



**STRUCTURAL, KINETIC, AND DYNAMICAL
ANALYSIS OF RECEPTOR-ACTIVATED
PROTEOLYSIS**

by

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Certificate

This is to certify that the thesis titled “**Structural, Kinetic, and Dynamical analysis of receptor-activated proteolysis**” being submitted by **Harika GL** to the Indraprastha Institute of Information Technology Delhi, for the award of the Master of Technology, is an original research work carried out by her under my supervision. In my opinion, the thesis has reached the standards fulfilling the requirements of the regulations relating to the degree.

The results contained in this thesis have not been submitted in part or full to any other university or institute for the award of any degree/diploma.

August, 2023



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Abstract

One of the challenging tasks in systems-level modeling is determining the bistable dynamics from the networks constructed from the experiments. A necessary but not sufficient condition for bistability is the presence of positive feedback motifs in the network. In many cases, one can easily identify positive feedback by visual inspection if the network is constructed properly from the experiments. But there are cases where positive feedback is implicit, and it is not straightforward to determine its presence in the network. A quick way to determine bistability from the biological network is to perform structural analysis using chemical reaction network theory (CRNT). CRNT is a powerful tool that can not only determine bistability but also provide kinetic parameters that one can use profitably to construct bifurcation diagrams to verify the presence of bistability numerically. However, the drawback in CRNT is that the kinetic parameters are not close to what is obtained from the experiments. Therefore, kinetic parameters must be separately determined from the experimental data. They can be obtained directly from the experiments or extracted by fitting the steady state or time series data. However, the estimated kinetic parameters need not give rise to bistability, and in that case, either the parameters have to be recalibrated, or one has to discard the old mechanism and propose a new one. We demonstrate the usefulness of this structural and kinetic analysis to a recently proposed signal transduction pathway for receptor-activated proteolysis (RAP), where this pathway is triggered during amino-acid starvation. We proposed many mechanisms for RAP network dynamics where the feedback loops are hidden in the network. Structural analysis indicated that all the mechanisms exhibited bistability. A subnetwork also exhibited transcritical bifurcation. However, to perform kinetic analysis, sufficient data were not available and with the available data, the parameters were recalibrated to exhibit bistability. Overall, all the mechanisms seem plausible, and we hope that we will arrive at a resolution about the right mechanism, structure, and dynamics when we get more data from the experiments in the near future.

Keywords: *Saccharomyces Cerevisiae*; SPS signaling pathway; ODE; Receptor activated proteolysis; phosphodegron.

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Abbreviations

RAP	R eceptor A ctivated P roteolysis
RIP	R egulated I nteramembrane P rocessing
AAP	A mino A cid P ermease
CRNT	C hemical R eaction N etwork T heory
SR	S pecies R eaction
COPASI	C OMplex P ATHway S IMulator
SPS	S sy1 P tr3 S sy5
SS	S teady S tate
LP	L imit P oint
XPPAUT	X windows P hase P lane A UTomatic bifurcation software
ODE	O rdinary D ifferential E quation
SDE	S tochastic D ifferential E quation

Chapter 1

Introduction

Biological reaction networks are complex and highly non-linear. Though there is increasing knowledge about molecular interactions, simulating large networks seldom gives deep insights. Instead, analyzing smaller and independently functioning networks called motifs has become a useful strategy. Milo et al. ([1]) focussed on gene regulatory networks leading to a number of circuits capable of generating certain input-output behavior like thresholds and ultrasensitivity ([2]; [3];[4]), foldchange detection ([5]; [6]; [7]), concentration robustness ([8],[9]; [10]) or multistability ([11]). Also, from an engineering perspective, methods from control theory network motifs were analyzed.

Even with the increasing number of methods, it is still challenging to understand the behavior of a larger network by putting together the smaller networks whose behavior is known. However, structural aspects such as multistability and certain types of robustness can be studied based on the algebraic properties of the network regardless of the kinetic values such as rates and parameters ([12]; [13]). These methods will still limit us to certain structural properties of the network without giving sufficient information in terms of the kinetic aspects. To understand the behavior of the network under biological conditions and to obtain the biological range of parameters, it is necessary to analyze the network kinetically using methods such as parameter estimation.

In this thesis, we will look into analyzing a network structurally and kinetically and apply the same to study Receptor Activated Proteolysis (RAP) dynamically. Identifying the bistable dynamics of a network is a challenging task. Multistability

is the capacity of the network to admit multiple steady states. Multistability plays an important role in acting as a checkpoint in most biological networks. Positive feedback is a necessary but not a sufficient condition for a network to exhibit bistability. Usually, positive feedback can be detected by visual inspection of the network. However, in some cases, the positive feedback is implicit, rendering it impossible to detect with mere visualization ([11]). Bistability can be efficiently detected using the chemical reaction network theory (CRNT). The key objective of CRNT is to understand the behavior of chemical systems solely based on the underlying network regardless of the values of the kinetic parameters.

1.1 Thesis Organisation

In Chapter 2, we explain the biology of Receptor activated proteolysis (RAP) in detail. Here we explain the building of the network based on the available experimental information in the literature. For ease of understanding, we divide the network into subnetworks based on the key events in RAP.

In Chapter 3, we provide the basic background and terminologies of CRNT. We also show how to structurally analyze a network with the help of CRNT-based tools such as CRNT Toolbox, BioSwitch, and CRNT4SBML. These toolboxes give information regarding the multistability of the biological network. Along with detecting multistability, these toolboxes also generate parameter sets for which the network shows multistability.

Apart from CRNT, Species-Reaction (SR) graphs is a graphical way of looking for the capacity of a network to exhibit bistability. Based on the properties of the SR graph of a network, it is possible to preclude bistability. This helps in filtering out the networks that do not have the capacity to admit multiple steady states. In Chapter 4, we show how to construct an SR graph from a given biological network, along with its terminologies. We further analyze the network using an SR graph based on a set of theorems to look for the network's capacity for multistability.

Though CRNT toolboxes provide parameter sets for bistability, often, these kinetic parameters are not close to the biologically feasible values. Hence to perform kinetic analysis and dynamically interpret a biological network, it is important to determine the kinetic parameters from the available experimental data separately. In Chapter

5, we will show how to extract data points from the images of experimental data using ImageJ software. We also show how to fit data using MATLAB and COPASI. We sequentially show how to perform parameter estimation in COPASI using the experimental data.

We apply these methods for structural and kinetic analysis, to a recently proposed signal transduction pathway of receptor-activated proteolysis (RAP). In Chapter 6, we construct multiple models for RAP where the feedback loops are implicit. By performing structural analysis using CRNT and SR graphs, we could detect that the network exhibits bistability. A subnetwork also revealed the presence of imperfect transcritical bifurcation. We also performed parameter estimation in COPASI using the available experimental time series data.

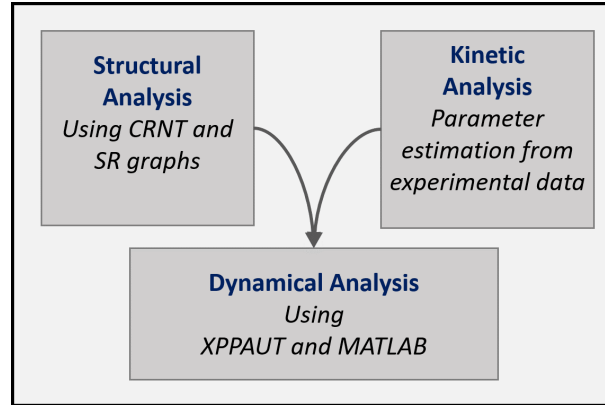


FIGURE 1.1: *Structural, Kinetic, and Dynamical Analysis.*

To study the dynamics of the biological network, it is necessary to perform structural and kinetic analyses of the network. The structural analysis is solely based on the structure of the network, irrespective of the kinetic parameters. The structural analysis of the network is done using CRNT and SR graphs. To perform a kinetic analysis of the network, we estimate kinetic parameters in the software COPASI using the available experimental data.

1.2 Statement of the thesis problem

1. The aim is to understand the dynamics of Receptor-activated proteolysis in *C. albicans* by constructing molecular circuits from the experiments and performing structural and kinetic analysis of the network.

2. We hypothesize multiple mechanisms based on the experimental data and compare their dynamics to understand which network is experimentally feasible.
3. To determine whether the network exhibits multistability, We perform structural analysis using CRNT and SR graphs.
4. To perform numerical bifurcation analysis to verify the presence of multistability, We perform kinetic analysis by extracting and fitting the model parameters from the experimental data using the software COPASI.

Chapter 2

Literature Survey - SPS sensing receptor-activated proteolysis

2.1 Introduction

In *Saccharomyces Cerevisiae*, plasma-membrane-localized SPS sensors play a critical role in sensing and responding to amino-acid present in the extracellular milieu. The sensors are important in controlling the expression of amino-acid permease that regulates the uptake of amino acids. The SPS signaling pathway is an amino-acid-induced signal transduction pathway ([14]) with an intricate regulatory network that controls two essential transcription factors, Stp1 and Stp2, present in the cytoplasm as a latent form.

The activation of latent transcription factors through receptor-activated proteolysis (RAP) is essential in triggering the gene expression and controlling the production of amino-acid permease through multiple upstream regulatory control in the SPS pathway. With the activation of the SPS sensing pathway, the transcription factors Stp1 and Stp2 undergo Ssy5-dependent proteolytic processing to enable their nuclear translocation ([15]).

We construct the detailed SPS network and model the regulatory dynamics of the system to capture the many important control points that lead to the activation of latent transcription factors in the presence of extracellular amino-acid. The differential equation model is based on the mass action kinetic laws that capture the

conformational switch in the SPS sensor pathway. We attribute the conformational switch to an ultrasensitive behavior. We look for the switch-like behavior in terms of multistability or the presence of transcritical bifurcation. We show that bifurcation dynamics provide a threshold control at each important step of the molecular pathway in controlling the activation of latent transcription factors. Our model is the first step towards the understanding of the inner working of RAP and may be useful to extend it to capture the similar dynamics seen in the virulent fungi *C. albicans*.

2.2 Receptor-mediated proteolysis

Cells respond to environmental changes by altering their gene expression. Understanding the mechanistic connections between the proteins that perceive environmental cues and the downstream components that regulate the activity of appropriate transcription factors is of major concern. Apart from being metabolites for protein synthesis, amino acids have an important role in nitrogen homeostasis, and yeast possesses sophisticated systems to import amino acids from the external environment efficiently. Yeast cells respond to low levels of extracellular amino acids by letting the amino acid induce its own uptake to enhance the regulation of the amino acid uptake.

In eukaryotes, the plasma-membrane-located signaling pathway is important to sense and respond to both internal and external cues ([15]). Amino-acid sensing pathway in the budding yeast *Saccharomyces Cervisiae* is a well-studied pathway that provides insight into the molecular mechanism of how amino-acid uptake is successfully executed under the adverse conditions that affect the cell growth and proliferation ([16]). One of the well-studied signaling pathways is the triggering of the SPS sensor pathway in response to the cell's inability to synthesize the important amino acids for survival. This results in the induction of the gene expression of many amino acid permeases that facilitates the uptake of the external amino acid and to signal ([16]). SPS sensing system is also important and interesting because of its unique proteolytic control process, which controls the processing of important substrates like transcription factors that are present as a latent form in the cytoplasm. Proteolytic processing is important in cell biology regulation; for example, this involves the processing of substrate and enzymes. The system either controls the regulation of the substrates or the enzymes. The enzymes are often constitutively expressed, and

the substrates are controlled. For example, proteases are constitutively active that process the available substrate through regulated intramembrane processing (RIP) and ubiquitin/proteasome-dependent processing (RUP). But there are cases where the control of enzymes takes place rather than the free available substrates. For example, the receptor controls the enzyme protease release and its enzymatic activity in RAP. The substrate activity is dependent on this receptor-controlled protease, and therefore, this is called receptor-activated proteolysis (RAP).

The regulation of amino acid uptake in yeast offers an attractive system to pursue signal transduction mechanisms. The signal transduction pathway that we have characterized is activated in response to extracellular amino acids and transduces signals directly from the plasma membrane to the nucleus without the apparent involvement of previously characterized signal transducing components, that is, kinase cascades, ubiquitin, and the proteasome.

Proteolytic processing is a mechanism used by eukaryotes to control the activity of latent transcription factors. For example, regulated intramembrane proteolysis (RIP) and ubiquitin/proteasome-dependent processing (RUP) are two mechanisms that employ proteolysis-dependent activation of latent membrane-anchored transcription factors.

RIP is catalyzed by membrane-bound proteases that cleave inactive precursor molecules to release active transcription factors from their membrane anchor. In the RUP mechanism, a polyubiquitylated membrane-anchored precursor is partially degraded by the 26S proteasome, releasing an active transcription factor. RIP and RUP rely on constitutively active proteases that process substrate transcription factors when they become available and bind the protease.

Consequently, in these pathways, signal transduction regulation occurs at the substrate availability level and not by direct regulation of the proteolytic activity of the processing protease.

RAP: In the yeast *Saccharomyces cerevisiae*, we have found that transcription factor activity can be regulated by directly controlling the enzymatic activity of an intracellular protease (3). Receptor-activated proteolysis (RAP) signifies the critical involvement of a plasma membrane receptor. RAP is distinguished from the

previously described protease-based signaling mechanisms, RIP and RUP, in that it does not rely on the modulation of substrate accessibility.

The signal transduction pathway governing this transcriptional response is known as the SPS sensing pathway. Signaling requires a functional Ssy1-Ptr3-Ssy5 (SPS) sensor, the SCFGrr1 ubiquitin ligase complex, casein kinase-dependent phosphorylation, and two homologous transcription factors, Stp1 and Stp2 (1, 17, 19, 27, 29, 34, 35).

Scaffold proteins: Scaffolds play a very significant role in cell signaling pathways. The scaffold proteins provide a platform to organize and bind to both kinase and its substrate. This helps facilitate the phosphorylation of the substrate by the kinase ([17]). Scaffold proteins also protect the bound substrates from the action of phosphatases. Ptr3 acts as a scaffold protein in the SPS sensing pathway to facilitate the intra-complex protein-protein interactions to activate Ssy5.

2.3 SPS sensing pathway

In this signal transduction pathway (Fig2.1), the sensor was found to be a plasma-membrane-localized complex that comprises Ssy1, Ptr3, and Ssy5 ([14]). This plasma-membrane-localized complex is known as an SPS sensor. Ssy1 does not itself transport amino acids; instead, it functions as a receptor for external amino acids, initiating signals that are transduced to the peripherally associated plasma-membrane proteins Ptr3p and Ssy5p ([16]).

Ssy1: Ssy1 is homologous to amino-acid permease (AAP). Ssy1 differs from the other members of the family as it contains an unusually long N-terminal domain ([16]). This Amino-acid permease homolog interacts directly with the extracellular amino acids, which act as a signaling molecule, but has no amino acid transporter activity. However, it helps recruit casein kinases YCK1 and YCK2 to hyperphosphorylate and activate downstream component PTR3 in response to amino acid stimulus.

Ssy5: Ssy5 exhibits homology to chymotrypsin-like serine proteases and is expressed as a zymogen that cleaves itself into an N-terminal prodomain and a C-terminal catalytic (Cat) domain. The Cat domain contains a conserved catalytic triad

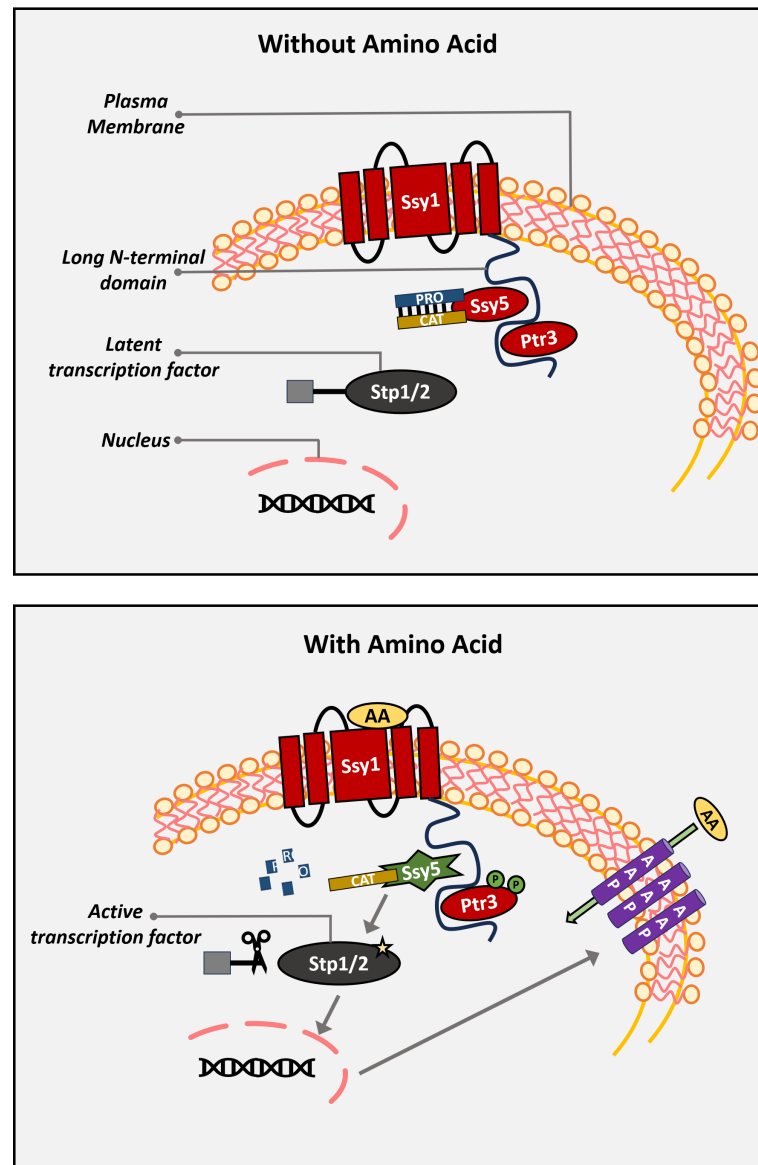


FIGURE 2.1: SPS sensing pathway.

SPS sensor is a plasma membrane-localized complex comprising Ssy1, Ssy5, and Ptr3. Without the induction of the extracellular amino acid, the prodomain remains non-covalently bound to the catalytic domain of the Ssy5 protease, inhibiting the activation of the latent transcription factors Stp1 and Stp2. In the presence of the amino acid, the Ssy1 protein bound to the plasma membrane senses the extracellular amino acid. This triggers a Ptr3-dependent multi-site phosphorylation and ubiquitination to degrade the prodomain and release the catalytic Ssy5. The activated Ssy5 protease cleaves the latent transcription factors Stp1 and Stp2, which are then translocated to the nucleus and bind to the amino acid permease (AAP) gene. This further regulates the uptake of amino acids via the AAP channel.

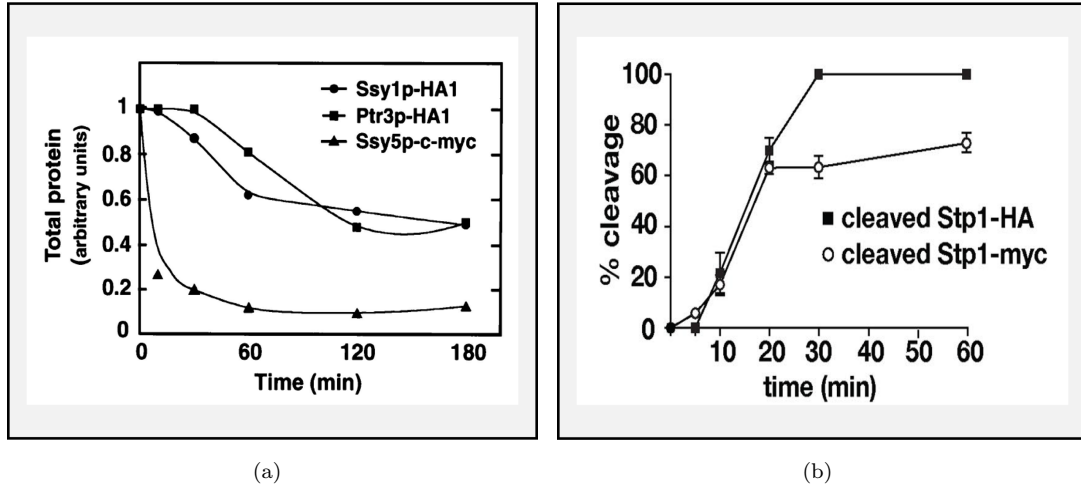
with a predicted active-site serine (S640) that is required for function. N-terminal sequencing of the Cat domain identified alanine 382 as the first residue, consistent with the notion that autoprocessing occurs between prodomain residue 381 and Cat domain residue 382.

Ptr3: Ptr3 is a phosphoprotein. With the induction of external amino acid sensed by Ssy1, Ptr3 gets hyperphosphorylated. This phosphorylation further aids in the consequent ubiquitination leading to the degradation of the prodomain and releasing the catalytic domain of Ssy5.

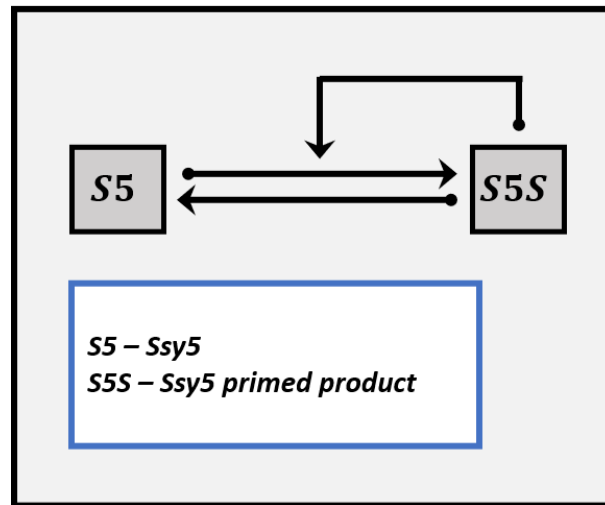
2.3.1 Mechanism:

The extracellular amino acids induce the SPS sensor by binding to the Ssy1 protein present in the plasma membrane. The Ssy1 protein then undergoes a conformational change which facilitates the activation of Ssy5 protease with the help of Ptr3. The Ssy5 is activated by degrading the prodomain to release the catalytic domain. The prodomain of Ssy5 is degraded by the hyperphosphorylation of Ptr3, which is followed by ubiquitination at the phosphorylated sites. The activated Ssy5 cleaves the N-terminal regulatory domains of the transcription factors Stp1 and Stp2. These cleaved transcription factors translocate from the cytoplasm to the nucleus to bind with the promoters of the amino acid permease (AAP) genes. This further regulates the amino acid permease channels for the uptake of extracellular amino acids. This way, the SPS sensing pathway enables the amino acids to induce their own uptake.

Based on the experimental information available for the SPS sensing pathway, the mechanism is modeled into a set of reactions. The reactions are divided into four steps which are discussed in the next section. Fig2.2 shows some of the experimental time series data for Ssy5, Ssy1, Ptr3, and activated Stp1. This data is further used for parameter estimation, which is discussed in Chapter 6.

FIGURE 2.2: *Experimental time series data.*

(a) Time series is taken from Figure 8 of [18]. The data from the curves *Ssy5p-c-myc* and *Ptr3p-HA1* were used to fit the transient concentrations of the species *Ssy5* (*S5*) and the complex *Ssy1-Ptr3* (*SP*), respectively. (b) The time series from Figure 2 of [19] is taken to fit the transient concentration of the activated transcription factor *STPa*.

FIGURE 2.3: *Autolysis of the protein Ssy5.*

The protein *Ssy5* represented by *S5* has a prodomain and a catalytic domain. The prodomain partially cleaves and binds to the catalytic domain forming the primed product *S5S*. The reactions show the conversion of *S5* to *S5S* with an autolytic step.

2.4 Steps involved in SPS sensing pathway

2.4.1 Autolysis of Ssy5

The first important step in the SPS network is the autolysis of the protein Ssy5. This protein, Ssy5, consists of both pro and catalytic domains. The partial cleavage of the pro-domain results in its non-covalent binding of the Cat-domain to give rise to the primed product protein S5S. This is an autolytic process, and presently the mechanism of this autolytic process is not clear. The function of the Cat-domain is to activate the latent transcription factors Stp1 and Stp2, and this process happens only when Ssy5-cat releases itself from the clutches of the Pro-domain. The prodomain is required for the maturation of the protease and subsequently functions as a potent inhibitor that prevents Ssy5 proteolytic activity in the absence of signaling. Consistent with an inhibitory function of the prodomain, a tight inverse correlation exists between prodomain levels and Stp1 processing. The processing of the prodomain within the zymogen is frequently associated with a conformational change that suffices to fully activate the protease (18, 41, 45). In contrast to this established concept, Ssy5 requires a second signal-mediated activation step. Fig2.3 shows the autolysis of Ssy5 protease.

