**Energy modeling of the EGFR pathway**

**Abstract:**

Rule-based modeling is motivated by modular domain structure and combinatorial complexity of macromolecules. In rule-based modeling languages, such as BioNetGen, macromolecules are characterized using structured objects that correspond to the domain structure of their biological counterparts, and their transformations are governed by reaction rules. Although rule-based modeling approach offers a framework for representing multiple reactions with a single reaction rule, multiple rules are required to describe cooperative interactions. Here, using an energy-based extension to BioNetGen, we address the problem of regulatory complexity brought on by the cooperative interactions between different sites in the Epidermal Growth Factor Receptor (EGFR) signaling pathway. Although a vast number of both experimental and computational studies focus on EGFR signaling, the mechanisms underlying many features of the kinetics of EGFR signaling remain unknown. In this study, we use a systematic approach to explore the behavior of the EGFR pathway. We first develop a rule-based and also an energy-based model of the EGFR signaling pathway using the BioNetGen language, which the latter contains a number of cytoplasmic target proteins and leads to multiple cooperative interactions. The presented models are then fitted to published experimental data demonstrating transient tyrosine phosphorylation of EGFR using parameter estimation approaches. The results reflect the significance of the remarkable reduction in binding affinity of Shc to EGFR upon phosphorylation, which is one of the key mechanisms responsible for the transient behavior of EGFR phosphorylation. In addition, we performed sensitivity analysis to gain a deeper understanding of how cooperative energy parameters influence the transient generation. [BRIEF DESCRIPTION OF RESULTS]

**Introduction:**

The Epidermal Growth Factor Receptor (EGFR) signaling is a significant signal transduction pathway actively involved in the cellular development, proliferation, and differentiation regulations [1, 2]. It has been found to be major driver of many types of cancers, and neurodegenerative disease rendering it a subject of medical interest [3]. EGFR activation is induced by ligand bindings such as Epidermal Growth Factor (EGF), which in turn leads to EGFR dimerization and several tyrosine residues auto-phosphorylation. The phosphorylation in the cytoplasmic receptor domain initiates a biochemical communication between the receptor-ligand combination and cytoplasmic target proteins such as Src homology and collagen domain protein (Shc), growth factor receptor-binding protein 2 (Grb2), and phospholipase C-γ (PLCγ) [4, 5]. These early processes, including binding and phosphorylation of monomers using their kinase domains stimulate proliferation and differentiation, and thus result in short-term responses within the EGFR signaling cascade.

Kholodenko et al. studied this short-term behavior using a combination of a computational model and experimental data [5]. They developed a detailed kinetic model of early signaling events that considered a fairly large number of components and interaction parameters. On the experimental side, they showed it was possible to measure dynamics at fairly high temporal resolution using standard biochemical methods with fairly high precision, especially in comparison to most data that is reported in the literature. Despite a sophisticated mathematical model of the factors governing the kinetics of the EGFR signaling pathway, the model outputs do not adequately explain the transient behavior of several model components in experimental data. Furthermore, due to the model's manual incorporation of thermodynamic constraints, some detailed balance constraints have not been satisfied.

The question of how molecular diversity which arises from multi-domain protein-protein interactions influences the functional behavior of the EGFR signaling components has been addressed in the model developed by Blinov et al [6]. This model is mainly based on what Kholodenko et al. presented, however it takes the protein tyrosines into account separately. Nonetheless, the model of Blinov et al. still has some major shortcomings. Like the Kholodenko model, they do not use a systematic parameter estimation approach to find the best fit to the data. While they distinguish between EGFR phosphorylation and its binding sites, they do not consider the possibility of between-site cooperativity. Furthermore, both models specify rate constants directly, leaving open the possibility of violating thermodynamic constraints.

Rule-based modeling represents the interconnected molecules as structured objects which are governed by specific rules. Several protein-protein interaction consequences, including the formation of heterogeneous protein complexes, protein degradation, and post-translational protein modification, are present in nearly all biological process networks[7-9]. Typically, proteins' modular domains, which comprise binding and catalytic functions, mediate these processes. Despite the importance of these interactions on comprehending the dynamics of the biological system, adopting standard methods for considering these details generally leads to a complex network model. However, Rule-based modeling approaches enable modeling the site-specific details of protein-protein interactions while reducing combinatorial complexity [8].

