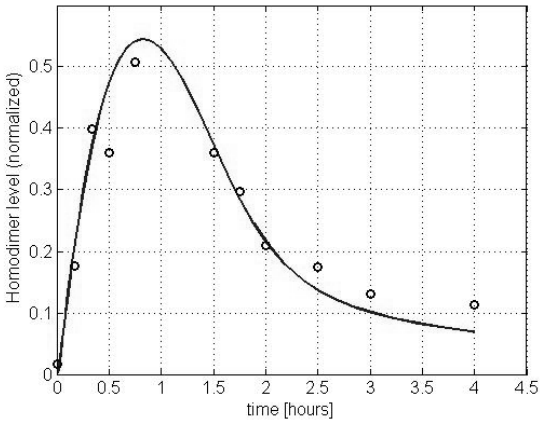

Fig. 7. Comparison of simulation and experimental data for the $STAT1$ homodimer level.

6. Conclusions

The paper shows how mathematical modeling can be applied to advance knowledge about regulatory mechanisms in signaling pathways. Though the model is simplified and only a numerical analysis was made, it helped us to reject two hypotheses about regulatory mechanisms. As a result, the number of experiments necessary to explain the phenomena obseved so far, and thus build a structural view of the pathway, was cut. It also suggested the process that controls the level of STAT1 homodimers in the nucleus, indicating the direction for new experimental work. Moreover, the framework for including unknown processes in the system description, by means of first-order time lag elements, was presented.

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