The following facts were taken from the experiments based on which the reactions were taken as shown in Fig2.3:

1. C-terminal region of Ssy5 has the amino acid triad H-histidine, D-aspartic acid, and S-serine), constituting the catalytic site for proteases and the distribution of the triad resulting in the loss of proteolytic activity ([20]).
2. Under Ssy5 overproduction, self-processing occurs independent of the other two plasma membrane proteins of the SPS sensor, namely, Ssy1 and Ptr3. The high dosage of Ssy5 seems to favor Ssy5-Ssy5 contacts to facilitate self-processing in a Ptr3-independent manner.
3. A sequence of Ssy5 region favors autolytic cleavage by Ssy5-Ssy5 interaction that is well conserved, and the integrity of this region is important for autolytic cleavage.

4. Ssy5 autolytic reaction and processing occur independent of external amino acid. Therefore, these reaction steps must occur upstream of amino acid-mediated SPS complex stabilization.

2.4.2 Stabilisation of SPS

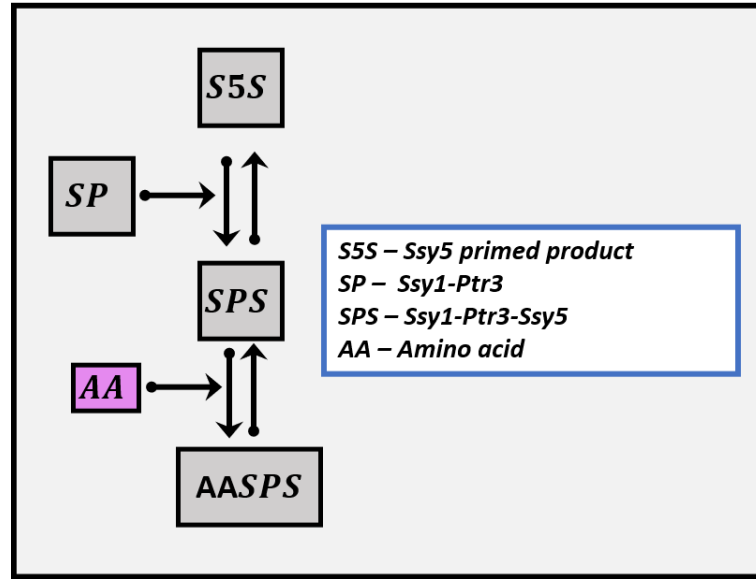


FIGURE 2.4: *Stabilisation of SPS.*

The complex of *Ssy1* and *Ptr3* is taken as *SP*. The binding of the inactive primed *Ssy5* with *SP* forms an unstable complex *SPS*. The binding of the external amino acid to the *Ssy1* receptor in *SPS* complex stabilized the complex.

We take the interactions between the receptor protein *Ssy1* and the adapter *Ptr3* together as one complex *SP*. This complex is the sensor for external amino acids. ([20]) The *Ssy5* primed product, *S5S*, obtained as a result of the autolysis, is inactive. The inactive *S5S* binds to *SP* to form an unstable, nonsignalling sensor complex *SPS* (*Ssy1-Ptr3-Ssy5*) as shown in Fig2.4. There is a reversible switch between the signaling and non-signaling complexes, which we have not captured in the reaction. The binding of amino acid *AA* to the signaling complex *SPS* forms a conformationally active stable complex *AASPS* that involves further in the signaling process.

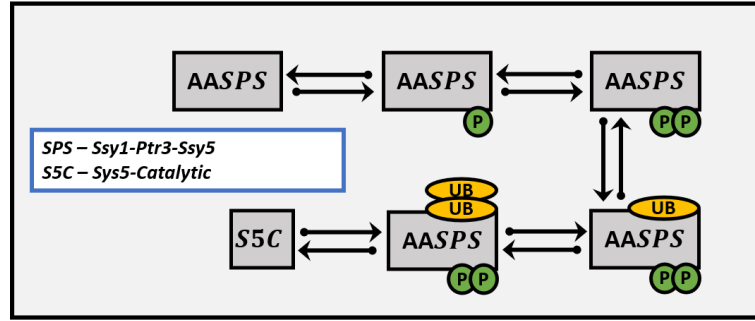


FIGURE 2.5: Release of catalytic Ssy5.

The amino acid-bound SPS complex undergoes phosphorylation. The Yck1/2-catalyzed phosphorylation has been shown to lead to Grr1-dependent polyubiquitylation and subsequent degradation of the prodomain by the 26S proteasome. This releases the catalytic Ssy from the clutches of the prodomain.

2.4.3 Release of Catalytic Ssy5

The release of the Ssy5 catalytic domain involves four critical steps in what is called the receptor-activated proteolysis (RAP) of the SPS sensor signaling system.

1. Autolysis of Pro-Ssy5 that non-covalently binds to Cat-Ssy5 side to form a primed Ssy5 and inhibit Ssy5-cat function.
2. Complexation of primed Ssy5 with Ssy1-Ptr3 to form a functional Ssy1-Ptr3-Ssy5 sensor that senses extracellular amino acid to form a complex, AA-SPS.
3. Phosphorylation-ubiquitylation reactions of the pro-domain in AASPS that releases the Ssy5-Cat to free from the pro-domain.
4. Finally, processing of the latent transcription factor Stp1 by the unfettered protease Ssy5-Cat.

The processing endoprotease Ssy5 is regulated via proteasomal degradation of its noncovalently associated N-terminal prodomain. We find that degradation of the prodomain requires a conserved phosphodegron comprising phosphoacceptor sites and ubiquitin-accepting lysine residues. Upon amino acid induction, the phosphodegron is modified in a series of linked events by a set of general regulatory factors involved in diverse signaling pathways. First, an amino acid-induced conformational change triggers phosphodegron phosphorylation by the constitutively active plasma membrane-localized casein kinase I (Yck1/2). Next, the prodomain becomes a

substrate for polyubiquitylation by the Skp1/Cullin/Grr1 E3 ubiquitin ligase complex (SCFGrr1). Finally, the modified prodomain is concomitantly degraded by the 26S proteasome. The phosphorylation and subsequent ubiquitination of the amino acid-bound SPS complex is shown in the Fig2.5.

Phosphodegrom: A conserved phosphodegrom comprises phosphoacceptor sites and ubiquitin-accepting lysine residues ([21])

Paradoxically, protein degradation can have activating functions. For example, the degradation of an autoinhibitory domain or regulatory inhibitory subunit within a protein complex can liberate an intrinsic catalytic activity. Understanding the mechanisms governing regulated proteolysis is an important task in cell biology.

2.4.4 Activation of Stp1/2

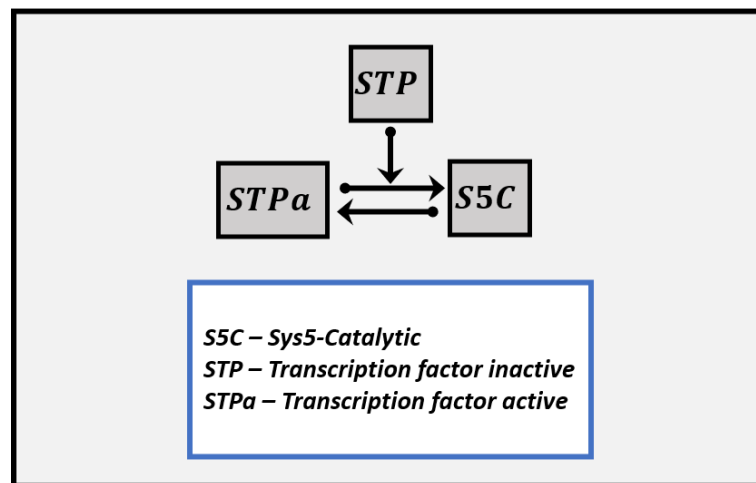


FIGURE 2.6: *Activation of transcription factor Stp1/2.*

The transcription factors *Stp1* and *Stp2* are represented by *STP*. The activated transcription factor is represented by *STPa*. The *Ssy5* Catalytic domain (*S5C*) activates the latent transcription factor for the regulation of the uptake of extracellular amino acids.

The final step involves the enzymatic activation of the latent transcription factor *STP* by the catalytic *Ssy5*, *S5C*. The active transcription factor *STPa* is the processed transcription factor that is required for the transport of extracellular amino acid to the cytoplasm by taking part in the gene regulatory interactions to release the amino

acid permease for the uptake of the external amino acid. Fig2.6 shows the reactions used for the activation of Stp.

2.5 Summary

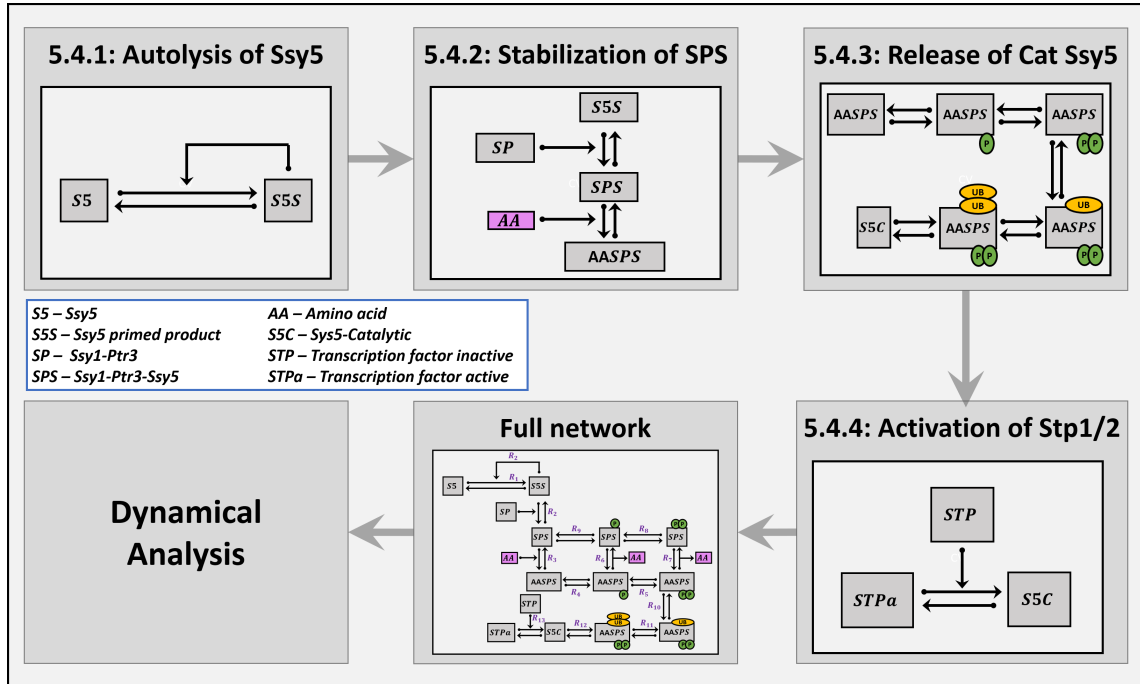


FIGURE 2.7: Steps involved in SPS sensing pathway.

(i) The SPS pathway starts with the autolysis of Ssy5. (ii) The SPS (Ssy1-Ptr3-Ssy5) complex is unstable and is stabilized by the external amino acid. (iii) The binding of the external amino acid triggers a series of reactions involving multi-site phosphorylation and polyubiquitination, which releases the catalytic domain of Ssy5. (iv) The Catalytic Ssy5 cleaves the latent transcription factor Stp1/2 facilitating its translocation to the nucleus to regulate amino acid permease for the uptake of extracellular amino acid.

The amino acid-induced activation of the transcription factor Stp1/2 in the SPS pathway of yeast cells follows four major steps. The first step is the autolysis of Ssy5 (section: 2.4.1), which happens prior to the interaction with the extracellular amino acids. In the presence of the amino acid, the complex SPS (Ssy1-Ptr3-Ssy5) is stabilized by binding with the amino acid (section: 2.4.2). The binding of the amino acid triggers the release of the catalytic domain of Ssy5 (section: 2.4.3). Finally, the released catalytic Ssy5 cleaves the transcription factor Stp1/2 (section: 2.4.4),

facilitating its translocation to the nucleus, where it can regulate the amino acid permease gene for the uptake of extracellular amino acid.

In Chapter 6, we construct a deterministic mathematical model based on the detailed molecular mechanism of receptor-associated proteolysis (RAP) that controls the catalytic part of the Ssy5 activation. The goal is to capture the occurrence of the Ssy5 conformational switch that releases the cat-domain from the pro-domain for processing of the latent transcription factor Stp1.

For each of the above steps, we first construct the molecular network of the SPS signaling pathway based on the available experimental data and convert the influence diagram into a nonlinear ordinary differential equations model based on the laws of the mass action kinetics. We also use chemical reaction network theory (CRNT) and SR graphs to determine whether our signaling network is capable of exhibiting multistability, independent of the kinetic parameters. It is also of interest to us to determine the role of each molecular step in the network in contributing to the activation of latent transcription factors.

Chapter 3

Chemical Reaction Network Theory (CRNT): Structural analysis of reaction networks

3.1 Introduction

Analyzing and understanding the response of the biological networks to input stimulus is important to understand the role of networks under normal and pathological conditions. Motifs are small biological networks that are part of the network yet function independently and have a function on their own. Examples of motifs are positive and negative feedbacks and feedforward networks that exhibit multistability, oscillations, and rapid response to the input stimulus. In this thesis, we look into the structural and kinetic aspects of the network using the tools of reaction network theory and dynamical systems and apply them to receptor-mediated proteolysis.

Chemical Reaction Network Theory (CRNT) is used to study the behavior of complex chemical reaction networks and to analyze the structure of the network based on a mathematical framework. The reactions are represented as a network, where nodes represent the species and edges represent the chemical reactions between them. The reactions are described in terms of mass action kinetics. The key objective of CRNT is to understand the behavior of chemical systems solely based on the underlying network regardless of the values of the kinetic parameters ([13]). However, the

theorems of CRNT only work for networks that follow mass action kinetics. CRNT provides theorems to determine the existence and multistability. CRNT Toolbox, BioSwitch, and CRNT4SBML are some of the tools which use the concepts of CRNT to perform structural analysis of a given network.

In this chapter, we first provide the background on the terminologies and basics of CRNT by taking an example, and then we compare the performances and analyze the pros and cons of various freely available CRNT toolboxes that give information about the multistability of the biological network.

3.2 Illustration: Signal transduction network

In a Signal transduction pathway, a series of molecular events converts the input stimulus to the cell's surface and converts a very specific response inside the cell Fig3.1. There are a number of steps that are elucidated in the signal transduction pathway through intense experiments over a period of time. Broadly, any signal transduction pathway starts with (I) input stimulus, usually hormones or ligands. The secreted ligands, a signaling molecule, bind to a cell-specific receptor protein that is perched on the top of the membrane. After binding of ligand to receptor results in a cascade of reactions through some proteins, generally called relay proteins, that reaches the cell target. Finally, end proteins activate the transcription factors that transcription of a specific gene. Finally, the translated protein performs the requisite function. Therefore, the signal transduction pathway is an important pathway that tells the cell about the external happenings to the cell so that it can adapt and prepare for any eventuality, be it good or bad. ([22]). Fig3.1.

We have taken a simple motif of the signal transduction pathway ([23]), as shown in Fig3.1, for an illustrative purpose to show how structural analysis is carried out by the CRNT.

In our network example, we have an activated protein S, activated and converted to protein Sp by an enzyme E1. The enzyme E2, in turn, deactivates Sp back to S. Also, the protein S self-activates to form a complex SSp. The following are the chemical reaction steps for the example signal transduction network that has only unimolecular and biomolecular reactions.

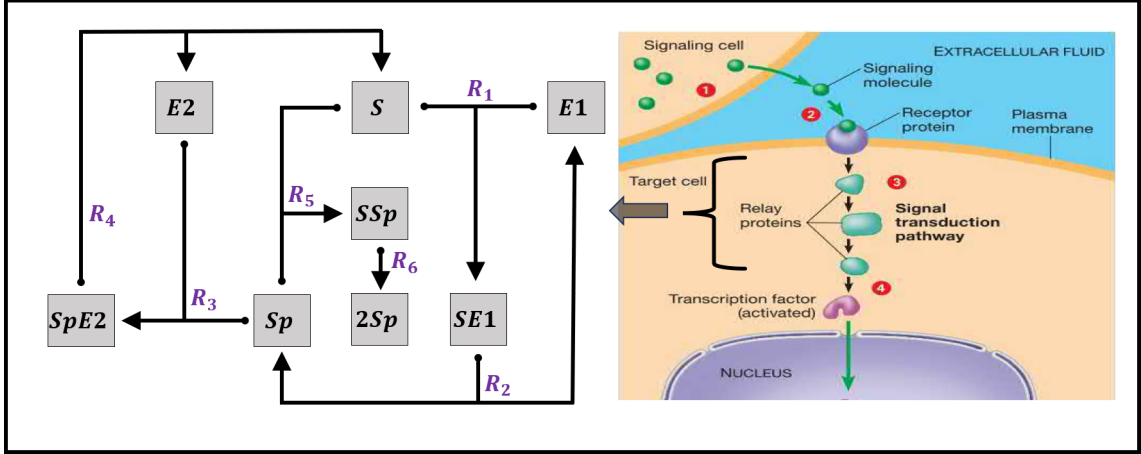
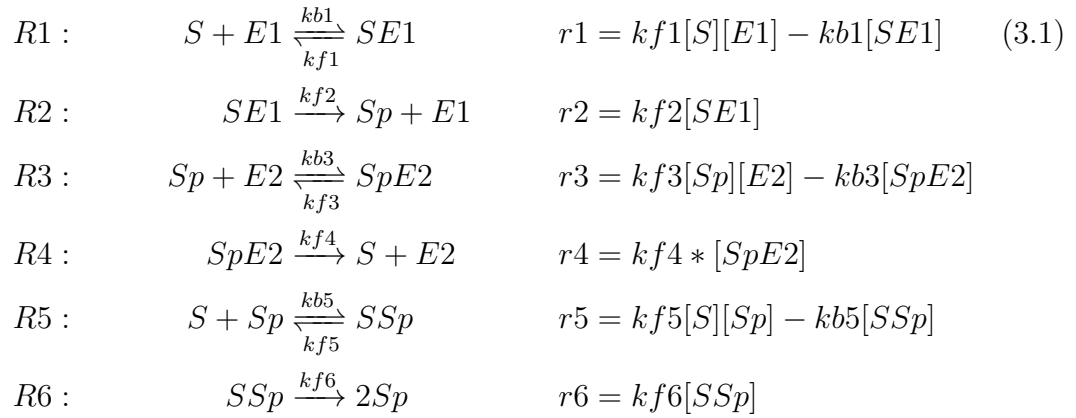


FIGURE 3.1: An illustrative network of signal transduction.

The network represents a closed signal transduction motif. The protein S is activated to protein Sp by the enzyme $E1$. The enzyme $E2$, in turn, deactivates Sp back to S . Further, the protein S activates itself by forming a complex SSp .

The reaction steps for the network shown in Fig3.1 are given below



In the above chemical steps, Ri 's are the reaction channels, and ri 's are the corresponding rates of the reactions with rate constants kfi 's.

Note that though we have provided all the chemical reaction steps, knowing the kinetic constants are not necessary for the structural analysis of the network. Importantly, our work aims to determine whether the structure exhibits multistability. In our network, by visual inspection, we can not find any positive feedback in the motif. However, various cases are known where the positive feedback is implicit and can lead to multistability. Markevich et al. are the first to show that the MPAK signal

transduction motif that is doubly phosphorylated has implicit feedback, and this motif is shown to exhibit bistability by Markevich et al. ([11]) It can also be said that if we can find the presence of bistability by the structural analysis of the motif using CRNT, then we can say for sure the presence of positive feedback in the motif. This is because the presence of positive feedback is the necessary but not a sufficient condition for bistability for the choice of kinetic parameters ([24]); therefore, CRNT analysis of biological network is an important and powerful tool that can be effectively employed for identification of multistability in a network. In the next section, we provide only necessary terminologies used in CRNT analysis to allow readers to follow the rest of the thesis. For in-depth details, the readers can look into [13].

3.3 CRNT terminologies

In this section, we present below some of the basic terminologies used in CRNT. We use the network in Fig3.1 as an example to describe the terms.

Complexes: The complex of a reaction network are the objects at the heads and tails of the reaction arrows. In the illustration 3.1, the complexes are $\{S + E1\}$, $\{SE1\}$, $\{Sp + E1\}$, $\{Sp + E2\}$, $\{SpE2\}$, $\{S + E2\}$, $\{S + Sp\}$, $\{SSp\}$ and $\{2Sp\}$.

Linkage classes: The linkage classes of a network are sets of complexes that make up a network. Two complexes C and C^* are directly linked if $C \rightarrow C^*$ or they can also be linked if there is a sequence of complexes $C1, C2, \dots, Cn$ such that $C \leftrightarrow C1 \leftrightarrow C2 \leftrightarrow \dots \leftrightarrow Cn \leftrightarrow C^*$. In the illustration 3.1, there are three linkage classes $S + E1, SE1, Sp + E1, Sp + E2, SpE2, S + E2$, and $S + Sp, SSp, 2Sp$.

Rank: The rank (r) of a reaction network can be defined as the number of linearly independent reactions of the network. It can be determined from the stoichiometric matrix of a given network. The stoichiometric matrix of the illustration 3.1 is given in table 3.1. The rank of the illustration network is 4.

TABLE 3.1: Stoichiometric matrix for the example network shown in Fig3.1

	S	Sp	E1	E2	SE1	SpE2	SSp
R1	-1	0	-1	0	1	0	0
R2	0	1	1	0	-1	0	0
R3	0	-1	0	-1	0	1	0
R4	1	0	0	1	0	-1	0
R5	-1	-1	0	0	0	0	1
R6	0	2	0	0	0	0	-1

Deficiency: The deficiency of a network given, the number of complexes (c), the number of linkage classes (l), and the rank of the entire network (r) is given by the equation 3.2.

$$\delta = c - l - r \quad (3.2)$$

3.4 Bistability

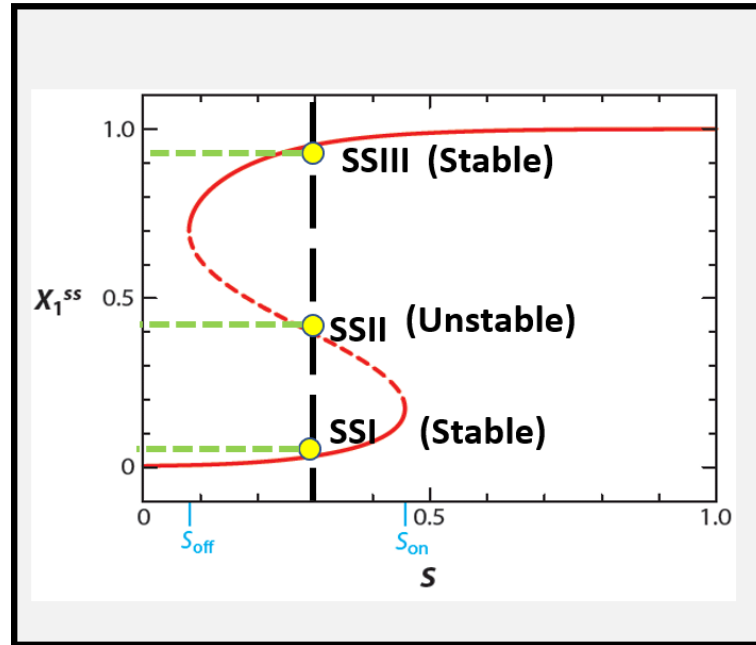


FIGURE 3.2: Bifurcation diagram with X_1 as the steady state and S as the parameter. The yellow dots represent the three steady states SSI, SSII, and SSIII. The steady states SSI and SSIII are stable, while SSII is unstable. The stable steady state SSI is the deactivated state, and SSIII is the activated state represented as S_{off} and S_{on} respectively.