Although a rule-based model provides a framework for demonstrating several reactions with a single reaction rule, it is constrained by the issues of detailed balance and regulatory complexity. The problem of detailed balance arises when reversible reaction rules are stated as though they are independently determined rather than mutually restricted by thermodynamic cycles [9]. Whereas cooperative interactions between the sites always lead to regulatory complexity issues, especially when multiple sites are used to govern various operations. Therefore, it takes numerous rules as well as, manually enforced detailed balance constraints to explain cooperative interactions and thermodynamic laws, which could result in a large model [9, 10].

Rule-based models can be specified in BioNetGen language (BNGL) for developing the modular and structure-based model of biochemical reaction networks. BioNetGen (BNG) is a rule-based software tool that uses a graph syntax and a set of reaction rules to define the molecular patterns and a large class of biochemical reactions, respectively [7, 8]. Moreover, it offers a concise framework for specifying a free energy accounting system based on the network generation methods for allosteric transitions. With the help of this BNG extension, known as eBNG, reaction rate laws may be calculated from reaction free energies and detailed balance constraints are automatically enforced. Furthermore, a system network with high order cooperative interactions could be handled efficiently by an energy-based model that is implemented in this formalism [9, 10].

In this study, we seek to rectify the weaknesses of the previous models by employing more systematic methodologies to explore the behavior of EGFR pathway. First, we attempt to map the relationships between cooperativities and the output of the system. In doing so, we are eager to gain a deeper understanding of how different model parameters influence signal transmission in EGFR pathway. Second, we aim to figure out what mechanism(s) cause transient in this system. To these ends, after demonstrating that the previously mentioned models are physically implausible due to violations of detailed balance, a rule-based version of the EGFR pathway model based on the Kholodenko’s paper is built in which detailed balance constraints are considered. Then, using an energy-based extension of BioNetGen (eBNG), we address the problem of regulatory complexity brought on by the cooperative interactions between different sites in the EGFR signaling pathway. Energy-based modeling has the advantage of enforcing thermodynamic restrictions automatically, as well as smaller number of parameters. Moreover, applying eBNG gives us the opportunity of easily expanding the model and bringing the model closer to reality by adding some interactions like simultaneous multiple binding of proteins to EGFR tyrosine residues. Finally, we apply parameter estimation methodologies to fit these versions of the EGFR signaling cascade model to the experimental data, and the results are used to infer a probable mechanism for transient production when it occurs.

**Methods:**

* **Building the rule-based model of EGFR signaling pathway**

The first presented model in this study is a rule-based version of the model in [5]. The aim of developing this model is not only providing the fundamental structure of energy modeling procedure, but also obtaining a set of optimal parameters utilizing parameter estimation techniques and imposing detailed balance constraints in order to have a fitted model of EGFR pathway against the experimental data which is believed to be plausible. The model, which is identical to that of Kholodenko et al. [5], has 50 parameters and 25 rules for creating the reaction network of the EGFR signaling cascade. However, 45 parameters are unknown as a result of the enforcement of detailed balance restrictions. More details on the model components, and biochemical reactions could be bound at [5]. The rule-based model was developed in BioNetGen which offers the opportunity of creating the multi-domain species resulting in a fewer number of model components [11].

**- The simple energy model**

In order to demonstrate some of the key features of the energy model, we first create a simple model containing a receptor, R, and two adaptors, A and B. The receptor could have two sites, one that binds A and one that binds B. Upon binding to R, A could become phosphorylated and B could bind to it. Using this simple model, We try to find parameters that would make it exhibit transient kinetics for A or B binding similar to what Kholodenko et al. [5] observed in their experiments. The model is developed both in rule-based and energy-based frameworks. These structures could be used to demonstrate how developing an energy model may aid in overcoming the challenges of detailed balancing constraints and regulatory complexity. It may also provide a simplified explanation of the potential mechanisms underlying EGFR transient behavior.