A bistable network is a reaction network with two coexisting stable, steady states separated by an unstable steady state. Consider a nonlinear ODE of the form given in equation 3.3 where equation 3.4 is the steady state equation, and S is the parameter. Fig3.2 shows the bifurcation diagram with X_1 as the steady state with S as the varying bifurcation parameter. The steady states SSI and SSIII are stable states which are separated by the unstable state SSII.

$$\frac{dx}{dt} = F(x_1, x_2, \dots, x_n; S) \quad (3.3)$$

$$F(x_1, x_2, \dots, x_n; S) = 0 \quad (3.4)$$

Multistability can be classified into reversible and irreversible transitions. Fig3.3 shows the classification of multistability. In reversible transitions, it is possible for the system to transit from one stable, steady state to the other from both directions. The Saddle Node bifurcation, where both the limit points are in the biologically feasible range (positive), comes under the reversible transition. Irreversible transitions have three cases, Type-1, Type-2, and the third case, where limit points are moved to infinity. Here, either one of the limit points is either negative or infinity (Type-1 and Type-2, respectively). In types 1 and 2, the transition is irreversible. Special cases of multistability, such as Mushrooms (four limit points) and Isolands (two limit points), also exist.

3.5 Deficiency in CRNT determines a network's presence or absence of multistability.

The capacity of a network to admit multistability is determined by the deficiency of the network. The deficiency of the network can be zero, one, or greater than one. Based on the deficiency of the network, deficiency theorems such as the deficiency zero, deficiency one, or higher deficiency theorem can be applied. The deficiency zero and one theorems are classified as lower deficiency theorems. The deficiency zero theorem states that, for any kinetics, the differential equations admit no positive equilibrium nor a periodic composition trajectory containing a positive composition, and hence,

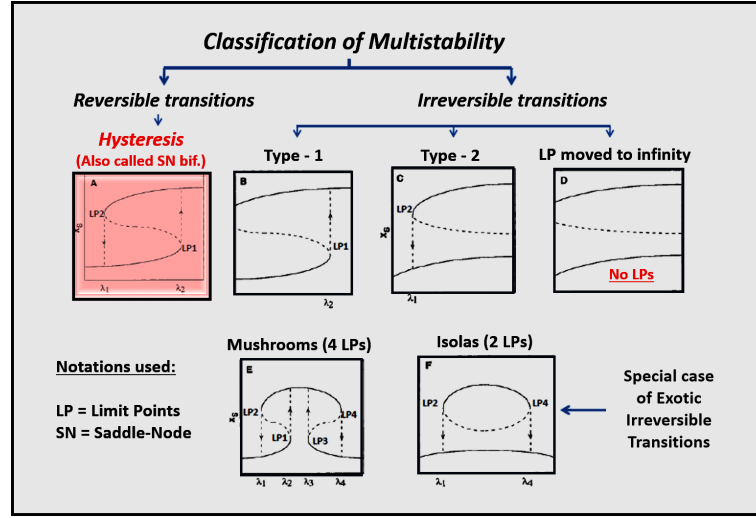


FIGURE 3.3: Classification of Multistability.

Multistability either follows reversible transitions or irreversible transitions. Hysteresis, also called the Saddle Node bifurcation, comes under the reversible transition. Irreversible transitions have three cases, Type-1, Type-2, and the third case, where limit points are moved to infinity. Special cases of multistability, such as Mushrooms (four limit points) and Isolals (two limit points), also exist.

TABLE 3.2: Networks and their capacity for bistability as detected by CRNT.

S.No	Network	Remark	Deficiency	Bistability	Ref
1	$E + S \leftrightarrow ES \rightarrow E + P$	Enzyme catalysis following Michaelis-Menten kinetics	0	NO	[12]
2	$E + S \leftrightarrow ES \rightarrow E + P$ $E + I \leftrightarrow EI$ $ES + I \leftrightarrow ESI \leftrightarrow EI + S$	Enzyme catalysis with mixed inhibition	1	NO	[12]
3	$E1 + S1 \leftrightarrow E1S1$ $E1 + S2 \leftrightarrow E1S2$ $E1S1 + E1 \leftrightarrow E1S1E1 \rightarrow E1S2 + E1$ $S2 + E2 \leftrightarrow E2S2 \rightarrow S1 + E2$	Subnetwork of G_1/S model	1	YES	[25]
4	$X \rightarrow X + P$ $P \rightarrow \phi$ $P + P \rightarrow PP$ $X + PP \leftrightarrow XPP$ $XPP \rightarrow XPP + P$	Positive autoregulatory network	2	YES	[26]

the network will not admit multistability. The deficiency theorem determines whether the network under mass action kinetics has the capacity for multiple steady states for deficiencies equal to and greater than one.

3.5.1 Toolbox-1: CRNT

In this section, we will illustrate how to use the freely available CRNT toolbox from the Feinberg group to perform CRNT analysis. The screenshot of the CRNT front end is shown in the Fig-3.4 (https://zenodo.org/record/5149266#.YSE_3d8pCUk).

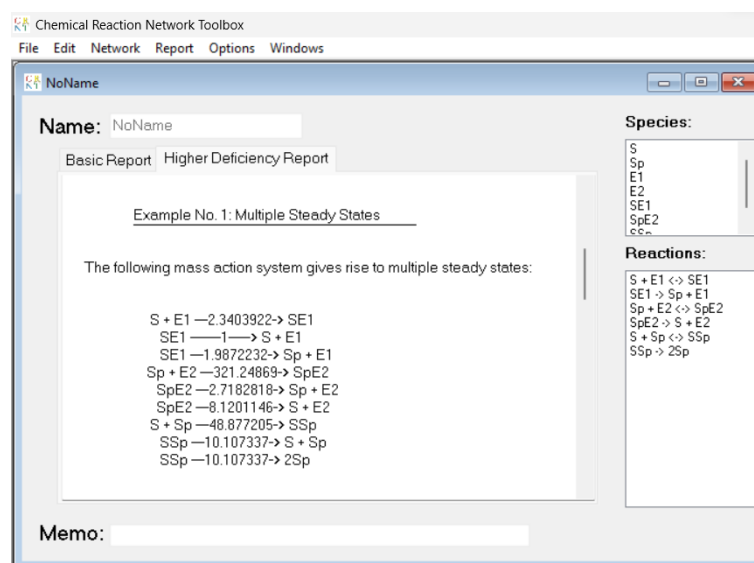


FIGURE 3.4: CRNT Toolbox Higher deficiency report.

When a network has a deficiency greater than 1, the CRNT toolbox allows the user to perform the Higher Deficiency report. This report shows whether the network is capable of multiple steady states. Also, the report generated a parameter set for which the network can give rise to bistability. This figure shows the parameter set generated by CRNT Toolbox in the Higher Deficiency report.

The input to the CRNT toolbox is the chemical steps of the reaction network without the kinetic constants. The toolbox automatically generates a file with an extension .NET. The toolbox also provides all the necessary information such as the number of complexes, linkage classes, rank, and the deficiency of the network. Based on the deficiency of the network, further analysis can be performed by generating the deficiency one or higher deficiency report.

This also provides information like when the deficiency of a network is zero, there is no set of ordinary differential equations for which the corresponding mass action kinetics of the network will exhibit multiple steady-states. When the deficiency of the network is one, the possibility that the system can admit multiple steady-states is given by the deficiency one report. For deficiency greater than one, the higher

deficiency report will give the possibility that the system admit multiple steady-states. In case the system admits multiple steady-states, both the deficiency one and higher deficiency reports will generate a plausible set of parameters for the network that gives rise to multistability.

Apart from the deficiency reports, we can also generate reports for Zero Eigenvalue, Mass Action Injectivity, Concordance, Strong Concordance, and Arbitrary Influences Concordance. It can also perform Network Degeneracy Test and gives the ODEs in LaTeX format.

CRNT toolbox, though robust and easy to work with is a DOS-based toolbox. The toolbox cannot take graph as an input. With the advent of many new graph representation of biological networks, one may possibly directly route the network as input to the CRNT toolbox for analysis. Many pathway graphs like KEGG ([27]) are available that may used for network analysis without writing the detailed chemical steps. Further, CRNT will not provide mass conservation or compute bifurcation diagrams. The next two toolboxes has such facilities which we explain in detail below.

3.5.2 Toolbox-2: BioSwitch

The software BioSwitch is a MATLAB-based toolbox. BioSwitch provides an automated multistability detection for gene-regulatory networks and signaling pathways ([28]). Bioswitch combines the methods introduced by [23] with the methods of CRNT for detection. For BioSwitch, the input network should be given as a C-graph. The function for the input network is given in the code 3.8.1 given below. The input network C-graph is given into the function *BioSWITCH_Evaluate*, which computes necessary information about the network, such as the mass conservation laws and deficiency. Further, *BioSWITCH_Lpsearch* searches for limit points and if the limit points are present, *BioSWITCH_Ncontin* computes the bifurcation diagram.

The analysis can be implemented for networks with (mass-conservation approach) or without mass conservation (semi-diffusive approach). The global optimization is used as the optimization method. Further, there is a provision to include known information regarding the parameters along with the solver options.

Mass conservation approach: The mass conservation approach is meant for closed systems where the input signal is the total amount of signal molecules. The decision vector is defined in terms of kinetic constants and concentrations.

Semi-diffusive approach: In the case of open systems, the semi-diffusive approach can be implemented. Unlike the mass conservation approach, the PCP should be the kinetic constant of the most influencing or key reaction, which serves as a signal to the motif.

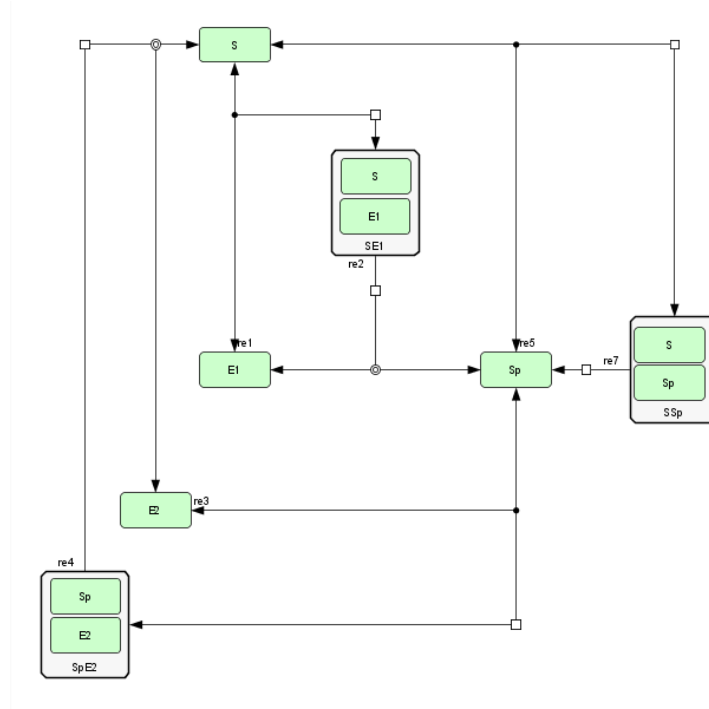
The illustration is a closed version of the signal transduction motif. Hence, the mass-conservation approach is used to perform the structural analysis. The MATLAB code (3.8.2) used to perform the analysis is given below.

3.5.3 Toolbox-3: CRNT4SBML

The CRNT4SBML is a Python package built based on the concept of CRNT. Similar to the BioSwitch in MATLAB, CRNT4SBML is designed exclusively to detect bistability in biochemical reaction networks. This tool is made to simplify the detection of bistability by automating the computational steps using the interface, systems biology markup language (SBML), which is a standard format for signaling pathways ([29]).

CRNT4SBML allows the implementation of mass conservation and semi-diffusive approaches using optimization and numerical continuation. The numerical continuation helps in identifying the stable and unstable branch points. The branches are identified by varying the principle continuation parameter (PCP). The PCP represents the input signal. This method also includes a multi-start optimization routine that is generalized for both approaches. Upon the detection of bistability, an input-response curve (bifurcation diagram) is generated.

CRNT4SBML requires Python version 3.7, so it is best to create a virtual environment to run CRNT4SBML. The input should be given as an SBML file. The SBML file can be generated using the software called CellDesigner. The network as given in Fig-3.1 in CellDesigner is shown in Fig-3.5. A basic report of the network can be generated. If the network has a deficiency as zero or one, the Low Deficiency Approach can be used to check if the Deficiency zero or one theorem is satisfied. In the presence of a

FIGURE 3.5: *Network representation in CellDesigner.*

To generate the SBML file for the network given in Fig 3.1, the complex must be interconnected in the CellDesigner, as shown in the figure. Here the reactions fall under two categories, Heterodimer Association (R1, R3, and R5) and Dissociation (R2, R4, and R6). The individual species were created using the icon *Generic Protein*, and the complexes were created using in icon *Complex*.

mass conservation relationship, the network can be analyzed using two approaches, Mass conservation and the General approach. In the case of the Mass Conservation Approach, the network should be uniterminal. A network is uniterminal when every linkage class of the network has only one terminal strong linkage class. If there is no mass conservation relation, and there is a presence of a boundary species the network can be analyzed using a Semi-diffusive Approach. The illustration given in Fig-3.1, is a uniterminal network, and hence, Mass Conservation Approach was used to analyze the network.

3.5.4 Results: CRNT Toolbox, BioSwitch, CRNT4SBML

3.5.4.1 Basic report

The network given in Fig3.1 consists of 7 species, 9 complexes, 3 linkage classes, 3 terminal strong linkage classes, and 3 non-terminal strong linkage classes. The rank of the entire network is 4, and the deficiency of the network is 2. The basic report generated by CRNT Toolbox, BioSwitch, and CRNT4SBML is summarised in table3.3. The basic report of CRNT Toolbox is comparatively more detailed than the other two software as it contains information about the strong linkage classes. However, CRNT Toolbox does not give any information regarding the mass conservation relationships.

- Species: S, Sp, E1, E2, SSp, SE1, and SpE2
- Complexes: $\{S + E1\}$, $\{SE1\}$, $\{Sp + E1\}$, $\{Sp + E2\}$, $\{SpE2\}$, $\{S + E2\}$, $\{S + Sp\}$, $\{SSp\}$ and $\{2Sp\}$
- Linkage classes: $\{S + E1, SE1, Sp + E1\}$, $\{Sp + E2, SpE2, S + E2\}$, and $\{S + Sp, SSp, 2Sp\}$
- Terminal Strong linkage classes: $\{Sp + E1\}$, $\{S + E2\}$, and $\{2Sp\}$
- Non-Terminal Strong linkage classes: $\{S + E1, SE1\}$, $\{Sp + E2, SpE2\}$, and $\{S + Sp, SSp\}$

There are three mass conservation relationships in the network.

1. Conservation law 1: $E2_T = E2 + SpE2$
2. Conservation law 2: $E1_T = E1 + SE1$
3. Conservation law 3: $S_T = S + SE1 + 2*SSp + Sp + SpE2$

TABLE 3.3: The Summary of reports generated by CRNT Toolbox, BioSwitch, and CRNT4SBML

	CRNT Toolbox	BioSwitch	CRNT4SBML
Species	×	✓	✓
Complexes	✓	✓	✓
Linkage Classes	✓	✓	✓
Terminal strong linkage classes	✓	×	×
Non-Terminal linkage classes	✓	×	×
Rank	✓	✓	✓
Deficiency	✓	✓	✓
Mass Conservation	×	✓	✓
ODEs	✓	✓	✓
Parameter sets for Bistability	✓	✓	✓
Parameter Units	×	×	✓
Bistability plots	×	✓	✓

3.6 Codimension-1 bifurcation of the reaction network based on all three toolboxes

CRNT Toolbox: The CRNT Toolbox generates a parameter set for bistability in the Deficiency One report ($\delta = 1$) and Higher Deficiency report ($\delta > 1$) in case the network has the capacity for multiple steady states. The parameters generated by CRNT Toolbox are not provided with units. Hence, we have assumed the concentration units to be molar(M) and the time in seconds (s). The bifurcation diagram obtained using the parameter set generated by this tool is shown in Fig3.6a.

BioSwitch: BioSwitch not only generates the parameter set for bistability but also gives the bistable plot as output. It also allows the user to select the continuation parameter and the species for which the bistable plots are required. It allows the user to set the upper and lower bounds and also lets the user enter the values of those parameters that are already known. The bifurcation diagram obtained using the parameter set generated by this tool is shown in Fig3.6b

When the MATLAB code for BioSwitch as given in 3.8.2, is run, the following output files are generated.

- Evaluation_illustration.txt

TABLE 3.4: Parameters for bistability generated by CRNT Toolbox, BioSwitch, and CRNT4SBML

Kinetic Constants	CRNT Toolbox	BioSwitch	CRNT4SBML
kf1 ($M^{-1}s^{-1}$)	2.34	0.1235	2e+6
kb1 (s^{-1})	1	0.0122	7.22e-4
kf2 (s^{-1})	1.99	0.1197	8.347e-2
kf3 ($M^{-1}s^{-1}$)	321.25	100	9.9e+7
kb3 (s^{-1})	2.72	0.0122	9.54e-4
kf4 (s^{-1})	8.12	31.0678	1e-1
kf5 ($M^{-1}s^{-1}$)	48.88	62.2934	5.2e-7
kb5 (s^{-1})	10.1	63.0112	6.92e-4
kf6 (s^{-1})	10.1	34.8846	3.76e-1
E1_T (M)	2.16	0.0205	2.8e-7
E2_T (M)	1.6	0.2439	4.9e-7
S_T (M)	5.9	1.1004	8.36e-7

- `illustration_input_cont_MassCon_default.m`
- `illustration_ode_MassCon.m`
- `illustration_input_lpsearch_MassCon_default_Results.m`
- `illustration_param_MassCon.m`
- `illustration_input_lpsearch_MassCon_default.m`
- `illustration_ObjF_DEF.m`
- `illustration_manifold.txt`

CRNT4SBML: In the presence of bistability, CRNT4SBML generates multiple sets of parameters for the network exhibit bistability. For the selected species and the parameter, CRNT4SBML also generates bifurcation diagrams for all the parameter sets. The parameter sets can be selected by using the code 3.8.4. The parameters given by CRNT4SBML have the concentration units as pico molar (pM) and the time in seconds (s). The bifurcation diagram obtained using the parameter set generated by this tool is shown in Fig3.6c. The parameters were converted to molar (M) to maintain uniformity.

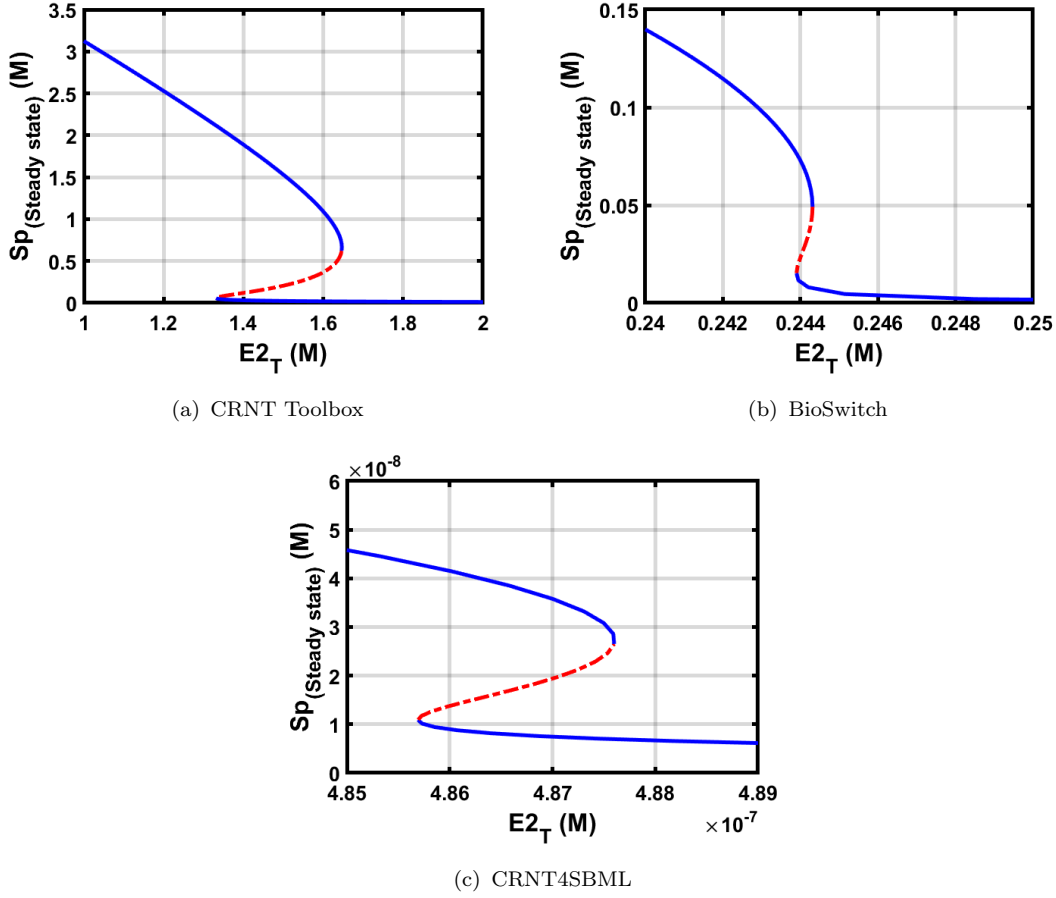


FIGURE 3.6: *Bistability diagram of Sp with total $E2$ ($E2_T$) as the parameter. The sets were obtained from CRNT Toolbox (a), BioSwitch (b), and CRNT4SBML (c). The bistability parameters for the network given in Fig 3.1 generated by the above-mentioned tools were used to make the figures. The figures were obtained from XPPAUT [30] and generated using MATLAB [31]*

3.7 Summary

1. CRNT Toolbox gives a detailed basic report which also includes information regarding the Terminal and Non-Terminal strong linkage classes.
2. Though CRNT Toolbox gives a detailed basic report, it does not give any information regarding the mass conservation relationship. Also, it does not generate bistability plots.

3. Unlike CRNT Toolbox, the other tools BioSwitch and CRNT4SBML, lets the user manually set the upper and lower bounds for the parameters based on the physiological range.
4. Both BioSwitch and CRNT4SBML generate a bistability plot based on the continuation parameter and species selected by the user. The plot is generated with the generated parameter set.
5. Additionally, CRNT4SBML generates multiple parameter sets that give rise to bistability along with a plot for every parameter set.
6. BioSwitch and CRNT4SBML have provisions for generating further results after the generation of parameters. However, CRNT Toolbox is a quick way to get the structural information along with the detection of bistability and generation of the parameters for any network.

3.8 Codes

3.8.1 Code: Bioswitch Input

MATLAB

```
% Function for input network in BioSwitch
function [ESP, COM, KNS] = illustration

ESP={'S','Sp','E1','E2','SSp','SE1','SpE2'};
COM={'S+E1','SE1','Sp+E1','Sp+E2','SpE2','S+E2','S+Sp','SSp','2*Sp'};
KNS={'k_1_2' 'k_2_1' 'k_2_3' 'k_4_5' 'k_5_4' 'k_5_6' 'k_7_8' 'k_8_7' 'k_8_9'};

end
```

3.8.2 Code: Run BioSwitch

MATLAB

```
BioSWITCH_Startup

display_message('Evaluate Network');
BioSWITCH_Evaluate('illustration') % To evaluate the network

display_message('Generate files for limit point search ...
(system with mass conservation)');
BioSWITCH_mkfiles_lpsearch_MassCon('illustration')
% creating files for limit point search

display_message('Search for limit points (deficiency approach)');
BioSWITCH_Lpsearch('illustration_input_lpsearch_MassCon_default')
% search for limit points

display_message('Generate files for continuation ...
(system with mass conservation)');
% indices to suppress from the odes
% Check Evaluation_illustration.txt to create the input vector.
% If the order of the species is changed, the input vector should be
% rewritten and the program should be run from the beginning

BioSWITCH_mkfiles_ncontin_MassCon('illustration', [4 3 1]) % MUST BE IN ORDER
```

```

display_message('Check steady state and perform continuation ...
(original network with conservation laws)');
% The file "illustration_input_cont_MassCon_default" can be edited to change the
% variable and the bifurcation parameter.
% The initial conditiions and the parameter values can be extracted from
% this file.
BioSWITCH_Ncontin('illustration_input_cont_MassCon_default')

% Steady state and parameter values
load illustration_input_lpsearch_MassCon_default_Results.mat
xbest = Results.xbest;
odefunc = illustration_ode_MassCon;
[ss, par] = illustration_param_MassCon(xbest);
[s,c,k] = illustration; % species, complex and rate constants
a = size(k);
a = a(2);
d = size(par) - size(k);
d = d(2);
for i = 1:d
    k{1,a+i} = string('C'+string(i));
end
display_message('Steady state concentration of the respective species');
disp(s)
disp(ss');
display_message('Respective parameter values');
disp(k)
disp(par')

```

3.8.3 Code: Run CRNT4SBML

Python

```

import crnt4sbml
network = crnt4sbml.CRNT("C:\Harika_IIITD\Thesis\CellDes\Millnew.xml")
approach = network.get_mass_conservation_approach()
print(approach.get_decision_vector())
print(approach.get_concentration_bounds_species())
bnds, conc_bnds = approach.get_optimization_bounds()
bnds
conc_bnds
num_itr = 100
import numpy
sys_min=numpy.finfo(float).eps
sd=0
prnt_flg=False
num_dtype=numpy.float64

```

```

params_for_global_min, obj_fun_val_for_params = approach.run_optimization \
    (bounds=bnds, \
     concentration_bounds=conc_bnds, iterations=num_itr, seed=sd, \
     print_flag=prnt_flg, numpy_dtype=num_dtype, sys_min_val=sys_min)
approach.generate_report()
numpy.save('params.npy', params_for_global_min)
params_for_global_min = numpy.load('params.npy')
print(approach.get_conservation_laws())
spcs="s2"
PCP_x="C2"
multistable_param_ind, plot_specifications = \
    approach.run_greedy_continuity_analysis(species=spcs, \
     parameters=params_for_global_min, dir_path="./stability_graphs_ill", \
     auto_parameters={'PrincipalContinuationParameter': PCP_x})
approach.generate_report()

```

3.8.4 Code: Parameter set selection in CRNT4SBML

Python

```

import crnt4sbml
import pandas
network = crnt4sbml.CRNT("C:\Harika_IIITD\Thesis\CellDes\Millnew.xml")
approach = network.get_mass_conservation_approach()
network.print_biological_reaction_types()
df = pandas.DataFrame(numpy.vstack([params_for_global_min[i] for i in \
    multistable_param_ind]).T, \
    columns=["set" + str(i + 1) \
    for i in \
    multistable_param_ind],

decision_vector_values = numpy.array(df['set1'])
decision_vector_values = params_for_global_min[0]
species_concentrations = [i(*tuple(decision_vector_values))\
    for i in approach.get_concentration_funs()]

print(species_concentrations)
print(approach.get_conservation_laws())
conservation_values = network.get_c_graph().get_b()*sympy.\
    Matrix([species_concentrations]).T
conservation_values

```

3.8.5 Code: XPPAUT file for signal transduction illustration**XPPAUT**

```

# XPPAUT FILE
# The file name is Sig_trans.txt

# Equations

# R1: S + E1 < - - - - - > SE1
# R2: SE1 - - - - - > Sp + E1
# R3: Sp + E2 < - - - - - > SpE2
# R4: SpE2 - - - - - > S + E2
# R5: S + Sp < - - - - - > SSp
# R6: SSp - - - - - > 2Sp

# Conservation relations
E1 = E1t-(SE1)
E2 = E2t-(SpE2)
S = St-((2*SSp)+SE1+Sp+SpE2)

# Rate of reactions
r1 = (kf1*S*E1)-(kb1*SE1)
r2 = (kf2*SE1)
r3 = (kf3*Sp*E2)-(kb3*SpE2)
r4 = (kf4*SpE2)
r5 = (kf5*S*Sp)-(kb5*SSp)
r6 = (kf6*SSp)

#Parameters
param E1t=0.0205 , E2t=0.2439, St=1.1004

param kf1= 0.1235
param kb1= 0.0122
param kf2=0.1197
param kf3=100.0000

```

```
param kb3=0.0122
param kf4=31.0678
param kf5=62.2934
param kb5=63.0112
param kf6=34.8846

# ODE's
Sp' = r2-r3-r5+(2*r6)
SE1' = r1-r2
SpE2' = r3-r4
SSp' = r5-r6

#auxiliaries aux E1=E1
aux E2=E2
aux S=S

aux E1total = E1+(SE1)
aux E2total = E2+(SpE2)
aux Stotal = S+((2*SSp)+SE1+Sp+SpE2)

# XPP settings @ meth=cvode, tol=9.99e-12,atol=9.99e-12, total=2000
@ bounds=100000000000, xp=t, yp=sp
@ xlo=0, xhi=200, ylo=0, yhi=200

#AUTO settings @ parmin=0, parmax=10, nmax=25000
@ autoxmin=0, autoymin=0, autoxmax=1 autoymax=50

done
```

Chapter 4

Network Analysis using SR graph

Introduction

A biological reaction network is usually highly non-linear and contains several parameters, such as kinetic constants. The major challenge of mathematical modeling is that most of these parameters are unknown. However, the structural properties of the model can be determined solely based on the network, irrespective of the values of the parameters. This was also shown in Chapter 3, where we analyzed the structure of the network and detect multistability with the help of tools based on the Chemical Reaction Network Theory.

Multistability is the capacity of the network to have multiple steady states. Multistability exhibits hysteresis, where the system resists transition from one state to another for small perturbations. The system maintains a threshold for the transition to occur. Multistability plays an important role in acting as a checkpoint in most biological networks, such as cell signaling and division.

In this chapter, we analyze a biological network using Species-Reaction (SR) graph. Based on a set of theorems, we will see how an SR graph can give information about a network's capacity for multistability. With the SR graph, it is easy to preclude the presence of bistability in networks. This helps in filtering out the networks that do not have the capacity to show bistability.

4.1 Basic Terminologies

An SR graph is a species reaction graph that represents a biological network. To understand the theorems based on the SR graphs, it is important to know a few terminologies which are used to describe the components and properties of an SR graph. A simple example of enzyme catalysis, as shown in equation 4.1, is used for illustration.

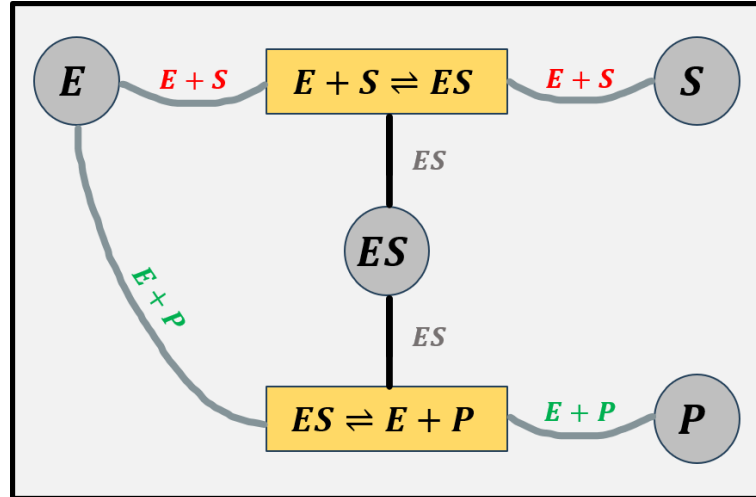
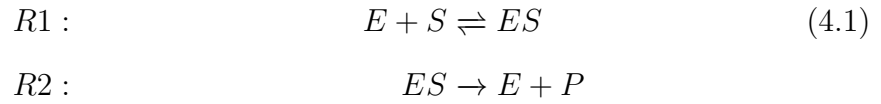


FIGURE 4.1: *SR graph for the enzyme catalysis reactions.*

An SR graph for the example 4.1. The species E , S , ES , and P are shown in grey circles, and the reaction $R1$ and $R2$ are shown in yellow boxes. The arcs connecting the species to reactions are labeled with the complexes. The reaction $R1$ is connected to the species E and S with arcs labeled as the complex $\{E+S\}$, and the specie ES is connected to reaction $R1$ with arc labeled as complex $\{ES\}$. For reaction $R2$, the species E , and P are connected with arcs labeled as the complex $\{E+P\}$, and the specie ES is connected to reaction $R2$ with arc labeled as complex $\{ES\}$. The arcs labeled with c -pairs are shown in grey color, and the arcs that do not contain c -pairs are shown in black. The c -pairs of the reactions $R1$ and $R2$ are shown in red and green colour respectively.

Species and Complexes: The species of the network given in equation 4.1 are E , S , ES , and P . The complexes are the objects that are present at the head and

tail of the reaction arrow. Here, the complexes are $\{E+S\}$, $\{ES\}$, and $\{E+P\}$. In the SR graph, the species are connected to the reactions with an arc and the arcs are labeled with the corresponding complexes. For the reaction R1 in example 4.1, the species E and S are connected to reaction R1 with arcs labeled as $\{E+S\}$. ES is connected to the reaction R1 with the arc labeled as $\{ES\}$.

C-pairs: A complex-pair (c-pair) in the SR graph is a pair of arcs that are adjacent to the same reaction symbol and are labeled with the same complex names. In Fig4.1, the complexes $\{E+S\}$ and $\{E+P\}$ shown in red and green color, respectively, are the two c-pairs present in this network. Note that the complex $\{ES\}$ is not a c-pair as it is not adjacent to the same reaction symbol.

Nodes and Edges: In the SR graphs, the species, and reaction symbols are nodes, and the arcs that connect the species and the reactions are called edges. Here, there are positive and negative edges. The edges that connect the reactants (species on the left of the reaction arrow) to the reaction symbols are called negative edges, and the edges that connect products (species on the right of the reaction arrow) to the reaction symbols are called positive edges.

Cycles in SR graph: A cycle is a closed path in which an arc or vertex is traversed twice. The example shown in Fig4.1 has a single cycle E-R1-ES-R2-E. This cycle has four edges, out of which two are positive (R1-ES and R2-E), and two are negative (E-R1 and ES-R2). Here on, we shall represent the total number of edges in a cycle as e and the number of positive and negative edges as p and q , respectively.

One cycle, Odd and Even cycles: The cycles in the SR graph is further classified into one-cycle, odd-cycle, and even-cycle. A one-cycle is a cycle in which the stoichiometric coefficient associated with each of its arcs is one. In this Fig4.1, the cycle is a one-cycle since the stoichiometric coefficients of all the reactions are one. The odd and even cycles can be identified in two ways, (1) based on the number of c-pairs ([12]) and (2) based on the number of edges ([32]). **Based on the number of c-pairs:** an odd-cycle has an odd number of c-pairs, and an even-cycle has an even number of c-pairs. **Based on the number of edges:** If $e/2$ is proportional

to q , then the cycle is an even-cycle else, it is an odd-cycle. For the cycle in Fig4.1 $e/2 = 2$ and $q = 2$. Since $e/2$ and q are proportional, it is an even-cycle.

Split c-pair: A c-pair that is split between two cycles such that each arc of the c-pair is present in two different cycles is called a split c-pair. Also, while counting the number of c-pairs in a cycle, if only one arc of the c-pair is present in the cycle, it is not taken into account. For example, the cycle in Fig4.1 does not have any c-pair as both the arcs of c-pair are not present in the cycle. However, since zero is an even number, it is an even-cycle.

4.2 Theorem for precluding multistability

Species-reaction (SR) graph of a given network helps in identifying the network's capacity for multiple steady states ([12]). This theorem says that for a network to have the capacity to admit multistability, it must satisfy a set of conditions.

The theorem states that the reaction network for which the SR graph satisfies both of the following conditions,

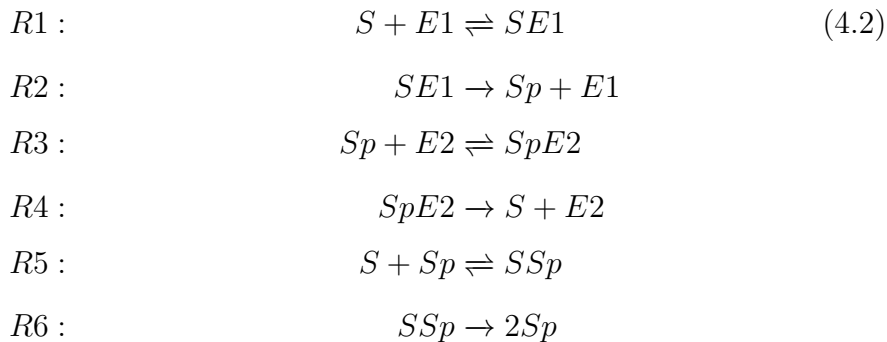
1. Each cycle in the SR graph is either a one-cycle, an odd-cycle, or both.
2. No c-pair is split by two even-cycles.

For such a reaction network, multistability is precluded. That is, the corresponding mass action differential equations cannot admit more than one positive steady state, no matter what (positive) values the rate constants, effluent coefficients, or species supply rates take.

This theorem effectively discriminates the networks that do not have the capacity for multistability. It is necessary for a reaction network to have a c-pair split by two even-cycles for reaction networks, where the stoichiometry of all the reactions is one, as all the cycles present in the network will be one-cycles. Also, for networks that have reactions where the stoichiometry is more than one, but the cycles which are not one-cycles happen to be odd-cycles, the network must have a c-pair split by two even-cycles for it to have the capacity to admit multistability.

4.3 Illustration

To illustrate the use of the theorem, we will use the same example as given in Chapter 3, Fig3.1. The reaction steps for the network are given in 4.2. The SR graph of the reaction network is given in Fig4.2.



In Fig4.2, the seven species are shown in grey circles. The reaction symbols for the reaction from R1 to R6 are shown in boxes. The arcs or edges connect the species to the reaction symbols. The colors of arcs containing the c-pair for reactions R1, R2, R3, R4, and R5 are green $\{S+E1\}$, blue $\{Sp+E1\}$, red $\{Sp+E2\}$, yellow $\{S+E2\}$, and purple $\{S+Sp\}$, respectively. The arcs that do not contain any c-pair are shown in black. The colors of all the reaction symbols are the same as their corresponding c-pairs. The color of the reaction symbol for R6 is black, as it does not correspond to any c-pair.

In this illustration, the reaction R6 has a stoichiometry of two. Hence the cycles which contain the edge $R6 - Sp$ will not be one-cycle. It is necessary to check whether these cycles are odd or even. If all the cycles are odd, then the capacity of the network to admit multistability can be precluded immediately. In the presence of even cycles, we must check for if a c-pair is split by two even cycles. The Python code 4.5.1 is used to find all the cycles present in the SR graph given in Fig4.2 and the output is shown in Fig4.3a. To find whether the cycles are odd-cycle or even-cycle, the Python code 4.5.2 is used. The output for the code 4.5.2 is given in Fig4.3b.

The properties of the SR graph given in Fig4.2 are provided in the table 4.1. There are a total of 15 cycles present in the SR graph. Of the 15 cycles, ten cycles are 1-cycle, and five cycles are 2-cycle (Cycles 7, 8, 11,13, and 14). However, all the

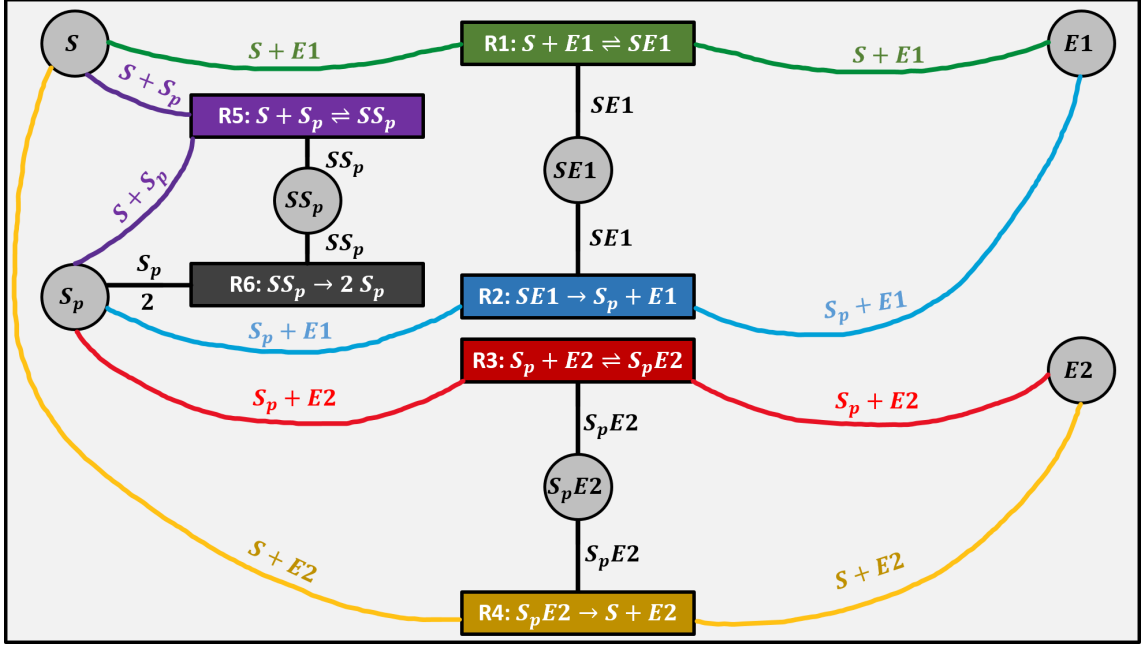


FIGURE 4.2: *SR graph for the network of signal transduction shown in Chapter 3. The seven species S , $E1$, $SE1$, S_p , $E2$, S_pE2 , and SS_p are shown in grey circles. The reaction symbols for the reaction from $R1$ to $R6$ are shown in boxes. The arcs or edges connect the species to the reaction symbols. The colors of arcs containing the c-pair for reactions $R1$, $R2$, $R3$, $R4$, and $R5$ are green $\{S+E1\}$, blue $\{S_p+E1\}$, red $\{S_p+E2\}$, yellow $\{S+E2\}$, and purple $\{S+S_p\}$, respectively. The arcs that do not contain any c-pair are shown in black ($SE1$, S_pE2 , SS_p , and S_p). The colors of all the reaction symbols are the same as their corresponding c-pairs. The color of the reaction symbol for $R6$ is black, as it does not correspond to any c-pair.*

2-cycles are Even, which does not satisfy the first point of the theorem, which says each cycle in the SR graph should either be a one-cycle, an odd-cycle, or both. Hence for this reaction network, multistability cannot be precluded. Additionally, the properties of this SR graph show that the second part of the theorem is also not satisfied. The second part of the theorem says that no c-pair should be split by two even-cycles. However, for this network, all the c-pairs $\{S_p+E2\}$, $\{S+E1\}$, $\{S_p+E1\}$, $\{S+E2\}$, and $\{S+S_p\}$ are split by two even-cycles as shown in table 4.1.

TABLE 4.1: Properties of SR graph

Cycle No.	Cycles	Stoichiometry	c-pairs	Odd or Even	Split c-pairs	
					c-pair	Cycle
Cycle 1	R1 - SE1 - R2 - S _p - R3 - S _p E2 - R4 - S	1-cycle	0	Even	S _p +E2	Cycle 15
					S+E1	Cycle 9
					S _p +E1	Cycle 9
					S+E2	Cycle 15
Cycle 2	R1 - E1 - R2 - S _p - R3 - S _p E2 - R4 - S	1-cycle	2	Even	S _p +E2	Cycle 15
					S+E2	Cycle 15
Cycle 3	R1 - SE1 - R2 - S _p - R3 - E2 - R4 - S	1-cycle	2	Even	S+E1	Cycle 9
Cycle 4	R1 - E1 - R2 - S _p - R3 - E2 - R4 - S	1-cycle	4	Even	-	-
Cycle 5	R1 - SE1 - R2 - S _p - R5 - S	1-cycle	1	Odd	S+E1	Cycle 9
					S _p +E1	Cycle 9
Cycle 6	R1 - E1 - R2 - S _p - R5 - S	1-cycle	3	Odd	-	-
Cycle 7	R1 - SE1 - R2 - S _p - R6 - SS _p - R5 - S	2-cycle	0	Even	S+E1	Cycle 9
					S _p +E1	Cycle 9
					S+S _p	Cycle 14
Cycle 8	R1 - E1 - R2 - S _p - R6 - SS _p - R5 - S	2-cycle	2	Even	S+S _p	Cycle 14
Cycle 9	R1 - SE1 - R2 - E1	1-cycle	0	Even	S+E1	Cycle 1,3,5,7
					S _p +E1	Cycle 1,5,7
Cycle 10	R3 - S _p E2 - R4 - S - R5 - S _p	1-cycle	1	Odd	S+E2	Cycle 15
Cycle 11	R3 - S _p E2 - R4 - S - R5 - SS _p - R6 - S _p	2-cycle	0	Even	S+E2	Cycle 15
Cycle 12	R3 - E2 - R4 - S - R5 - S _p	1-cycle	3	Odd	-	-
Cycle 13	R3 - E2 - R4 - S - R5 - SS _p - R6 - S _p	2-cycle	2	Even	S+S _p	Cycle 14
Cycle 14	R5 - S _p - R6 - SS _p	2-cycle	0	Even	S+S _p	Cycle 7,8,13
Cycle 15	R3 - S _p E2 - R4 - E2	1-cycle	0	Even	S _p +E2	Cycle 1,2
					S+E2	Cycle 1,2,10,11

```

Cycle Number 1: R1 - SE1 - R2 - Sp - R3 - SpE2 - R4 - S
Cycle Number 2: R1 - E1 - R2 - Sp - R3 - SpE2 - R4 - S
Cycle Number 3: R1 - SE1 - R2 - Sp - R3 - E2 - R4 - S
Cycle Number 4: R1 - E1 - R2 - Sp - R3 - E2 - R4 - S
Cycle Number 5: R1 - SE1 - R2 - Sp - R5 - S
Cycle Number 6: R1 - E1 - R2 - Sp - R5 - S
Cycle Number 7: R1 - SE1 - R2 - Sp - R6 - SSp - R5 - S
Cycle Number 8: R1 - E1 - R2 - Sp - R6 - SSp - R5 - S
Cycle Number 9: R1 - SE1 - R2 - E1
Cycle Number 10: R3 - SpE2 - R4 - S - R5 - Sp
Cycle Number 11: R3 - SpE2 - R4 - S - R5 - SSp - R6 - Sp
Cycle Number 12: R3 - E2 - R4 - S - R5 - Sp
Cycle Number 13: R3 - E2 - R4 - S - R5 - SSp - R6 - Sp
Cycle Number 14: R5 - Sp - R6 - SSp
Cycle Number 15: R3 - SpE2 - R4 - E2

```

(a) Output: Cycles

```

Cycle Number 1: Even cycle
Cycle Number 2: Even cycle
Cycle Number 3: Even cycle
Cycle Number 4: Even cycle
Cycle Number 5: Odd cycle
Cycle Number 6: Odd cycle
Cycle Number 7: Even cycle
Cycle Number 8: Even cycle
Cycle Number 9: Even cycle
Cycle Number 10: Odd cycle
Cycle Number 11: Even cycle
Cycle Number 12: Odd cycle
Cycle Number 13: Even cycle
Cycle Number 14: Even cycle
Cycle Number 15: Even cycle

```

(b) Output: Finding Odd or Even cycles

FIGURE 4.3: Output images for the codes (a) 4.5.1, and (b) 4.5.2.

(a) The output shows the number of cycles along with the cycles. There are 15 cycles present in the SR graph given in Fig 4.2. (b) The output classifies the cycles into odd-cycle or even-cycle.

4.4 Summary

The SR graph is mainly used for systems following mass action kinetics. The systems following mass action kinetics are more precise and transparent than the other kinetic approaches, which use an approximation. The fine details of the reactions involved in the system have a great influence on the structure and dynamics of the system. The

theorem gives conditions for the properties of an SR graph to preclude multistability. This theorem is an extension of the theorem given by Schlosser and Feinberg ([33]). The network of signal transduction used in this chapter is already known to have bistability from CRNT analysis. Using this network, we are able to show that this theorem can be effectively used to preclude bistability while building models.