Here is a brief overview of the energy-based modeling principle and its implementation in BioNetGen. Each reaction should be primarily expressed in free energy space, where free energy variations () are associated to the equilibrium constants () as follows:

in which and are universal gas constant and temperature, respectively. could be scaled by . Therefore, reaction free energy is stated as the equation below:

and are the forward and backward reaction rates in a reversible chemical reaction, respectively [9]. In eBNG, a rate law function is expressed by Arrhenius theory of reaction rates for a reaction class containing all the reactions that share the similar rate law function. Then, and for any reaction in a class might be derived from the following equations based on linear transition state theory [12]:

In the above equations, denotes activation energy and is the rate distribution parameter which has a constant value between 0 and 1 and controls the contribution of in and [9].

Energy-based modeling in BioNetGen involves assigning free energies to molecular complexes using energy patterns. These energy patterns enable users to designate energy terms to various molecular states, which then allows for the computation of rate constants for model reactions based on the energy patterns and activation energies for those reactions. By utilizing energy patterns, cooperativities can be represented as energetic terms associated with higher-order complexes or states.

The model in rule-based structure has 8 reaction rules including 14 reversible and 1 non-reversible reaction, as well as 15 parameters. Due to the presence of one thermodynamic restriction along a cyclic set of reactions, there is a detailed balance constraint which reduces the number of independent reaction rates to 14. However, building the equivalent energy model needs only 5 reaction rules and 11 parameters. Besides having fewer number of model components, energy-based modeling framework eliminates the need to worry about enforcing the detail balance restrictions, because it implements this principle automatically.

…….The role of this simple model in describing the hypothesis about EGFR transient behavior……

* **Building the energy-based model in some steps**

The proposed energy model of EGFR signaling pathway in this study is developed in several steps. Each step involves the incorporation of one or more chemical species, as well as their related parameters and reactions. The model in the first step is comprised of three species: EGF, EGFR, and Shc. Therefore, the reaction rules define EGF binding to EGFR, EGFR dimerization, Shc binding to EGFR, transphosphorylation and dephosphorylation of tyrosines in Shc and the cytoplasmic portion of EGFR. Two more model elements, Grb2 and Son of Sevenless (SOS), are used in steps 2 and 3. Step 2 considers Grb2 binding to EGFR and SOS, while the adaptor role of phosphorylated Shc for enabling indirect Grb2-EGFR binding is modeled in step 3. In addition to the above mentioned interactions, PLCγ phosphorylation, activation, and binding to EGFR are the other early events which have been modeled in step 4. The kinetic parameters of the model at these four steps are adjusted so that the final model in step 4 recovers the model outputs in [5]. It's worth noting that the model in step 4 has substantially less components than the model in the Kholodenko et al.’s study [5], with only 19 reaction rules and 34 parameters.

Finally, the energy-based model is extended by discarding some restrictive assumptions in the Kholodenko et al.’s model [5]. This leads to a more realistic model by adding some interactions including simultaneous binding of multiple proteins to their target phosphotyrosine residues, and allowing the phosphorylated receptors to undimerize. Despite the addition of these interactions that were not taken into account in the original Kholodenko et al.’s model [5], the subsequent model in step 5 also has fewer parameters than the original model. The energy-based models in different steps can be found at: ….

* **Parameter estimation and Markov chain Monte Carlo sampling using PEtab format and pyPESTO**

The EGFR pathway models given in this study, including rule-based and energy-based models, are fitted to the experimental data in [5] using the python parameter estimation package pyPESTO [13]. It provides an algorithm for multi-start optimization and uncertainty analysis in computational biology problems which help to find the global optimum utilizing various optimization methods. For problem specification, we use pyPESTO linked to PEtab which is a standardized data format that defines measurement noise, parameter bounds, model outputs, and experimental conditions to prepare data for parameter estimation problems [14]. Using the applicable features of pyPESTO, we further evaluate the properties of the optimization problem using uncertainty analysis and Markov chain Monte Carlo sampling techniques which is a widely used method to estimate the uncertainty of the parameter estimate and assess the performance of the model in fitting the observed data.

* **Sensitivity Analysis**

In this study, we used forward sensitivity analysis to investigate the sensitivity of the energy-based model of EGFR pathway to changes in its parameters. Specifically, we utilized the AMICI python package [15] to perform the analysis. AMICI provides an efficient interface to the SUNDIALS suite of numerical solvers, which enables efficient computation of both model solutions and their sensitivity analysis. Forward sensitivity analysis is used to analyze the sensitivity of models to changes in their parameters. We first defined the cooperative free energy parameters of the model as a set of parameters that we wanted to investigate and then varied each parameter individually while keeping the others constant. We then computed the corresponding changes in the model output and used these to calculate the sensitivity coefficients. These coefficients enabled us to quantify the relative impact of each parameter on the model output and to identify the parameters that had the largest effect on the model behavior.

**Results:**

**- Addressing Detailed Balance Violations in Previous Models**

There are three distinct thermodynamic cycles including free EGFR, ShcP, Grb2, SOS, and their respective complexes. One of these cycles consists of the association of ShcP-Grb2 and EGFR to give EGFR-ShcP-Grb2; the association of EGFR-ShcP-Grb2 and SOS to give EGFR-ShcP-Grb2-SOS. Subsequently, the dissociation of EGFR-ShcP-Grb2-SOS leads to the formation of EGFR and ShcP-Grb2-Sos. Finally, the cycle concludes the dissociation of ShcP-Grb2-Sos to yield the initial species in the loop. In order to adhere to the principle of detailed balance, the multiplication of equilibrium constants around this particular cycle must equate to 1. Nonetheless, the multiplication of relevant equilibrium constants surpasses the value of 1, suggesting that the assumptions of the Kholodenko and Blinov model are not thermodynamically feasible. This lack of feasibility is demonstrated in the subsequent equation, which is derived from Eq.10 in [5]:

The other cyclic pathways that are deemed unlikely have been illustrated in Eq.8 and Eq.12 of [5], and their veracity can be promptly verified.

So, we have developed a rule-based version of the EGFR signaling pathway that incorporates the constraints of detailed balance. In this model, the number of unknown parameters decreases by five. This is because there are five cyclic pathways within the model that must be taken into account as they impose thermodynamic constraints on the parameters. Consequently, we obtain a rule-based model consisting of 25 reactions and 45 unknown parameters which are estimated to demonstrate a desirable agreement with the experimental data, a feature that was not observed in the original model presented in [5].

* **Fitting the rule-based version of the Kholodenko’s model to the data**

1. **parameter estimation of the rule-based model of EGFR signaling pathway**

In this paper, the rule-based model of the EGFR signaling pathway which can be found in the supplementary files, has been created in BioNetGen. To estimate the parameters, we employed a multi-start optimization method using pyPESTO [13]. Figures 1a and 1b display a comparison between the experimental data and the model outputs of the original EGFR signaling pathway model in [5] and the rule-based model, respectively. These figures illustrate the short-term expression levels of multiple species including the total phosphorylated EGFR, Shc, and PLCγ, as well as the total Grb2 co-precipitated with Shc and bound to EGFR. Looking at these figures, we are able to compare the results obtained from the original model of Kholodenko presented in [5] with those obtained from our rule-based model.

Based on the results illustrated in Figures 1b and 1b, it is evident that the rule-based model with estimated parameters demonstrates superior ability in reproducing experimental data. This improved performance can be attributed to the utilization of multi-start approaches in finding the global optimum shown in supplementary figure S1 (Waterfall plot). One of the key reactions implicated in the transient behavior of the EGFR, as reported in [5], is the tyrosine phosphorylation of Shc bound to EGFR. Upon phosphorylation, the binding affinity of Shc to EGFR markedly diminishes, a fact that is reflected in the optimized parameter set. This reaction thus represents a promising candidate for further exploration through energy-based modeling techniques.

Furthermore, according to the kinetic model, the way in which signals are propagated in the EGFR pathway is heavily influenced by the comparative quantities of the molecular factors present. It seems that changes in the relative levels of signaling proteins have a greater impact on the timing of phosphorylation/activation responses to EGF than most variations in the kinetic constants [5]. Therefore, in this section, we try to determine the initial concentration of the model species using a parameter estimation approach. Then, the optimization problem consists of four more unknown parameters including the initial concentration of EGFR, Grb2, Shc, and PLCγ. Using the same methods, the comparison between the model output using the optimized values and the experimental data can be found in figure 2. Also, figure S2 represents the likelihood function values of the estimated parameters at the end of each optimization run. The results demonstrate that allowing the concentration values to vary leads to an improvement in optimization by reducing of the cost function value and increasing the number of iterations that converge to the global optimum.