4.5 Codes

4.5.1 Code: Finding Cycles

Python

```
# To find the number of cycles in SR graph
# Write the dictionary to label the species a number
names = {'1':'R1','2':'R2','3':'R3','4':'R4','5':'R5',
         '6':'R6','7':'S','8':'E1','9':'SE1','10':'Sp',
         '11':'E2','12':'SpE2','13':'SSp'}

# Write all the edges in the graph using the numbers mentioned in the dictionary
graph = [[1, 7], [1, 8], [1, 9], [2, 9], [2, 10], [2, 8], [3, 10], [3, 11], \
         [3, 12], [4, 12], [4, 7], [4, 11], [5, 7], [5, 10], [5, 13], \
         [6, 13], [6, 10]]

cycles = []

# Main to print the cycles
def main():
    global graph
    global cycles
    cnum = 1
    for edge in graph:
        for node in edge:
            findCycles([node])
    for cy in cycles:
        path = [names[str(node)] for node in cy]
        s = " - ".join(path)
        print("Cycle Number %d: " % (cnum), s)
        cnum = cnum+1

# Defining findCycles to find all the cycles
def findCycles(path):
    start_node = path[0]
    next_node = None
    sub = []

    # Visiting each edge and each node of each edge
```

```

for edge in graph:
    node1, node2 = edge
    if start_node in edge:
        if node1 == start_node:
            next_node = node2
        else:
            next_node = node1
    if not visited(next_node, path):
        sub = [next_node]
        sub.extend(path)
        findCycles(sub);
    elif len(path) > 2 and next_node == path[-1]:
        # Cycle
        p = rotate_to_smallest(path);
        inv = invert(p)
        if isNew(p) and isNew(inv):
            cycles.append(p)

def invert(path):
    return rotate_to_smallest(path[::-1])

# Arranging the nodes of the cycle
def rotate_to_smallest(path):
    n = path.index(min(path))
    return path[n:]+path[:n]

def isNew(path):
    return not path in cycles

def visited(node, path):
    return node in path

main()
cyclenumber = len(cycles)

```

4.5.2 Code: Identifying Odd or Even cycles

Python

```

# dictionary graph along with weights
graph = {
    '1': [('7',-1), ('8',-1),('9',1)], '2': [('9',-1), ('10',1), ('8',1)],
    '3': [('10',-1), ('11',-1), ('12',1)], '4': [('12',-1), ('7',1), ('11',1)],
    '5': [('7',-1), ('10',-1), ('13',1)], '6': [('13',-1), ('10',1)],
    '7': [('1',-1), ('4',1),('2',-1)], '8': [('1',-1), ('2',1)],
    '9': [('1',1), ('2',-1)], '10': [('2',1), ('3',-1),('5',-1),('6',1)],

```

```

    '11': [('3',-1),('4',1)], '12': [('3',1),('4',-1)],
    '13': [('5',1), ('6',-1)]
}

# Dictionary to store edge weights
weights = {
    ('1', '7'):-1, ('1', '8'):-1, ('1', '9'):1, ('2', '9'):-1,
    ('2', '10'):1, ('2', '8'):1, ('3', '10'):-1, ('3', '11'):-1,
    ('3', '12'):1, ('4', '12'):-1, ('4', '7'):1, ('4', '11'):1,
    ('5', '7'):-1, ('5', '10'):-1, ('5', '13'):1, ('6', '13'):-1,
    ('6', '10'):1, ('7', '1'):-1, ('8', '1'):-1, ('9', '1'):1,
    ('9', '2'):-1, ('10', '2'):1, ('8', '2'):1, ('10', '3'):-1,
    ('11', '3'):-1, ('12', '3'):1, ('12', '4'):-1, ('7', '4'):1,
    ('11', '4'):1, ('7', '5'):-1, ('10', '5'):-1, ('13', '5'):1,
    ('13', '6'):-1, ('10', '6'):1
}

# Counting total number of edges and number of negative edges
edge = []
nedge = []
for i in range(0,cyclenumber):
    c = cycles[i]
    s = len(c)
    count = 0
    ncount = 0
    for j in range(0,s):
        if j < s-1:
            w = abs(weights[(str(c[j]),str(c[j+1]))])
            nw = weights[(str(c[j]),str(c[j+1]))]
            count = count+w
            if nw < 0:
                ncount = ncount+1
            else:
                ncount = ncount
        else:
            w = abs(weights[(str(c[j]),str(c[0]))])
            nw = weights[(str(c[j]),str(c[0]))]
            count = count+w
            if nw < 0:
                ncount = ncount+1
            else:
                ncount = ncount
    edge.append(count)
    nedge.append(ncount)
print(edge)
print(nedge)

# Defining odd_or_even to classify the cycles
def odd_or_even(a, b):
    even = 'Even cycle'
    odd = 'Odd cycle'
    if a>b:
        c = a/b

```

```
    if c.is_integer() == True:
        r = even
    else:
        r = odd
else:
    c = b/a
    if c.is_integer() == True:
        r = even
    else:
        r = odd
return r

# Function to print the cycles
def print_result(cn,r):
    print("Cycle Number %d:" % (cn+1), r)

for i in range(0,cyclenumber):
    p = edge[i]
    q = nedge[i]
    r = odd_or_even(p/2,q)
    print_result(i,r)
```

Chapter 5

Kinetic Analysis: Extraction, Estimation, and Fitting of the kinetic models

5.1 Introduction

Most of the biological systems that are modeled using ordinary differential equations (ODEs) involve species that represent the concentration or number of molecules of the chemical species at the molecular level, such as the proteins. Once the network is modeled for the kinetic analysis, the rate equations are written equations can be written based on the kinetic laws such as the mass action kinetics (MA) and Michaelis-Menten kinetics (MM). These rate equations are based on several parameters that have biological meaning. These parameters include association or complex dissociation constants, production or decay rates, etc. These parameters influence the behavior of a network. The challenging part of modeling a biological system is that most of these parameters are unknown.

In the structural analysis seen in Chapter 3 and Chapter 4, we did not require the values of the kinetic parameters. However, for kinetic analysis, which involves quantitatively predicting the behavior of a biological system, the parameters need to be estimated. The parameters can either be obtained directly from the literature or can be estimated by fitting the simulations of the model with the observed

experimental data, such as steady state, or time series data obtained from experiments, such as fluorescent binding assays or western blotting.

In this chapter, we will see how to extract data from the images of the experimental results using the software ImageJ. We will also see how to use this data information to fit the equations. We will show a simple example of a target ligand binding assay to fit the data in MATLAB using the function `fittype()`. Finally, we will also see how to use the software COPASI (Complex Pathway Simulator) to estimate parameters using the time series data.

5.2 Data Extraction

Data extraction is the first step for parameter estimation. It involves obtaining quantitative measurements from the images of a given experimental data, such as time series data or steady-state data. The time series data contains measurements of the concentration of species over the course of time. The steady-state data contains the measurements of the concentration of species at equilibrium.

ImageJ is an open-source software that provides tools and functionalities to analyze and extract data from images manually. ImageJ can be downloaded from the link <http://imagej.nih.gov/ij/download.html>. ImageJ is a platform-independent Java-based software. The download is available for Windows, Linux, and Mac OS X. ImageJ by itself is not a powerful tool. However, its functionality can be extended by numerous freely available plugins. One such plugin that we use for data extraction is `Figure_Clibration.class`. Given below are the steps to get the x and y coordinates of the experimental data points from an image. The time series data ([34]) shown in Fig5.1 is used as an example.

- Use the Rectangle tool to select the area of the plot, as shown in Fig5.2a.
- To calibrate the x and y, use the Plugin Figure Calibration as shown in Fig5.2b and enter the range of the x and y axis as shown in Fig5.2c. Note that to use the plugin for Figure Calibration, the `Figure_Calibration.class` must be downloaded.

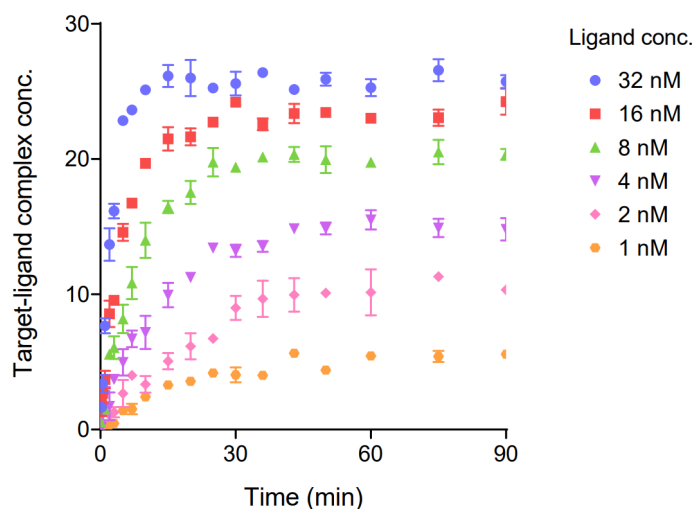


FIGURE 5.1: *Time series data for the target-ligand association.*

The Y axis of the data represents the concentration of the target-ligand complex. The time is taken in minutes. There are six sets of time series data, each corresponding to different ligand concentrations. The ligand concentrations taken were 1, 2, 4, 8, 16, and 32 nM.

- Unselect the Rectangle by clicking out of the frame and then the tool Multi-point.
- Use the cursor to select the data points for which the coordinates are to be extracted.
- Once all the data points are selected, Press CTRL+M, and the Results table will pop up showing the coordinates of the data points selected, as shown in Fig5.2d.
- Finally, the results can be saved as a Results.csv file.

5.3 Analysing Kinetic Binding Data

Here, we'll be analyzing the kinetics of ligand-target interaction. This interaction usually involves the association of the reactants into a complex and the further dissociation of the complex. The association and dissociation rate constants help us

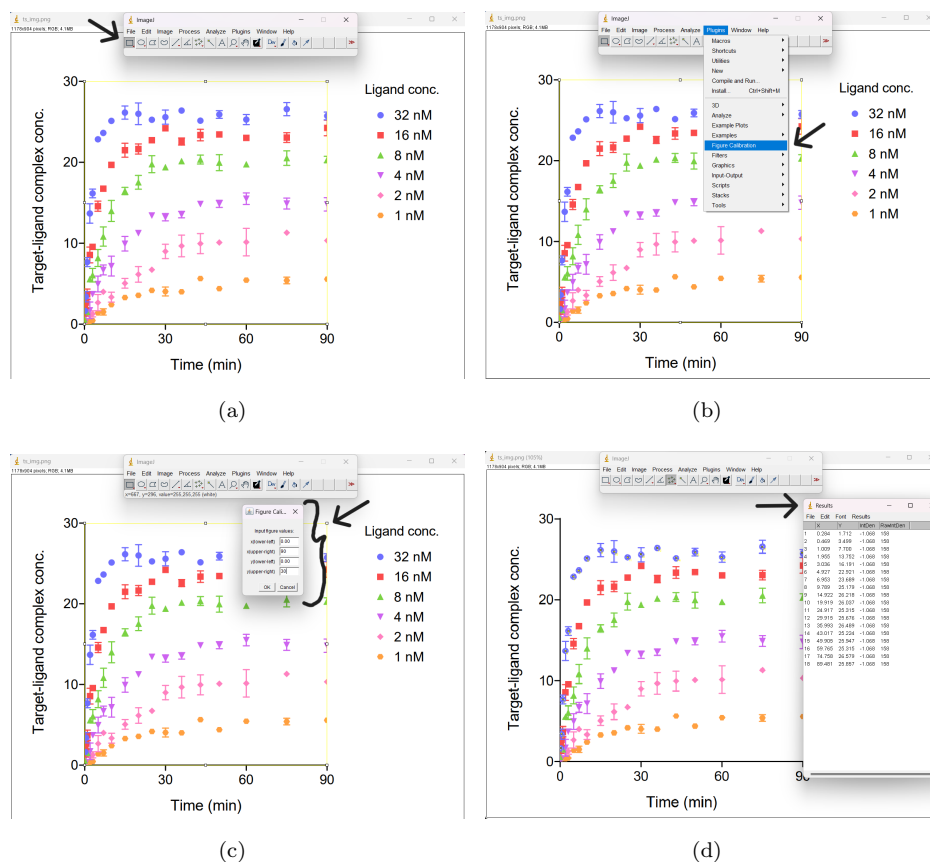


FIGURE 5.2: Steps for data extraction using ImageJ software.

Figures (a)-(d) show the screenshots to represent the steps to extract data points from an image using the ImageJ software in sequence. (a) The Rectangle icon is used to select the area of the plot. (b) The Figure Calibration plugin is selected to calibrate the axes of the plot. (c) The range of the X and Y axes should be entered in the Figure Calibration dialog box. (d) Select the icon Multi-point and click on the data points using the cursor. Press CTRL+M for the results table.

quantify these interactions. These rate constant values can be calculated using the time series data. “ImageJ” and “MATLAB” are the software used for data extraction and curve fitting.

5.3.1 Direct Target-Ligand Binding



$$Rf = k1 * R * L(Ms^{-1}) \quad (5.2)$$

$$Rd = k2 * RL(Ms^{-1}) \quad (5.3)$$

The equation 5.1 is the basic bimolecular interaction where R is the target, L is the ligand, and RL is the complex. k1 represents the association rate constant, and k2 is the dissociation rate constant. In equation 5.3, Rf and Rd represent the rate of association and the rate of dissociation, respectively. From the rates given in equation 5.3, the units of k1 and k2 can be deduced as $M^{-1}s^{-1}$ and s^{-1} respectively.

5.3.1.1 Association rate constant from Direct Target-Ligand Binding Assays

It involves measuring the rate constants from assays that directly quantify the interaction of the ligand with the target. Assays such as Fluorescence and Bioluminescence that gives real-time continuous read modality are more promising for this method. The rate of change of RL can be written as shown in equation 5.4. Here, L is taken in excess so that it can be considered as a constant, making it a pseudo-first-order reaction. R can be written in terms of RL considering the conservation relation of the targets where the total target concentration (Rt) is constant.

$$\frac{d[RL]}{dt} = (k1 * [R] * [L]) - (k2 * [RL]) \quad (5.4)$$

$$Rt = R + RL$$

$$R = Rt - RL \quad (5.5)$$

Substituting equation 5.5 in 5.4,

$$\begin{aligned} \frac{d[RL]}{dt} &= (k1 * (Rt - [RL]) * [L]) - (k2 * [RL]) \\ \frac{d[RL]}{dt} &= (k1 * Rt * [L]) - [RL](k1 * [L] - k2) \end{aligned} \quad (5.6)$$

In the equation 5.6, the terms $(k1 * Rt * [L])$ and $(k1 * [L] - k2)$ are constants, and hence, the equation 5.6 is in the form of a Product-Decay equation as given in equation 5.7

$$\frac{dx}{dt} = I - \gamma x \quad (5.7)$$

The solution of the Product-Decay equation is given by

$$x(t) = \frac{I}{\gamma} - \left(\frac{I}{\gamma} - x_o\right)e^{-\gamma t}$$

where $x(0) = x_o$, $I, \gamma > 0$ (constants) (5.8)

The solution of equation 5.6 based on equation 5.8 can be written as

$$RL(t) = \frac{(k1 * Rt * [L])}{(k1 * [L] - k2)} - \left(\frac{(k1 * Rt * [L])}{(k1 * [L] - k2)} - RL_o\right)e^{-(k1*L-k2)t}$$

As RL is a complex, its initial concentration is known to be zero. Hence, $RL(0) = RL_o = 0$.

$$RL(t) = \frac{(k1 * Rt * [L])}{(k1 * [L] - k2)} - \left(\frac{(k1 * Rt * [L])}{(k1 * [L] - k2)}\right)e^{-(k1*L-k2)t}$$

$$RL(t) = \frac{(k1 * Rt * [L])}{(k1 * [L] - k2)}(1 - e^{-(k1*L-k2)t}) \quad (5.9)$$

At infinite time, RL reaches a steady state, and so, $RL(infinity) = RL_{ss}$

$$RL_{ss} = \frac{(k1 * Rt * [L])}{(k1 * [L] - k2)} \quad (5.10)$$

$$k_{obs} = k1 * L - k2 \quad (5.11)$$

By substituting equation 5.10 in equation 5.9 gives,

$$RL(t) = RL_{ss}(1 - e^{-(k1*L-k2)t})$$

$$RL(t) = RL_{ss}(1 - e^{-(k_{obs})t}) \quad (5.12)$$

Here we are taking the time course data from [34]. The target-ligand association data for each concentration of the ligand is measured. From the given graph (Fig5.1), the corresponding X and Y coordinates were extracted using ImageJ software, as

shown in Fig 5.2. These values were taken and then fit into the equation 5.12 using the function `fitype()` in MATLAB. After fitting, the constant k_{obs} for every ligand concentration was taken and fitted into the equation 5.11. As it is linear, the fit gives a straight line whose slope corresponds to k_1 . The code for fitting is given in 5.6.1. The k_1 and k_2 , as given by the fit, are $1e+7 \text{ M}^{-1}\text{min}^{-1}$ and 0.037 min^{-1} , respectively.

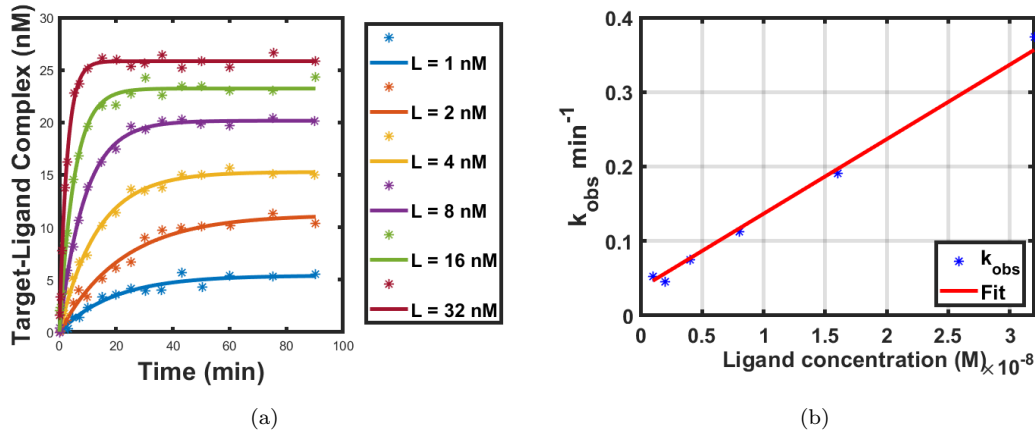


FIGURE 5.3: Measurement of rate constants for the target-ligand binding.

The measurement of the rate constants is done in two parts. In the first part (a), six time-series data are fit to the equation 5.12, and the k_{obs} for different ligand concentrations are obtained. In the second part (b), the k_{obs} was plotted against the respective ligand concentration, and the points were fitted using the equation 5.11 to obtain k_1 (slope) and k_2 values. The k_1 and k_2 , from the fit, are $1e+7 \text{ M}^{-1}\text{min}^{-1}$ and 0.037 min^{-1} , respectively. The fitting was performed using the `fitype()` function in MATLAB (code: 5.6.1).

5.3.1.2 Dissociation rate constant from a Direct Target-Ligand binding assay

In order to measure the concentration of the complex during dissociation, the target and ligand are pre-incubated to form the complex. Then, the concentration is noted during the dissociation, and the experiment is set in such a way that the association of the labeled ligand is blocked. The dissociation reaction is shown in equation 5.13.



The rate of change of RL from the reaction 5.13 is given as

$$\frac{d[RL]}{dt} = -k_2 * RL$$

By integration, we get

$$RL(t) = RL_o * e^{-(k_2 * t)}$$

(5.14)

where $RL(0) = RL_o$

The data of the dissociation curve is extracted using ImageJ software. The data is then fit into the equation 5.14 using the function `fitype()` in MATLAB as shown in Fig5.4. The k_2 , as obtained from the fit, is 0.084 min^{-1} .

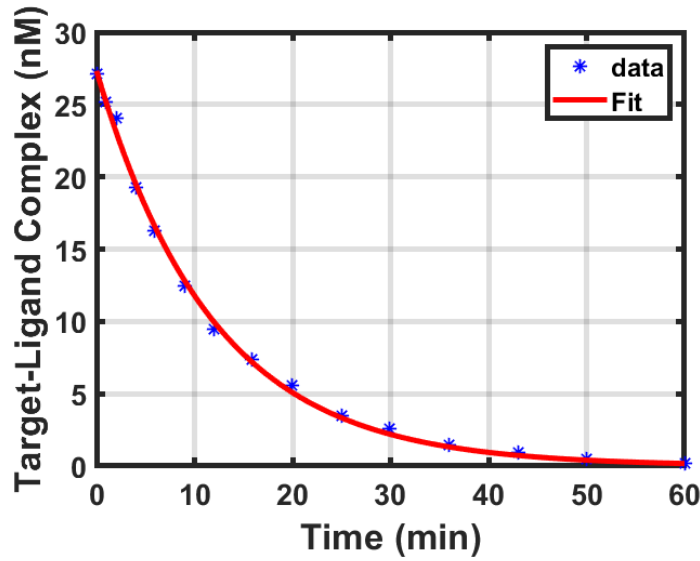


FIGURE 5.4: *Time-series fit for the dissociation of target-ligand complex (RL).*
The time series data for the dissociation of RL shown by blue stars were fit using the equation 5.14 to get k_2 value. The k_2 obtained from the fit is 0.084 min^{-1} . The fitting was performed using the `fitype()` function in MATLAB (code: 5.6.2).

5.3.2 Riccati equation

The solution methods for the linear differential equations are more developed than the nonlinear ODEs, which is why most of the differential equations used for modeling biological interactions are tried to be approximated to linear differential equations. However, due to various complexities and nonlinearities in biological systems, often nonlinear differential equations occur, such as the one obtained in the total QSSA.

Riccati equation is a form of nonlinear differential equation which has importance in modeling biological interactions.

The general form of the Riccati equation can be written as

$$\frac{dy}{dt} = a(t)y^2 + b(t)y + c(t) \quad (5.15)$$

where $a(t)$, $b(t)$ and $c(t)$ are continuous functions of t .

5.3.2.1 Riccati equation in Receptor Ligand Binding

For the Receptor-Ligand binding given in reaction 5.1, the differential equation for the complex RL is given in equation 5.6. The differential equation for RL can be written in terms of Riccati's equation and can be solved as follows.

$$\frac{d[RL]}{dt} = (k_1 * (R_T - [RL]) * [L]) - (k_2 * [RL])$$

Let $K_D = k_2/k_1$

The equation 5.6 can be rewritten in terms of K_D .

$$\frac{1}{k_1} \frac{d[LR]}{dt} = L_T R_T - (L_T + R_T + K_D)[LR] + [LR]^2 \quad (5.16)$$

where $L_T = L + RL$ and $R_T = R + RL$

$$\frac{1}{k_1} \frac{d[LR]}{dt} = A - Bx + x^2 \quad (5.17)$$

where $A = L_T R_T$, $B = L_T + R_T + K_D$, and $x = [LR]$

The equation 5.17 is in the general form of Riccati's equation 5.15.

Considering the RHS of the equation 5.17 and completing the square

$$\begin{aligned} & A - Bx + x^2 + \frac{B^2}{4} - \frac{B^2}{4} \\ &= A + \left(x^2 - \frac{B}{2}\right)^2 - \left(\frac{B}{2}\right)^2 \\ &= \left(x - \frac{B}{2}\right)^2 - \left(\frac{B^2}{4} - A\right) \end{aligned}$$

Let us consider u and v as the following

$$u = x - \frac{B}{2} \implies du = dx \quad (5.18)$$

$$v^2 = \frac{B^2}{4} - A \implies v = \pm \sqrt{\frac{B^2}{4} - A} \quad (5.19)$$

By substituting equation 5.18 in equation 5.17, we get

$$\frac{1}{k1} \frac{du}{dt} = u^2 - v^2 \quad (5.20)$$

$$\int \frac{du}{u^2 - v^2} = \int k1 dt \quad (5.21)$$

$$\frac{1}{2v} \ln \left| \frac{u-v}{u+v} \right| = k1t + C \quad (5.22)$$

$$\ln \left| \frac{u-v}{u+v} \right| = 2v(k1t + C) \quad (5.23)$$

$$\frac{u-v}{u+v} = e^{2v(k1t+C)} \quad (5.24)$$

In the equation 5.20, let us assume y to be

$$y = 2v(k1t + C) \quad (5.25)$$

By substituting y (5.25) in equation 5.20, we get

$$\frac{u-v}{u+v} = e^y \quad (5.26)$$

$$u - v = ue^y + ve^y \quad (5.27)$$

$$u - ue^y = ve^y + v \quad (5.28)$$

$$u = \frac{v(1 + e^y)}{(1 - e^y)} \quad (5.29)$$

Substituting equation 5.25 in 5.26, we get

$$u = \frac{v(1 + e^{\frac{t}{\tau_s}})e^{2vC}}{(1 - e^{\frac{t}{\tau_s}})e^{2vC}} \quad (5.30)$$

where $\tau_s = \frac{1}{2vk1}$

In equation 5.30, e^{2vC} is the term with integration constant. To calculate the integration constant, let us consider the initial condition of x and u . From equation 5.18,

$$x(t=0) = 0 \implies u(t=0) = -\frac{B}{2} \quad (5.31)$$

Substituting equation 5.31 in equation 5.30

$$u(t=0) = -\frac{B}{2} = \frac{v(1 + e^{2vC})}{(1 - e^{2vC})} \quad (5.32)$$

$$e^{2vC} = \frac{B + 2v}{B - 2v} \quad (5.33)$$

By substituting equation 5.32 and u from equation 5.18 in equation 5.30, we get

$$\begin{aligned} x - \frac{B}{2} &= \frac{v(B - 2v + e^{\frac{t}{\tau_s}}(B + 2v))}{(B - 2v - e^{\frac{t}{\tau_s}}(B + 2v))} \\ x &= \frac{v(B - 2v + e^{\frac{t}{\tau_s}}(B + 2v))}{(B - 2v - e^{\frac{t}{\tau_s}}(B + 2v))} + \frac{B}{2} \\ x &= \frac{B^2 - 4v^2 - (B^2 - 4v^2)e^{\frac{t}{\tau_s}}}{2((B - 2v) - e^{\frac{t}{\tau_s}}(B + 2v))} \\ &\quad \text{divide Nr. and Dr. with } e^{\frac{t}{\tau_s}} \\ x &= \frac{(B^2 - 4v^2)(e^{\frac{-t}{\tau_s}} - 1)}{2((B - 2v)e^{\frac{-t}{\tau_s}} - (B + 2v))} \\ (B^2 - 4v^2) &\text{ can be written as } (B + 2v)(B - 2v) \\ x &= \frac{(B + 2v)(B - 2v)(e^{\frac{-t}{\tau_s}} - 1)}{2((B - 2v)e^{\frac{-t}{\tau_s}} - (B + 2v))} \\ &\quad \text{divide Nr. and Dr. with } (B + 2v) \\ x &= \frac{(B - 2v)(e^{\frac{-t}{\tau_s}} - 1)}{2(\frac{(B - 2v)}{B + 2v}e^{\frac{-t}{\tau_s}} - 1)} \\ x &= \frac{(B - 2v)(e^{\frac{-t}{\tau_s}} - 1)}{2(\frac{(B - 2v)}{B + 2v}e^{\frac{-t}{\tau_s}} - 1)} \end{aligned} \quad (5.34)$$

In equation 5.34, by substituting B and v from equations 5.17 and 5.18 respectively in the term $\frac{B}{2} \pm v$, we get

$$\frac{B}{2} \pm v = \frac{L_T + R_T + K_D}{2} \pm \sqrt{\frac{(L_T + R_T + K_D)^2}{4} - L_T R_T} \quad (5.35)$$

$$= LR_{\pm} \quad (5.36)$$

By substituting the terms $\frac{B}{2} \pm v$ and x from equations 5.35 and 5.17, respectively in equation 5.34, we get

$$LR(t) = \frac{LR_{-}(1 - e^{\frac{-t}{\tau_s}})}{(1 - e^{\frac{-t}{\tau_s} \frac{LR_{-}}{LR_{+}}})} \quad (5.37)$$

5.4 Parameter estimation using Copasi

In Copasi, the parameter estimation is done on the basis of the given experimental dataset. Here, the time series data for the target ligand association extracted from Fig5.1 was taken and fitted. Copasi measures the distance between the model and data based on the weighted sum of squares as given in the equation 5.38.

$$E(P) = \sum_{i,j} w_j \cdot (x_{i,j} - y_{i,j}(P))^2 \quad (5.38)$$

In equation 5.38, “P” is the parameter being tested, $x_{i,j}$ is the point from the dataset, and $y_{i,j}(P)$ is the simulated value. Here, i and j denote the rows and columns of the dataset. The weight for each data column is given by w_j . The weight “Mean Square” as given in the equation 5.39 was used. “Evolutionary Programming” was used as the optimization method. The obtained fit of the time series is given in Fig().

$$w_j = \frac{1}{< x_j^2 >} \quad (5.39)$$

The input file to load the reactions into Copasi can either be given as an SBML file, or the reactions can also be entered manually. In Copasi, multiple experimental data can be used simultaneously. Also, both the steady-state and time series data can be

used together. The experimental data should be in delimited form (tab or comma). The steps to carry out the parameter estimation using Copasi, given time series data, are given below.

- The model can either be loaded using an SBML file by going to File → Import SBML. Or, the model can also be manually entered in COPASI → Model → Biochemical → Reactions.
- Once the reactions are loaded, go to Reactions → Parameter Overview. Here, the default values for the parameters such as Initial species values and Kinetic Parameters, will be given, but they can be modified based on the known parameters.
- For estimating the parameter, got to COPASI → Tasks → Parameter Estimation.
- To load the experimental data, select the Experimental Data button. An Experimental Data dialogue box pops up. Load the experimental data either as tab or comma-delimited form and select the options based on the data. Make sure the data is given with the header so that it becomes easy to select the type of column once the data is loaded.
- For times series data, select the column containing time points as Time and the column containing the concentration of the species as either a dependent or independent variable. In this illustration, the column represents the target-ligand complex (RL) which is a dependent variable. In case there are columns that do not contain any relevant information, they can be classified under the Type-ignored.
- After selecting the type, select the Model Object. In this illustration, it is the transient concentration of the complex RL.
- After loading the experimental data, select the parameters that are unknown. Here, the unknown parameters for the reaction 5.1 are k_1 , k_2 , and the initial concentration of R.
- The Lower Bound, Upper Bound, and the Start Value of the parameters can be adjusted based on the experiment. Also, the optimization method must be selected. Here, we use Evolutionary Programming. The output plots, such as

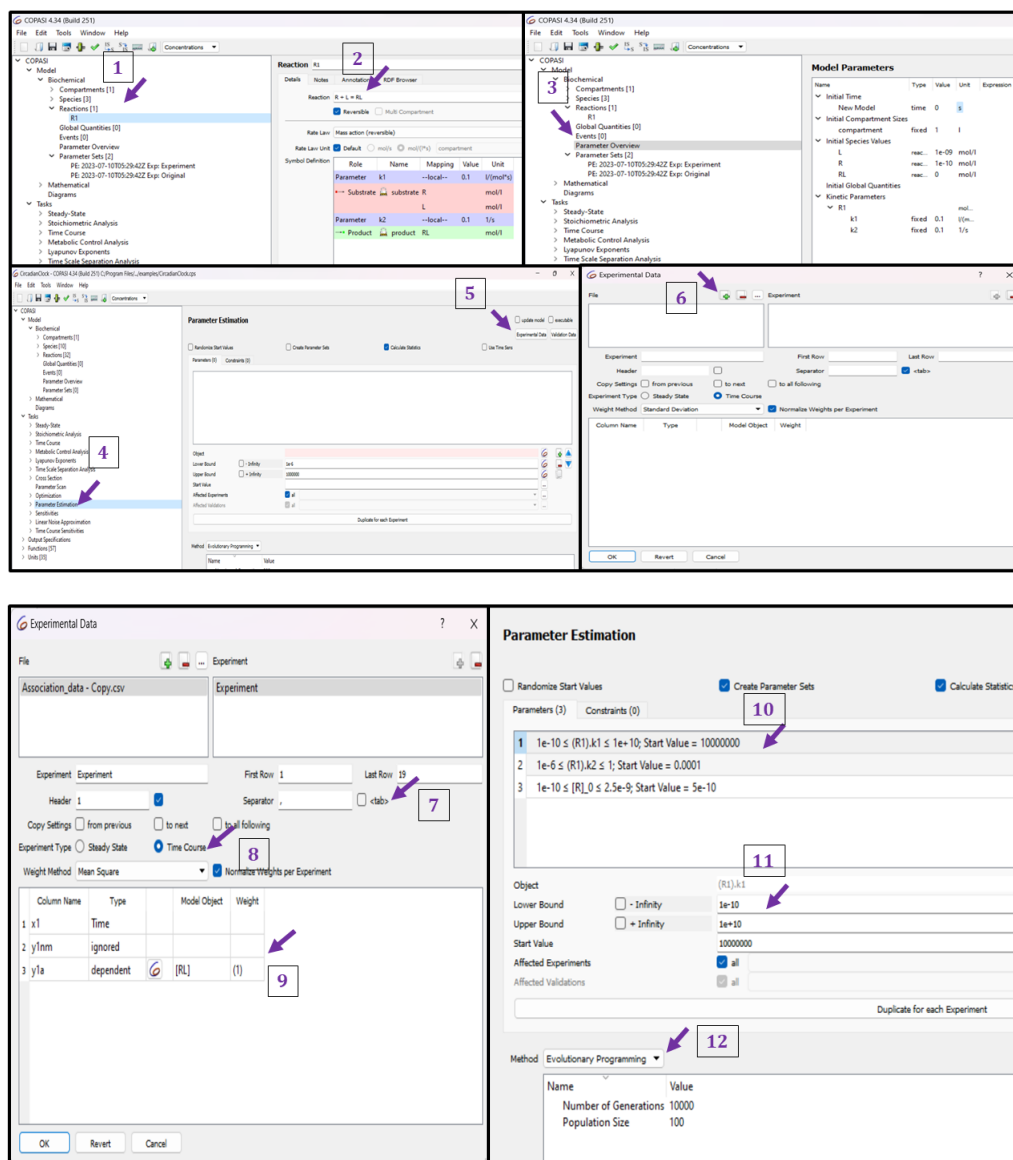


FIGURE 5.5: Steps to perform parameter estimation using Copasi.

The numbered arrows show the sequence of steps to perform parameter estimation. (1,2) Go to Reaction and enter the reactions of the model. These steps can be replaced by loading the model from an SBML file. (3) Go to Parameter Overview and modify the parameters. (4,5) Go to Parameter Estimation under Tasks, and select the Experimental Data button to load the experimental data. (6-9) Steps involved to load the experimental data. (10) Select the parameters that are to be estimated. (11) Set the lower and upper bounds for the parameters. (12) Select the method of optimization before running the task.

the Parameter Estimation Result and Progress of Fit, can be selected using Output Assistant.

- Press Run to perform the fitting task.

5.4.1 Results

The time-series data to perform parameter estimation in Copasi was obtained from Fig5.1. The data corresponding to the ligand concentration 1nM was taken. Once the fitting task was performed, the output plots, Parameters Sets, and the result were generated by Copasi. The plots that are generated are shown in Fig5.6. The results can be examined from Tasks → Parameter Estimation → Result. There is also an option to save the results. The Results page has statistical information such as the root mean square and standard deviation. The k_1 and k_2 , estimated by Copasi, are $1.2\text{e}+7 \text{ M}^{-1}\text{min}^{-1}$ and 0.02 min^{-1} , respectively.

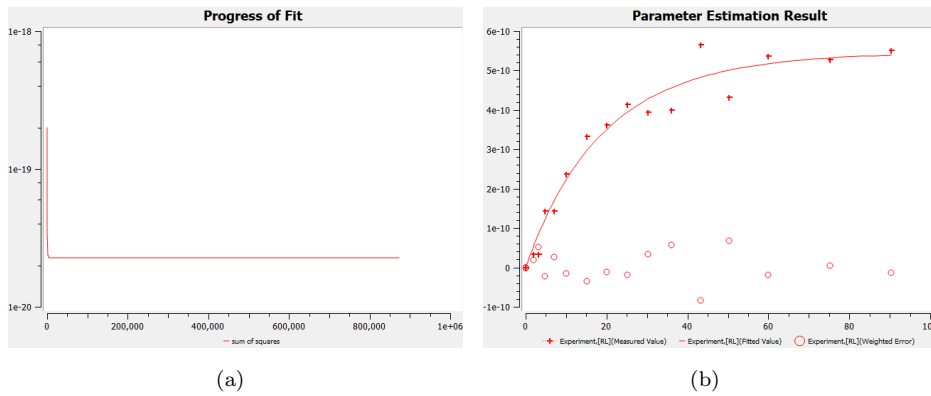


FIGURE 5.6: *Output plots generated by Copasi.*

(a) The *Progress of Fit* shows the objective value, the sum of squares plotted against the number of iterations. Flattening of this curve means that the optimization has reached the least sum of squares. (b) *Parameter Estimation Result* shows the final fit along with the actual data points (+) and the errors for each data point (o).

5.5 Summary

1. The data from the experimental images can be extracted using the software ImageJ.
2. The extracted data can be fit using the *fittype* function in MATLAB by given the corresponding function of the model.

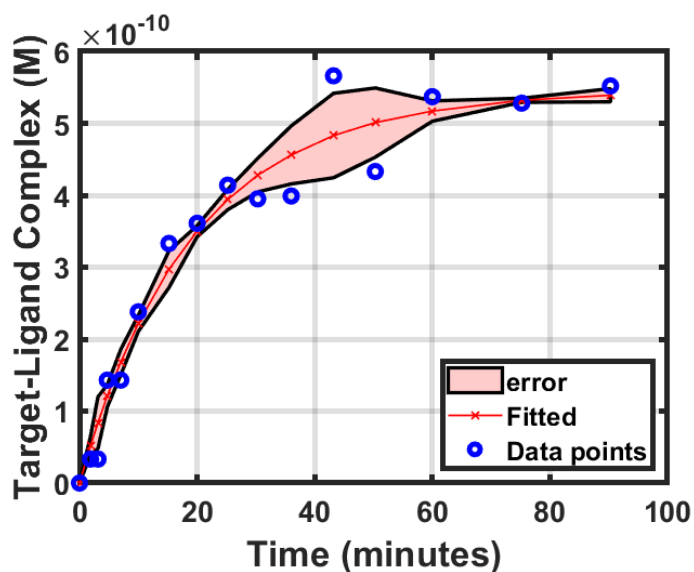


FIGURE 5.7: *Parameter estimation result.*

The Parameter Estimation Results downloaded from Copasi was used to generate the fit image, along with the error band, in MATLAB (code: 5.6.3). The k_1 and k_2 , estimated by Copasi, are $1.2e+7 \text{ M}^{-1}\text{min}^{-1}$ and 0.02 min^{-1} , respectively.

3. In case of multiple reactions where it is cumbersome to define the function, the software COPASI can be used to fit multiple experimental data, such as the time series and steady-state data, simultaneously.
4. The results of the fit obtained are taken and plotted in MATLAB along with the error bands for better visualization.

5.6 Codes

5.6.1 Code: Fitting association data

MATLAB

```
clc,clear
close all
% importing data from excel sheet
[data,hd,row] = xlsread("assoc_data.xlsx");
% seperating data into x and y
```

```

x1 = data(:,1); y1 = data(:,2); x2 = data(:,3);
y2 = data(:,4); x3 = data(:,5); y3 = data(:,6);
x4 = data(:,7); y4 = data(:,8); x5 = data(:,9);
y5 = data(:,10); x6 = data(:,11); y6 = data(:,12);
j = 1;
colour = ['r','b','m','k','y','g'];
colour_data = ['*r';'*b';'*m';'*k';'*y';'*g'];

% for loop to fit all the data
for i = 1:2:12
    g = fittype('a*(1-exp(-k*x))'); % defining fit type
    % fitting data using fit type g
    f = fit(data(:,i),data(:,(i+1)),g,'Startpoint',[1,0.01]);
    % taking kobs from the fit f
    kobs(j) = f.k;
    % ligand concentration from fit f
    RL(j) = f.a;
    figure(1)
    plot(f,colour(j),data(:,i),data(:,(i+1)),colour_data(j))
    j = j+1;
    hold on
end
legend('data (L = 1) ','L = 1','data (L = 2) ','L = 2',...
       'data (L = 4) ','L = 4','data (L = 8) ','L = 8',...
       'data (L = 16) ','L = 16','data (L = 32) ','L = 32')
ylabel("Target-Ligand Complex conc"); xlabel("Time (min)")

hold off

% fitting kobs values
L = (10^(-9)).*[1,2,4,8,16,32];
h = fittype('a*x+b');
f2 = fit(L,kobs,h,'Startpoint',[10^7,0.0001]);
kf = f2.a;
kb = f2.b;
figure(2)
plot(f2,L,kobs);
legend('data kobs', 'kobs')
xlabel("Ligand concentration (M)"); ylabel("kobs (min^-1)")
% Displaying important results
disp('Ligand concentrations taken:')
disp(L)
disp('The values of kobs are:')
disp(kobs)
disp('The value of k1 obtained from the fitting kobs is:');
disp(kf)
disp('The intercept of kobs plot which gives k2 is:')
disp(kb)

```

5.6.2 Code: Fitting dissociation data

MATLAB

```
clc,clear
close all
% importing data from excel sheet
[data,hd,raw] = xlsread("dissociation_data.xlsx");
% seperating data into x and y
x = data(:,1); y = data(:,2);
g = fitttype('a*(exp(-k*x))'); % defining fit type
% fitting data using fit type g
f = fit(x,y,g,'Startpoint',[1,0.01]);
% taking kobs from the fit
% ligand concentration from fit f
kb = f.k;
figure(1)
plot(f,x,y)
legend('data','data fit')
xlabel("Time (min)"); ylabel("Target-Ligand Complex conc")

disp('The value of k2 obtained:');
disp(kb)
```

5.6.3 Code: Error band

MATLAB

```
clear
close all
[data,hd,raw] = xlsread("C:\Harika_IITD\MTechThesis\chap2\result.xlsx");
x = data(:,2); m = data(:,3); f = data(:,4);
x = x(~isnan(x));
m = m(~isnan(m));
f = f(~isnan(f));

e = std([m';f']);
e = e';
figure(1)
hold on
lo = f - e;
hi = f + e;
hp = patch([x; x(end:-1:1); x(1)], [lo; hi(end:-1:1); lo(1)], 'r');
hold on;
```

```
h1 = plot(x,f);

set(hp, 'facecolor', [1 0.8 0.8], 'edgecolor', 'k','linewidth',2);
set(h1, 'color', 'r', 'marker', 'x','linewidth',1);
scatter(x,m,45,'b','linewidth',3);
xlabel('Time (minutes)','fontweight','bold','fontsize',18);
ylabel('Target-Ligand Complex','fontweight','bold','fontsize',18);
legend('error','Fitted','Data points','Location','southeast');
box on
```

Chapter 6

Structural, Kinetic, and Dynamical Analysis of Receptor activated Proteolysis

6.1 Introduction

The activation of the SPS signaling pathway starts with the activation of a zymogen protease Ssy5 that has an N-terminal pro-domain and C-terminal catalytic domain. The pro-domain is partially cleaved and non-covalently attaches itself to the catalytic domain to inhibit its activity. This process is independent of the presence of amino acids. However, the mechanism of this autolytic process is not clear. The function of the catalytic domain is to activate the latent transcription factors Stp1 and Stp2, and this process happens only when the catalytic Ssy5 is released from the Pro-domain. This takes place by receptor-mediated proteolysis (RAP), signaled by the extracellular amino acid. In the presence of the amino acid, the receptor Ssy1 binds to Ptr3 to form a complex. Ptr3 is an adapter that attracts Ssy5 to form a ternary complex sensor SPS (Ssy1-Ptr3-Ssy5) on the plasma membrane. Ptr3 is also important in facilitating signal propagation by coupling Ssy1, an amino acid sensing protein, and Ssy5, an endoprotease, that cleaves its inhibiting pro-domain and frees the Cat-domain, to catalyze the activation of the latent transcription factors Stp1/2 in the cytoplasm.

In this Chapter, we construct a mathematical model based on the detailed molecular mechanism of receptor-associated proteolysis (RAP) that controls the catalysis of the transcription factor via the Ssy5 activation. We model this to capture the occurrence of the switch that releases the cat-domain from the pro-domain of Ssy5 for processing the latent transcription factor Stp1.

We first construct the molecular network of the SPS sensing pathway. The nonlinear ordinary differential equations (ODEs) are written based on the network using the laws of mass action kinetics. We also use the chemical reaction network theory (CRNT) and the theorems of the SR graph to determine whether the network is capable of exhibiting multistability, independent of the kinetic parameters.

6.2 Statement of the thesis problem

1. In this chapter, we begin with constructing the molecular circuits for *C. albicans* from the experiments.
2. We perform structural analysis using CRNT and SR graphs to determine the presence of multistability.
3. We hypothesize multiple mechanisms and compare their dynamics to understand which network is experimentally feasible.
4. We also perform kinetic analysis by extracting and fitting the parameters of the model from the experimental data using the software COPASI.

6.3 Autolysis of Ssy5



The first step of the SPS sensing pathway involves the autolysis of the protein Ssy5 (S5). The protein S5 has a pro-domain and catalytic domain. In this step, the

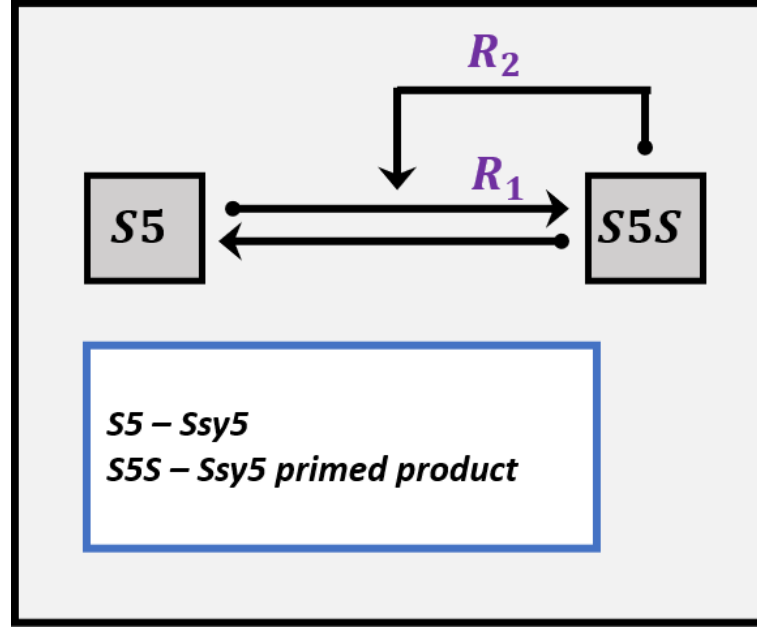


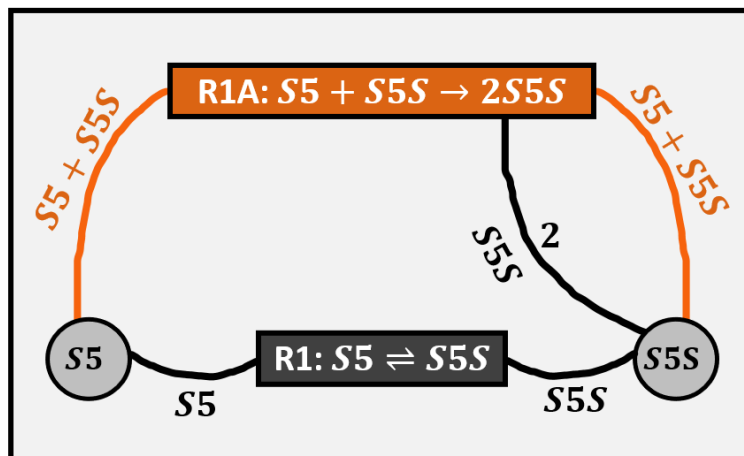
FIGURE 6.1: Autolysis of the protein Ssy5.

The protein Ssy5 represented by $S5$ has a prodomain and a catalytic domain. The prodomain partially cleaves and binds to the catalytic domain forming the primed product $S5S$. The reaction R_1 shows the conversion of $S5$ to $S5S$ and the reaction R_2 represents the autolysis step.

pro-domain partially cleaves and forms a non-covalent binding with the catalytic domain forming a primed product $S5S$. In this section, we took only the autolytic part of the network to look for the dynamical behavior that can generate switch-like behavior. We, therefore, took only the autolytic part of the network to study its behavior. The autolytic part of the network consists of the two reactions R_1 and R_2 as given in the equation 6.1. In the chemical steps, R_i 's are the reaction, and ri 's are the corresponding rates of the reactions with kfi 's as the forward rate constants and kbi 's as the reverse rate constants where i represents the number of the reaction. The corresponding ODE and mass conservation are given below.

$$\begin{aligned} \frac{d[S5S]}{dt} &= r_1 + r_1 A \\ &= -kf_1 A [S5S]^2 + (kf_1 A (S5T) - kb_1 - kf_1) [S5S] + kf_1 (S5T) \end{aligned}$$

Mass conservation: $S5 = S5T - S5S$

FIGURE 6.2: SR graph for autolysis of *Ssy5*.