1. **MCMC sampling and convergence diagnostics of the optimized rule-based model**

We employed MCMC sampling to estimate the parameters uncertainty of the optimized rule-based model. We used the adaptive Metropolis-Hastings algorithm with parallel tempering to generate a large number of samples (2 million) from the posterior distribution of the parameters. Furthermore, we utilized convergence diagnostics such as Geweke [16] and Gelman-Rubin [17] tests to ensure that the MCMC chains had converged to the stationary distribution.

Using MCMC sampling, we obtained a well-defined and reasonable shaped posterior distributions for each parameter which suggest the likely ranges of parameter values. Moreover, convergence diagnostics indicated that the MCMC chains has converged to the stationary distribution. The burn-in index which is computed using Geweke test and helps to determine when an MCMC chain has converged to the target distribution for these chains are 800,000. This index also indicates the number of iterations that should be discarded before the chain can be considered to have converged, and should be used to assess the convergence of an MCMC chain along with other tests, such as the Gelman-Rubin. The Gelman-Rubin diagnostic which compares the within-chain and between-chain variability to determine if the chains have converged, showed that the potential scale reduction factor was close to one for all parameters, indicating that the chains had converged. The results of these analyses can be found in the supplementary files.

The parameter identifiability analysis indicates that the original EGFR signaling pathway model is capable of describing the transient behavior of the EGFR and other constituents of the model. However, the absence of a systematic approach for parameter estimation has been addressed in this paper through the implementation of a parameter estimation method followed by MCMC sampling on an optimized set of parameters in a rule-based version of the model.

* **Energy-based model of EGFR pathway**

The energy model for the EGFR signaling pathway, proposed in this study, is constructed through a series of steps. In each step, one or more chemical species, along with their associated reactions, are integrated into the model. During the various steps of modeling as described in the methods section, the parameters in the model are regulated such that the outputs of the original model in [5] can be recovered. However, as previously noted, the outputs obtained from the energy-based model do not align with the experimental data. Additionally, in the process of constructing the energy-based model, various parameters and additional reactions are included, such as the binding of multiple proteins to target phosphotyrosine residues, to create a more realistic scheme. These parameters need to be estimated. Thus, we employ similar techniques used in the rule-based model to fit the energy-based model to the experimental data.

* **Fitting the energy-based version of the EGFR model to the data**

The energy-based model of EGFR signaling pathway consists of 19 reactions and 37 parameters which required to be estimated to accurately simulate the dynamics of the pathway. Here,

pyPESTO [13] was utilized to estimate the optimal values for the model parameters to provide best match of the experimental data with the simulated model. Figure 3 illustrates the optimization results, including a comparison between the energy-based model outputs and the experimental data. Moreover, the ordered likelihood function values of the estimated parameters at the conclusion of each optimization run are displayed in figure S3. The presence of a plateau at the initial points of the figure indicates that the optimization was successful in discovering a good global optimum. Also, the validity of the assumption that the binding affinity of Shc to EGFR significantly decreases upon phosphorylation was confirmed by the acceptable fit of the energy-based model to the experimental data. This was supported by the positive estimated value for the corresponding free energies associated with this reaction.

1. **MCMC sampling results**

**….**

* **Sensitivity analysis results**

The sensitivity analysis results indicate that the cooperative free energy parameters play a critical role in the transient behavior of EGFR phosphorylation. Notably, our results demonstrate that the free energy parameters for multiple bindings of Shc, Grb2, and PLCγ to EGFR are highly sensitive. This highlights the significance of these essential reactions that were previously overlooked in the EGFR signaling pathway models. Additionally, the free energies of binding for Shc to EGFR while Grb2 is bound to Shc, the binding of SOS to the Shc-Grb2 complex, and the parameters associated with the binding of phosphorylated Shc and PLCγ to EGFR are also significant contributors to the system's sensitivity. The latter two parameters mentioned in [5] as being responsible for the transient behavior of EGFR phosphorylation have been identified as highly sensitive in this study. The sensitivity analysis results that provide sensitivity indices for all cooperative free energy parameters with respect to time, and their average values are depicted in figures S4 and S5.