The SR graph for the reactions $R1$ and $R1A$ is given in Fig 6.1. The species $S5$ and $S5S$ are shown in grey circles. The reaction symbols for the reactions $R1$ and $R1A$ are shown in boxes. The arcs or edges connect the species to the reaction symbols. The color of the c-pair containing reaction $R1A$ is orange. The reaction $R1$ which does not contain any c-pair, is shown in black. There is only one c-pair $\{S5+S5S\}$ in this graph. The cycle $R1A$ - $S5S$ (via complex $S5S$)- $R1$ - $S5$ - $R1A$ is a 2-cycle and an even cycle.

To check for bistability, we studied the properties of the SR graph and did the CRNT analysis. The SR graph is given in Fig 6.2. There is only one c-pair $\{S5+S5S\}$ in this graph. The cycle $R1A$ - $S5S$ (via complex $S5S$)- $R1$ - $S5$ - $R1A$ is a 2-cycle and an even cycle. Hence bistability cannot be precluded. To confirm bistability, we did a CRNT analysis. The network has four complexes, two linkage classes $\{S5, S5S\}$ and $\{S5+S5S, 2S5S\}$, the rank of the entire network is 1, and the deficiency of the network is 1. However, the Deficiency One Report showed that the network cannot admit multiple positive steady states. This eliminates the presence of bistability in the autolytic part of the network.

Since the bistability is ruled out for the network, we look for the presence of any other bifurcation. This one-dimension nonlinear ODE has 4 parameters. Since this is a 1D nonlinear ODE, we want to reduce it to a dimensionless form to compare it with the generic forms of the nonlinear ODEs that exhibit various types of bifurcations. To do this, we start by scaling the variables $[S5S]$ and the time t by taking $[x] = \frac{[S5S]}{[S5S]_0}$ and $\tau = \frac{t}{t_0}$. We take the units of concentration and time as nM and s, respectively. On plugging the scaling variables into the ODE above, we get

$$\begin{aligned}
\frac{[S5S]_0}{[t_0]} \frac{d[x]}{d\tau} &= -kf1A[S5S]_0^2[x]^2 + (-kb1 - kf1 + kf1A(S5T))[S5S]_0[x] + kf1(S5T) \\
&= -kf1A[S5S]_0^2[x]^2 \left(\frac{t_0}{[S5S]_0}\right) + (-kb1 - kf1 + kf1A(S5T))[S5S]_0[x] \left(\frac{t_0}{[S5S]_0}\right) \\
&\quad + kf1(S5T) \left(\frac{t_0}{[S5S]_0}\right) \\
&= -kf1A[S5S]_0[x]^2 t_0 + (-kb1 - kf1 + kf1A(S5T))[x] t_0 + kf1(S5T) \left(\frac{t_0}{[S5S]_0}\right)
\end{aligned}$$

Taking the scaling constants as $[S5S]_0 = S5T$ and $t_0 = \frac{1}{kf1}$, with the parameters $a = \frac{kf1A}{kf1} S5T$, $r = a - (1 + \frac{kb1}{kf1})$, and $h = 1$, we get the following dimensionless equation

$$\frac{d[x]}{d\tau} = r [x] - a [x]^2 + h$$

This has a form of transcritical bifurcation, provided $h = 0$. However, here $h \neq 0$, and for $h = 1$, this leads to an imperfect bifurcation. We perform below the steady state analysis and identify the condition for the parameter r at which the bifurcation can occur or not. The XPPAUT code for this network is given in code:[6.9.1](#)

6.3.1 Steady state analysis

The steady states are

$$\frac{d[x]}{d\tau} = 0 \implies r [x] - a [x]^2 + h = 0$$

$$a [x]^2 - r [x] - h = 0$$

$$[x]_{ss} = \frac{r \pm \sqrt{r^2 + 4 a h}}{2a}$$

The jacobian is

$$\frac{d[\dot{x}]}{dx} = r - 2a[x]$$

The eigenvalue λ of the jacobian around the steady state from the secular determinant is

$$\frac{d[\dot{x}]}{dx} - \lambda = r - 2a[x]_{ss} - \lambda = 0$$

So the eigenvalue is

$$\lambda = r - 2a[x]_{ss}$$

For the steady state, $[x]_{ss}$, the eigenvalue is

$$\lambda = r - 2a \left(\frac{r \pm \sqrt{r^2 + 4 a h}}{2a} \right)$$

For $h = 1$, this leads to

$$\lambda = \pm \sqrt{r^2 + 4 a}$$

Therefore for $[x]_{ss} = \frac{r + \sqrt{r^2 + 4a}}{2a}$, $\lambda = \sqrt{r^2 + 4a}$, > 0 , is unstable. For $[x]_{ss} = \frac{r - \sqrt{r^2 + 4a}}{2a}$, $\lambda = -\sqrt{r^2 + 4a}$, < 0 is stable. So one of the fixed points is always positive, and stable, while the other one is always negative, and it is unstable.

6.3.2 Phase-plane plot: No transcritical bifurcation point for $h > 0$

A phase-plane plot of $\dot{x}$ vs x shows that for $h > 0$, the curves are shifted up so that there will always be two zeros; i.e., the curves cut the x-axis at two places. One of them is stable, and the other is unstable. To determine the nature of bifurcation, we carry out a graphical analysis to show that there is no bifurcation for $h > 0$. The condition for transcritical bifurcation is that the two fixed points collide with each other to form only one fixed point, where the exchange of stability takes place, after which two fixed points are once again created. In the phase plane, as we change the bifurcation parameter, the two fixed points come to coalesce with each other,

collide, and become tangential to the x-axis, and then once again, two fixed points are created.

For that, we write the above ODE in the following way

$$a [x]^2 - r [x] - h = 0$$

Taking the left(L) and right (R) of the equation as

$$\underbrace{a [x]^2 - r [x]}_L = \underbrace{h}_R$$

From the plot of L and R on the same axis, the intersection provides a steady state. We also get the bifurcation point when L is tangential to R. To find the value of r at which the bifurcation occurs. We invoke the tangency condition: $L = R$ and $\frac{dL}{dx} = \frac{dR}{dx}$. This gives

$$a [x]^2 - r [x] = h$$

and

$$2 a x - r = 0 \text{ and } x_{max} = \frac{r}{2 a}$$

To get the bifurcation point, substituting x_{max} in the $L = R$ equation gives

$$a \left(\frac{r}{2a} \right)^2 - r \left(\frac{r}{2a} \right) = h$$

This gives

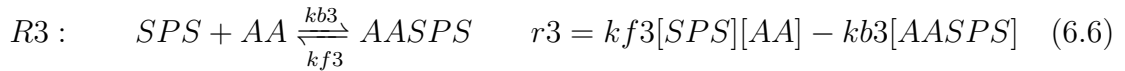
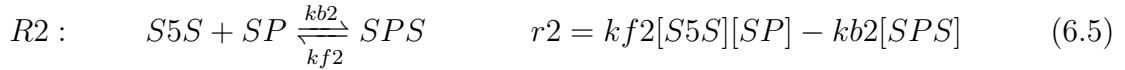
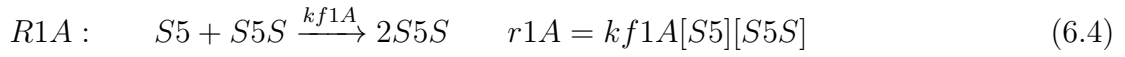
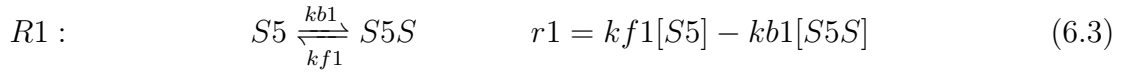
$$r^2 = -4ah \implies r = \pm 2i\sqrt{ah}$$

So there is no bifurcation point for any values of r in this model. If suppose we take $h = 0$, this leads to $r = 0$ and $x = 0$ as the bifurcation point since this is the steady state that exists all the time, and the exchange of stability takes place at this point. This corresponds to a transcritical bifurcation. However, in our case, $h = 1$, this parameter is called the imperfection parameter that destroys the transcritical bifurcation. We call this bifurcation as the imperfect transcritical bifurcation. We show in the phase-plane plot in Figure-6.3 the imperfect bifurcation for $h = 1$.

Note that the y-intersection always happens at $h = 1$ in the phase-plane plot. The MATLAB code for the rate balance plot and the imperfect transcritical bifurcation is given in codes:6.9.2.

6.4 Minimal model: Transcritical bifurcation with amino acid sensing

Having shown the presence of imperfect transcritical bifurcation, we next proceed to determine the role of S5S in binding to SP to form a complex SPS, and the stabilization of this complex by amino acid. For this, we added two more reactions as shown in Fig6.4. After the formation of the primed product S5S, it binds with the complex Ssy1-Ptr3 (SP) to form SPS. The complex SPS is unstable and is stabilized by the external amino acid (AA). The reactions taken are shown below. We perform numerical bifurcation analysis to find what happens to the imperfect bifurcation.



The ODEs are

$$\begin{aligned} \frac{d[S5S]}{dt} &= r1 + r1A - r2 \\ \frac{d[SPS]}{dt} &= r2 - r3 \\ \frac{d[AASPS]}{dt} &= r3 \end{aligned}$$

with the same S5T as the parameter. The mass conservation relation are

$$S5 = S5T - (S5S + SPS + AASPS)$$

$$SP = SPT - (SPS + AASPS).$$

The XPPAUT code for the network is given in code:6.9.4

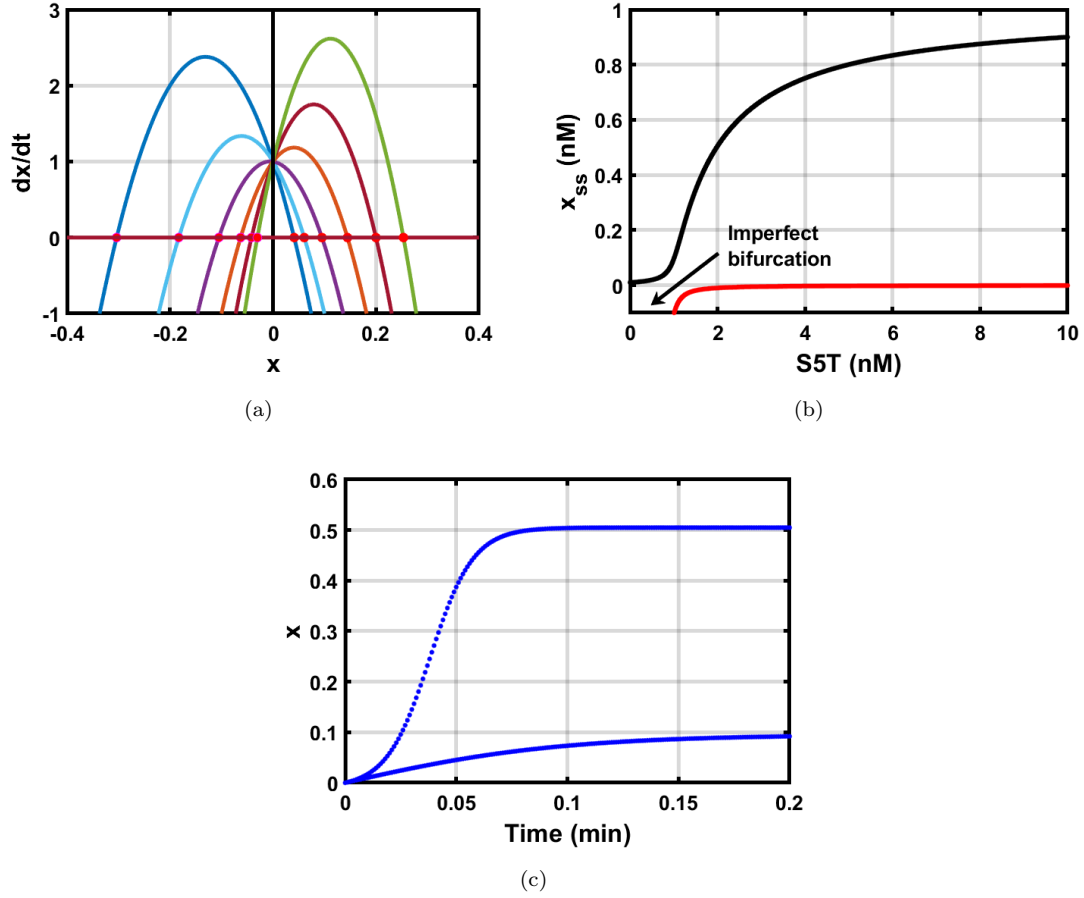


FIGURE 6.3: Bifurcation of imperfect transcritical bifurcations.

(a) Phase plane plot of imperfect transcritical bifurcation (Imperfect TB) for various values of $S5T$ ($S5T = 0.8$ to 1.3 in steps of 0.1 as we move from left to right). There is no collision of steady states and exchange of stability for any value of $S5T$. All the curves meet at y -intercept 1 , the value of imperfect parameter h . For a curve, there are two fixed points. The red and magenta colored points are the stable and unstable steady states for a taken $S5T$ value. The left fixed point in magenta color is unstable, and the right one in red color is a stable fixed point. We take the kinetic constants $kf1 = 0.0001$, $kf2 = 0.01$, and $kf3 = 0.01$, and the parameter $h = 1$. We use these values to get the dimensionless a and r . Both a and r are the functions of $S5T$. Therefore, we take $S5T$ as the bifurcation parameter that amounts to varying the parameter r . (b) Numerical analysis of imperfect bifurcation for $h=1$ where the black and red points indicate stable and unstable steady states. (c) The times series for $S5T=1$ is close to the imperfect bifurcation point and has no inflection point. The times series for $S5T=5$ is far away from the imperfect bifurcation point and has an inflection point.

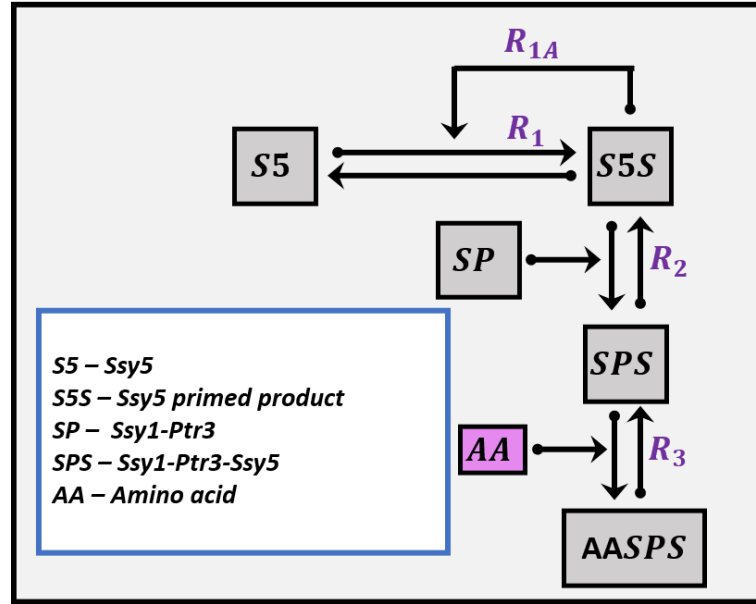


FIGURE 6.4: Amino acid sensing by SPS.

After the autolysis of $S5$, the primed product $S5S$ then binds with the complex $Ssy1-Ptr3$ (SP) to form SPS . The complex SPS is unstable and is stabilized by the external amino acid (AA). The reactions $R1$ and $R1A$ are the same as autolysis. The reaction $R2$ represents the binding of $S5S$ to SP , and the reaction $R3$ is the binding of AA with SPS forming the stable complex $AASPS$.

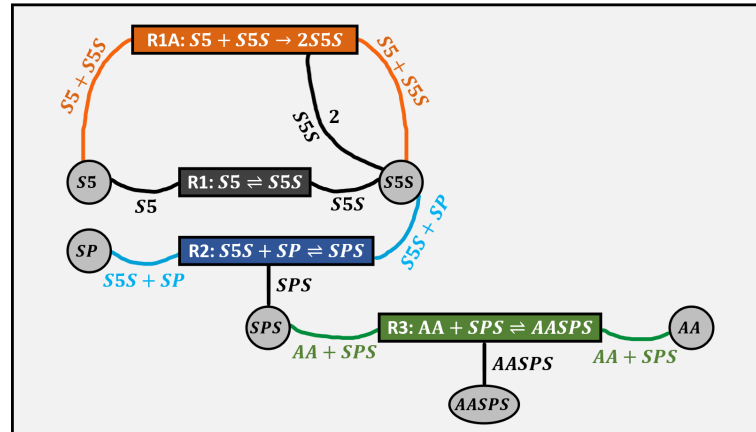


FIGURE 6.5: SR graph for the minimal model for SPS stabilization (Fig6.4).

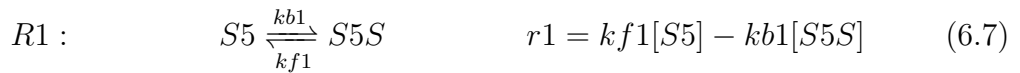
The species $S5$, $S5S$, SP , SPS , AA , and $AASPS$ are shown in grey circles. The reaction symbols for the reactions $R1$, $R1A$, $R2$, and $R3$ are shown in boxes. The arcs or edges connect the species to the reaction symbols. The colors of the c-pair containing reactions $R1A$, $R2$, and $R3$ are orange, blue, and green, respectively. The reaction $R1$ which does not contain any c-pair, is shown in black. There are 3 c-pairs $\{S5+S5S\}$, $\{S5S+SP\}$, and $\{AA+SPS\}$ in this graph. The cycle $R1A-S5S$ (via complex $S5S$)- $R1-S5-R1A$ is a 2-cycle and an even cycle.

The numerical bifurcation of the above equation still retains the imperfect transcritical bifurcation (Fig6.6a), but a delayed bifurcation (Fig6.6b). This also creates the inflection point, and the system therefore is ultrasensitive; i.e., it creates a switch that we attribute to the amino acid stabilization to a conformationally stable complex AASPS from an unstable SPS complex (Fig6.6c and d). Also, this is a cascade of reactions, where active S5S triggers complex formation SPS, followed by AASPS complex stabilization. The MATLAB code for the transcritical bifurcation is given in code:6.9.5

6.5 SPS sensing pathway: Network

In this section, we explain the construction of the molecular network for the whole SPS sensing pathway from the formation of the primed product S5S to the activation of the transcription factor STP. Their corresponding rates based on mass action kinetic law and the formation of corresponding nonlinear ODEs are also given. In the chemical steps, Ri 's are the reaction, and ri 's are the corresponding rates of the reactions with kfi 's as the forward rate constants and kbi 's as the reverse rate constants where i represents the number of the reaction. All the reactions are considered to be reversible.

Reaction R1



The protein Ssy5 (S5) has two domains, the pro-domain, and the catalytic domain. This step consists of the autolysis of the S5. The pro-domain partially cleaves, and it forms a non-covalent binding with the catalytic domain forming a primed product S5S. The conversion of S5 to S5S is independent of the external amino acid. Hence this reaction occurs upstream to the amino acid mediated stabilisation of the SPS complex. In the reaction, R1, the conversion of the protein Sys5 (S5) to the primed product (S5S) is taken as a reversible reaction.

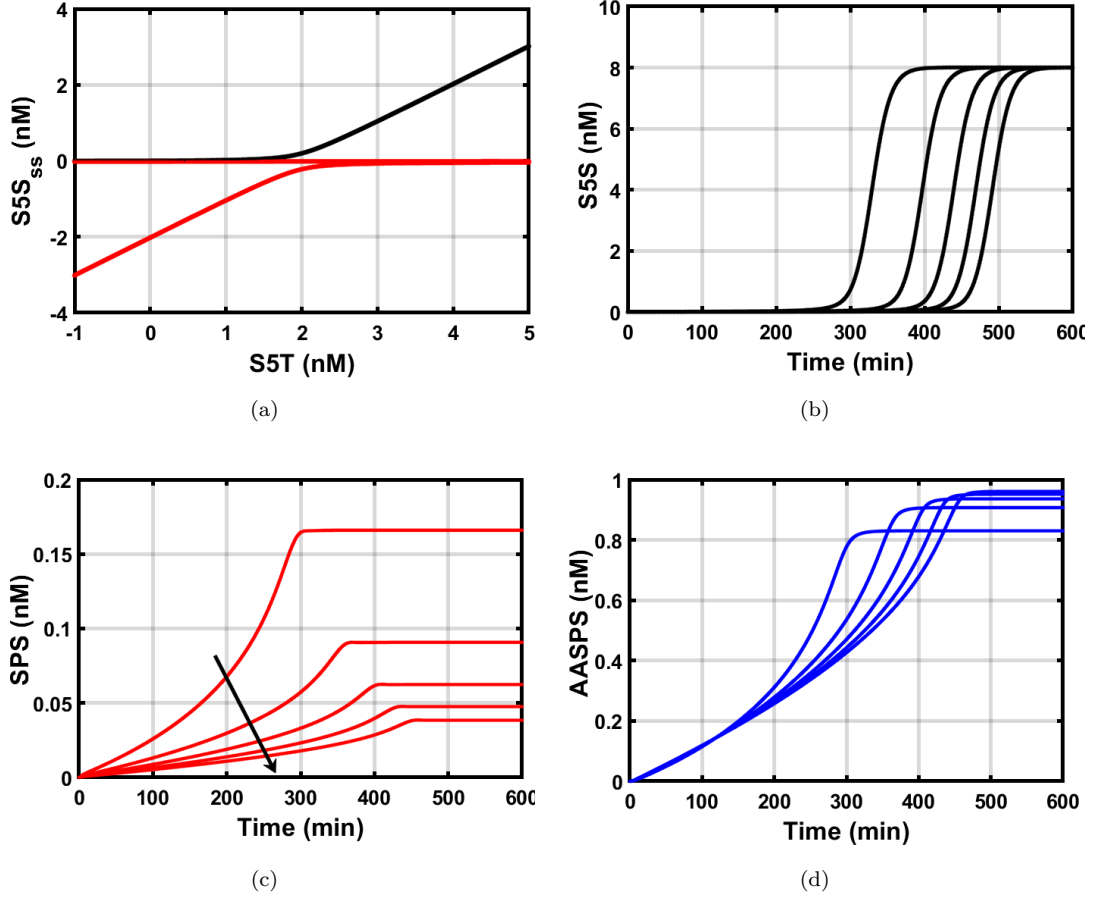
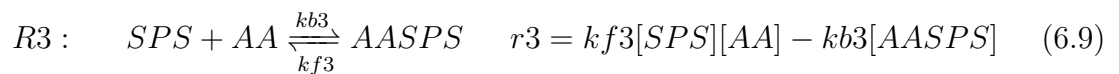
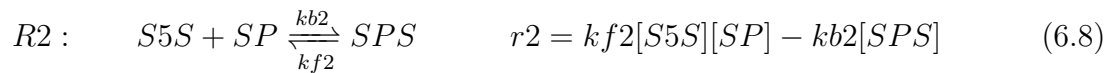


FIGURE 6.6: Codimension-1 bifurcation of imperfect transcritical bifurcations of amino acid stabilized SPS and Time series of the amino acid stabilized SPS.

(a) Imperfect transcritical bifurcation (Imperfect TB) for the bifurcation parameter $S5T$. We take the kinetic constants $kf1 = 0.0001, kf2 = 0.01, kb1 = 0.01, kf3 = 0.5, kb3 = 0.1, kf4 = 0.5, kb4 = 0.1, SPT = 1, AA = 1$. The inclusion of SPS complex and amino acid stabilization delays the imperfect bifurcation. (b,c,d) Time series indicates the presence of inflection points, due to which there is a finite induction period seen in all the variables. To simulate, we use the same kinetic constants, with $S5T=10$. The effect of the amino acid increase is to stabilize the formation of the complex AASPS. When we increase the amino acid AA from 1 to 5 in steps of 1, the induction period delays in all the variables (in the SPS time series in red, the black arrow shows the direction of AA increase).