**Discussion:**

The dynamics of the EGFR signaling pathway are intricate, and numerous attempts have been made to model its behavior. Previous studies have concentrated on cooperativity as the principal factor influencing observed behavior, examining both binding and phosphorylation cooperativity, as well as cooperativity between binding at different sites on molecules. These studies seem to achieve reasonable agreement with experimental data using ad hoc parameters that do not appear to fit the data proficiently. Moreover, the lack of a systematic approach for finding parameters for these models raises concerns about their reliability. Kholodenko et al. [5] do not provide a rigorous fit of their model parameters to experimental data, resulting in uncertainty regarding whether their parameters provide the best fit to observation. Furthermore, their model conflates all tyrosine phosphorylation sites on EGFR, without considering the possibility of multiple effectors binding at different sites on the same molecule. Although, the presented model in [6] can predict experimental outcomes beyond the scope of the pathway-like model by incorporating more molecular details than the pathway-like model in [5], both of these models lack plausibility due to their violation of thermodynamic restrictions.

In this study, we began by utilizing a rule-based version of the model presented in [5]. However, we improved upon this model by considering the detailed balance constraints in each cyclic pathway. This modification resulted in a more robust and reliable model. Then, employing a more rigorous and systematic approach, we determined the model parameters and their likely ranges using parameter estimation and MCMC sampling techniques. The optimization results showed a better agreement with the experimental data, especially when we allowed the initial concentration of specific molecules to vary. Moreover, the MCMC sampling provided well-defined posterior distributions for each of the model parameters. The convergence of the MCMC chains to the stationary distribution further validates the reliability of our results..

Then, we addressed the problem of regulatory complexity brought on by the cooperative interactions between different sites in the EGFR signaling pathway using an energy-based version of the EGFR signaling pathway model. Despite having fewer parameters, the provided model automatically enforced thermodynamic constraints. Moreover, utilizing the energy-based extension of BioNetGen for modeling EGFR signaling pathway allows us to include some developmental interactions, such as simultaneous binding of multiple proteins to the tyrosine residues of the EGFR. Following that, a parameter estimation method and an MCMC sampling methodology were used to determine the parameters of the developed energy-based model as well as their uncertainties. The results give us a set of optimum parameters that may be able to account for the changes in experimental data brought on by various doses of EGF.

Previous model of EGFR pathway in [5] has revealed that the transient dynamics of EGFR phosphorylation are influenced by the preservation of phosphotyrosine residues against dephosphorylation, provided that a target protein is bound to EGFR. This implies a reduction in binding affinity (as expressed by ) of phosphorylated Shc and PLCγ to EGFR, indicating negative cooperativity. Such negative cooperativity is reflected in the energy-based model optimization results. Additionally, the sensitivity analysis results have highlighted the crucial role of the cooperative free energy terms of phosphorylated Shc and PLCγ.

For instance, the role of binding affinities of Shc to EGFR in both phosphorylated and dephosphorylated states in the transient behavior of EGFR phosphorylation is endorsed by the positive estimated value for the corresponding free energy which shows the significant decrease for the affinity upon phosphorylation.

Sensitivity of energy-based parameters

Responsible mechanisms using SA

Some comparison of ode model and energy-based model

Although the comparison of likelihood function values for the energy-based and rule-based models of the EGFR signaling pathway does not reveal significant differences, the energy-based model stands out for its ability to describe critical reactions using fewer parameters. Additionally, the energy-based model automatically takes into account certain factors, such as thermodynamic constraints, that must be manually incorporated in rule-based models.

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**Figure captions:**

**Figure 1: a) The Kholodenko's model outputs vs experimental data.** The experimental data and the model outputs of the original EGFR signaling pathway model in [5]. **b) The rule-based model outputs vs experimental data.** The figures illustrate the short-term expression levels of multiple species including the total phosphorylated EGFR, Shc, and PLCγ, as well as the total Grb2 co-precipitated with Shc and bound to EGFR. The experimental data has been shown using dashed line while the simulation results have been illustrated as solid lines. The experimental data are available at 0, 15, 30, 45, 60 and 120 seconds.