Reaction R2 and R3



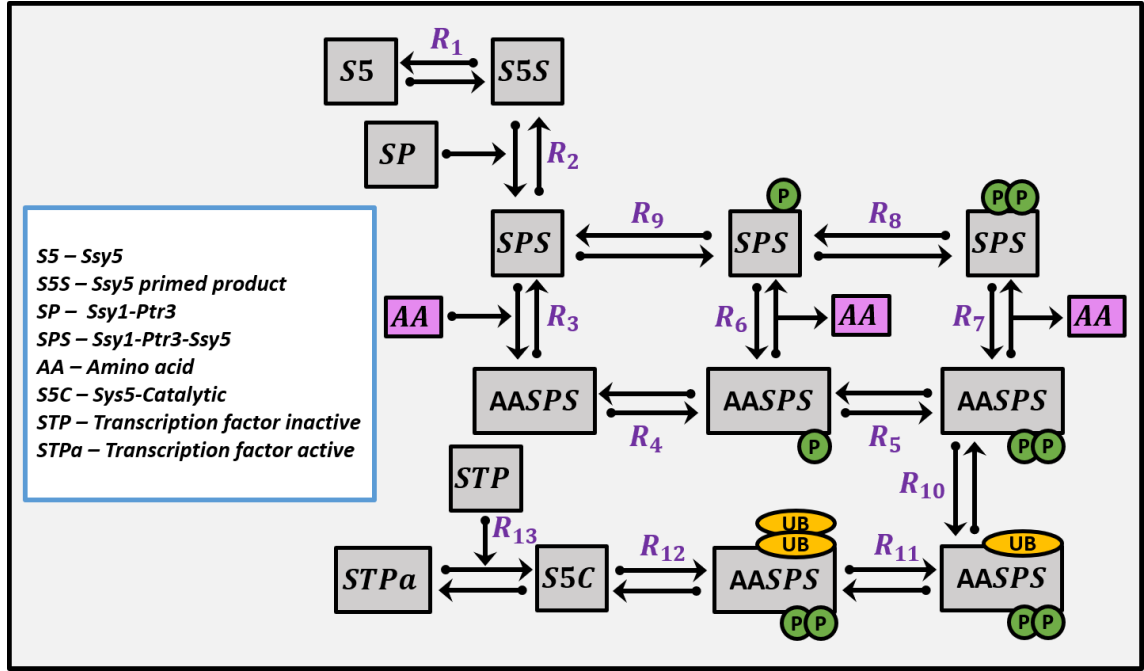
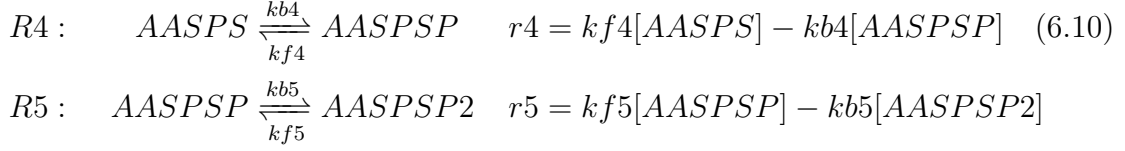


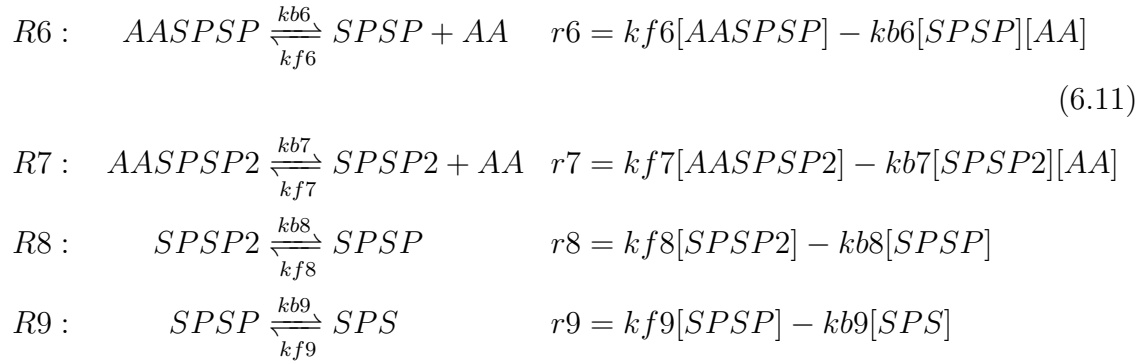
FIGURE 6.7: Biological network of SPS sensing pathway.

The grey boxes represent the protein molecules present in the pathway. The amino acid, which acts as a signal molecule in this pathway, is represented in purple boxes. The phosphorylated species are attached to P in a circle, and ubiquitinated species are attached to UB in an oval. In the first reaction, the protein Ssy5 represented by S5, undergoes an autolytic cleavage to give S5S. The species SP represents the complex of Ssy1 and Ptr3. S5S binds to SP to form an unstable complex SPS, which is stabilized by the binding of an external amino acid forming AASPS. The amino acid-bound SPS further undergoes multisite phosphorylation. We have assumed the phosphorylation to be sequential, first forming AASPSP and then AASPSP2. The phosphorylated product AASPSP2 further undergoes polyubiquitination at the two phosphorylated sites in a sequence, forming AASPSP2UB first and then AASPSP2UB. The polyubiquitination is followed by the degradation of the prodomain in S5S, releasing the catalytic Sys5 (S5C). S5C then catalyses the activation of the inactive transcription factor STP to STPa.

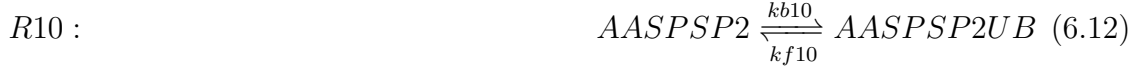
We take the complex of the receptor protein Ssy1 and the adaptor protein Ptr3 directly as SP. The primed product S5S, which is inactive, binds to SP to form the complex SPS (Sys1-Ptr3-Ssy5). SPS is the signaling complex. It is unstable and is stabilized with the binding of amino acid (AA). The reaction R2 shows the formation of the signaling complex SPS with the binding of S5S and SP. The reaction R3 shows the binding of AA to SPS and forming a stable complex AASPS.

Reaction R4 and R5

The binding of AA to SPS triggers phosphorylation, followed by ubiquitination. The phosphorylation takes place at both Ptr3 and Sys5 independently. Since there is a lack of experimental evidence for the cooperative effect, we assume sequential phosphorylation. The reactions R4 and R5 show sequential phosphorylation and dephosphorylation. Since there are two phosphorylation sites, R4 shows phosphorylation of AASPS at one of the sites giving AASPSP. In the reaction, R5, the other site, is also phosphorylated, giving AASPSP2.

Reaction R6 - R9

Here, we assume that the phosphorylated products AASPSP and AASPSP2 will release AA, forming SPSP and SPSP2, respectively. The reactions R6 and R7 show the release of AA from AASPSP and AASPSP2, respectively. The reactions R8 and R9 show the subsequent dephosphorylation of SPSP2 to SPSP in reaction R8 and SPSP to SPS in reaction R9. All the reactions mentioned above are taken as reversible reactions.

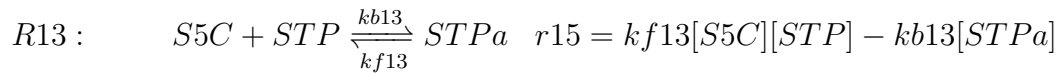
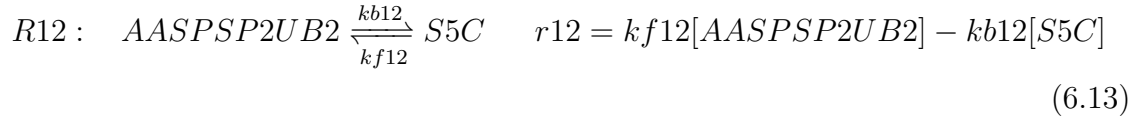
Reaction R10 and R11

$$r10 = \quad kf10[AASPSP2] - kb10[AASPSP2UB]$$



$$r11 = \quad kf11[AASPSP2UB] - kb11[AASPSP2UB2]$$

The phosphorylation is followed by the polyubiquitination of the phosphorylated product AASPSP2. This process leads to the proteasomal degradation of the Ssy5 prodomain. In the reactions, the ubiquitination is taken in a sequential manner where the phosphorylated product AASPSP2 undergoes ubiquitination to form AASPSPUB in reaction R10. AASPSP2UB further undergoes ubiquitination to give AASPSP2UB2 in reaction R11. The reactions are taken as reversible.

Reaction R12 and R13

After the polyubiquitination step, the proteasomal degradation of the Ssy5 prodomain occurs, releasing the catalytic part of Ssy5. The reaction R12 shows the degradation of ubiquitinated complex AASPSP2UB2 to release the catalytic part of Ssy5 (S5C). This catalytic part of Ssy5 involves in the enzymatic activation of the inactive transcription factor STP into STPa. The activated transcription factor can take part in the gene regulatory interactions to release the amino acid permease for the uptake of the external amino acid. The reaction R13 is the final step which shows the activation of STP by S5C.

The mass conservation relation is as follows

$$STP_T = STP + STPa \quad (6.14)$$

$$AA_T = AA + AASPS + AASPSP + AASPSP2 \quad (6.15)$$

$$+ AASPSP2UB + AASPSP2UB2 + S5C + STPa$$

$$SP_T = SP + AASPS + AASPSP + AASPSP2 \quad (6.16)$$

$$+ AASPSP2UB + AASPSP2UB2 + S5C + SPS$$

$$+ SPSP + SPSP2 + STPa$$

$$S5_T = S5 + AASPS + AASPSP + AASPSP2 \quad (6.17)$$

$$+ AASPSP2UB + AASPSP2UB2 + S5C + S5S$$

$$+ SPS + SPSP + SPSP2 + STPa$$

The corresponding 15 differential equations are

$$\frac{d[S5]}{dt} = -r1 \quad (6.18)$$

$$\frac{d[S5S]}{dt} = r1 - r2 \quad (6.19)$$

$$\frac{d[SP]}{dt} = -r2 \quad (6.20)$$

$$\frac{d[SPS]}{dt} = r2 - r3 + r9 \quad (6.21)$$

$$\frac{d[AA]}{dt} = -r3 + r6 + r7 \quad (6.22)$$

$$\frac{d[AASPS]}{dt} = r3 - r4 \quad (6.23)$$

$$\frac{d[AASPSP]}{dt} = r4 - r5 - r6 \quad (6.24)$$

$$\frac{d[AASPSP2]}{dt} = r5 - r7 - r10 \quad (6.25)$$

$$\frac{d[SPSP]}{dt} = r6 + r8 - r9 \quad (6.26)$$

$$\frac{d[SPSP2]}{dt} = r7 - r8 \quad (6.27)$$

$$\frac{d[AASPSP2UB]}{dt} = r10 - r11 \quad (6.28)$$

$$\frac{d[AASPSP2UB2]}{dt} = r11 - r12 \quad (6.29)$$

$$\frac{d[S5C]}{dt} = r12 - r13 \quad (6.30)$$

$$\frac{d[STP]}{dt} = -r13 \quad (6.31)$$

$$\frac{d[STPa]}{dt} = r13 \quad (6.32)$$

6.5.1 Analysis using CRNT and SR graph

6.5.1.1 CRNT

To analyze the structural properties of the network, we use the Chemical Reaction Network Theory (CRNT). As shown in Chapter 3, the structural analyses using CRNT can be performed without any prior knowledge about the kinetic parameters. In this network, we look for the presence of bistability. In a bistable system, the two stable, steady states are separated by an unstable steady state. The colliding of

the unstable and stable steady states gives rise to the saddle-node bifurcation. The bistable dynamics act as a checkpoint in the system.

For the network shown in Fig6.7, the CRNT shows that the network can exhibit multistability. CRNT also gives information about the number of complexes (n), linkage classes (l), the rank of the entire network (r), and the deficiency of the network ($\delta = n - l - r$). The network has 17 complexes and four linkage classes ($\{S5, S5S\}$, $\{S5S + SP, SPS, SPSP, SPS2P\}$, $\{AA + SPS, AASPS, AASPSP, AASPSP2, SPSP + AA, SPSP2 + AA, AASPSP2UB, AASPSP2U2, S5C\}$, and $\{S5C + STP, STPa\}$). The rank of the entire network is 11, and the deficiency is 2.

The Higher Deficiency Report generated parameters for which the system gives rise to bistability. We have provided the parameters generated by CRNT Toolbox in the table 6.1. Since CRNT does not give any units for the parameters, we have taken nanomolar (nM) for concentration and seconds for time. The reactions R3 to R9 contribute to the bistability of the network as these reactions are independently capable of generating bistability. However, by removing any of the reactions between R3 to R9, the system loses bistability.

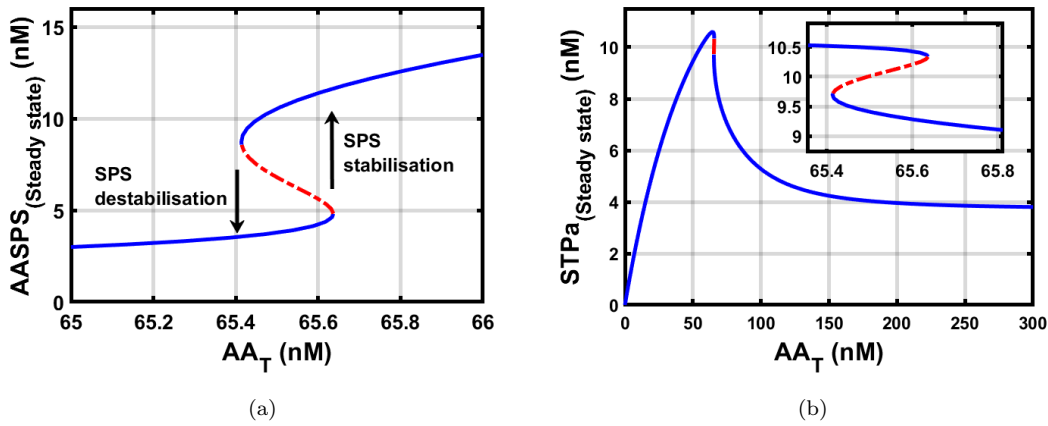


FIGURE 6.8: *Bifurcation diagram of the stabilized SPS (AASPS) and the activated transcription factor STPa against the total amino acid concentration. The parameters are taken from the CRNT Toolbox as given in table 6.1. The total concentration of amino acid is varied while keeping the total STP, SP, and S5 constant at 20 nM, 130 nM, and 130 nM, respectively.*

TABLE 6.1: Kinetic constants of the model from CRNT Toolbox

Reactions	kf1	kb1
R1	179.53489 s^{-1}	49.005949 s^{-1}
R2	1 $nM^{-1}s^{-1}$	154.25198 s^{-1}
R3	2780.6126 $nM^{-1}s^{-1}$	8914.8457 s^{-1}
R4	190.60145 s^{-1}	2909.6212 s^{-1}
R5	643.67283 s^{-1}	3801.0543 s^{-1}
R6	24757.413 s^{-1}	18388.243 $nM^{-1}s^{-1}$
R7	417.70116 s^{-1}	5856.5854 $nM^{-1}s^{-1}$
R8	1552.5808 s^{-1}	4426.9112 s^{-1}
R9	540.47995 s^{-1}	1331.2542 s^{-1}
R10	417.70116 s^{-1}	149.16326 s^{-1}
R11	149.16326 s^{-1}	113.69093 s^{-1}
R12	113.69093 s^{-1}	417.70116 s^{-1}
R13	39.749521 $nM^{-1}s^{-1}$	198.41674 s^{-1}

6.5.1.2 SR Graph

Along with the CRNT Analysis, we also study the properties of the model for the SPS sensing pathway to show that the model has the capacity for bistability. Fig6.9 shows the SR graph for the model. All the species are shown in grey circles, and the reaction symbols are shown in the boxes. The species and the reactions symbols constitute the nodes of the SR graph. The edges or arcs that connect the species to the reaction symbols are labeled with the corresponding complexes. Of the 17 complexes, only 5 complexes that correspond to the reactions R2, R3, R6, R7, and R13 form c-pairs.

The theorem to preclude bistability gives two conditions based on the properties of the SR graph. The first condition says that each cycle in the SR graph should either be a 1-cycle, an odd cycle, or both. The second condition is that no c-pair should be split by two even cycles. If the SR graph satisfies both conditions, the network will not exhibit bistability. However, if either of the condition is not satisfied, bistability cannot be precluded.

The SR graph (Fig6.9) has fifteen cycles, and all the cycles are 1-cycles, as the stoichiometry for all the reactions is one. The cycles and their properties are mentioned in table 6.2. Since all the cycles are 1-cycles, it satisfies the first point of the theorem. Hence for bistability to be possible, the SR graph must not satisfy

the second condition. That is, there should at least be one c-pair that is split by two even cycles. Here, of the fifteen cycles, nine cycles (Cycles 2, 4, 5, 7, 9, 11, 13, 14, and 15) are even. Cycles 2, 4, and 14 do not split any c-pair. The even-cycle 5 splits the c-pair $\{AA+SPS\}$ with the even-cycles 9, 11, and 12 and splits the c-pair $\{SPSP2+AA\}$ with the even-cycles 5 and 15. Hence the second condition of the theorem is not satisfied, and we can say that the network has the capacity to admit multiple steady states.

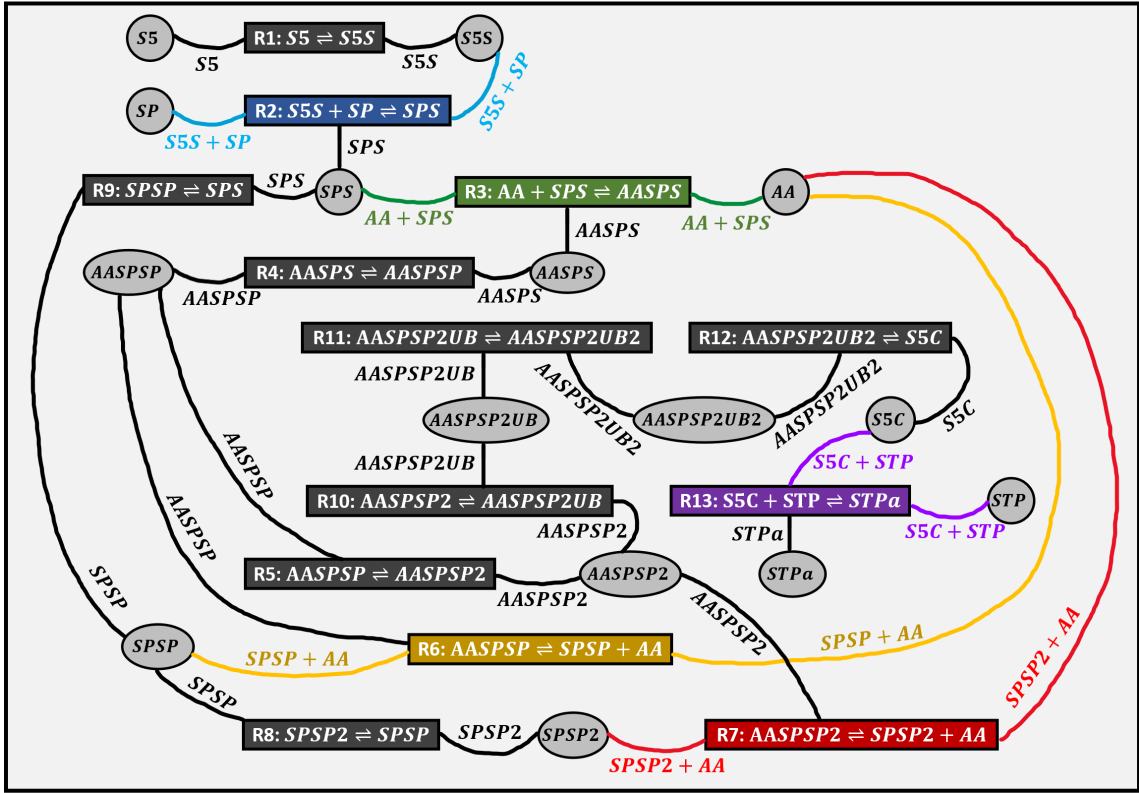


FIGURE 6.9: SR graph for the network of SPS sensing pathway.

The fifteen species are shown in grey circles. The reaction symbols for the reactions from R1 to R13 are shown in boxes. The arcs or edges connect the species to the reaction symbols. The colors of arcs containing the c-pair for reactions R2, R3, R6, R7, and R13 are blue $\{S5S+SP\}$, green $\{AA+SPS\}$, yellow $\{SPSP+AA\}$, red $\{SPSP2+AA\}$, and purple $\{S5C+STP\}$, respectively. The arcs that do not contain any c-pair are shown in black. The colors of all the reaction symbols are the same as their corresponding c-pairs. The color of the reaction symbols for R1, R4, R5, R8, R9, R10, R11, and R12 is black, as they do not correspond to any c-pair. All the cycles present in this SR graph are 1-cycles as the stoichiometry of all the reactions is one.

TABLE 6.2: Properties of the SR graph

Cycle No.	Cycles	Stoichiometry	c-pairs	Odd or Even	Split c-pairs	
					c-pair	Cycle
Cycle 1	R3 - SPS - R9 - SPSP - R8 - SPSP2 - R7 - AASPSP2 - R5 - AASPSP - R6 - AA	1-cycle	1	Odd	SPSP+AA	Cycle 3,7,12,15
Cycle 2	R3 - SPS - R9 - SPSP - R6 - AA	1-cycle	2	Even	SPSP2+AA	Cycle 3,6,11,13
Cycle 3	R3 - SPS - R9 - SPSP - R6 - AASPSP - R5 - AASPSP2 - R7 - AA	1-cycle	1	Odd	-	-
Cycle 4	R3 - SPS - R9 - SPSP - R8 - SPSP2 - R7 - AA	1-cycle	2	Even	SPSP+AA	Cycle 1,8,9,13
Cycle 5	R3 - SPS - R9 - SPSP - R8 - SPSP2 - R7 - AASPSP2 - R5 - AASPSP - R4 - AASPSP	1-cycle	0	Even	-	-
Cycle 6	R3 - SPS - R9 - SPSP - R6 - AA - R7 - AASPSP2 - R5 - AASPSP - R4 - AASPSP	1-cycle	1	Odd	AA+SPS	Cycle 9,10,11,12
Cycle 7	R3 - SPS - R9 - SPSP - R6 - AASPSP - R4 - AASPSP	1-cycle	0	Even	SPSP2+AA	Cycle 3,6,11,13
Cycle 8	R3 - SPS - R9 - SPSP - R8 - SPSP2 - R7 - AA - R6 - AASPSP - R4 - AASPSP	1-cycle	1	Odd	AA+SPS	Cycle 9,10,11,12
Cycle 9	R3 - AASPSP - R4 - AASPSP - R6 - AA	1-cycle	0	Even	SPSP+AA	Cycle 1,8,9,13
Cycle 10	R3 - AASPSP - R4 - AASPSP - R5 - AASPSP2 - R7 - SPSP2 - R8 - SPSP - R6 - AA	1-cycle	1	Odd	AA+SPS	Cycle 9,10,11,12
Cycle 11	R3 - AASPSP - R4 - AASPSP - R5 - AASPSP2 - R7 - AA	1-cycle	0	Even	SPSP+AA	Cycle 3,7,12,15
Cycle 12	R3 - AASPSP - R4 - AASPSP - R6 - SPSP - R8 - SPSP2 - R7 - AA	1-cycle	1	Odd	AA+SPS	Cycle 5,6,7,8
Cycle 13	R5 - AASPSP - R6 - AA - R7 - AASPSP2	1-cycle	0	Even	SPSP2+AA	Cycle 3,6,11,13
Cycle 14	R6 - AA - R7 - SPSP2 - R8 - SPSP	1-cycle	2	Even	AA+SPS	Cycle 1,5,10,15
Cycle 15	R5 - AASPSP - R6 - SPSP - R8 - SPSP2 - R7 - AASPSP2	1-cycle	0	Even	SPSP+AA	Cycle 5,6,7,8
					SPSP2+AA	Cycle 1,8,9,13
					-	-
					SPSP+AA	Cycle 1,5,10,15
					SPSP2+AA	Cycle 3,6,11,13

6.5.2 Results

From the CRNT analysis and the SR graph properties, it is evident that this network of the SPS sensing pathway is capable of exhibiting bistability. We used the parameters generated by CRNT Toolbox for the simulations. We generated the bifurcation diagrams using total amino acid AA_T as a parameter. We chose this parameter because, in the SPS sensing pathway, the external amino acid acts as a signal molecule and stabilizes the SPS complex by forming AASPS. The complex AASPS is responsible for releasing the catalytic S5 (S5C) for catalyzing the activation of the transcription factor STP.

Fig6.8 shows the bifurcation diagram of AASPS and the activated transcription factor STPa with varying total amino acid concentration (AA_T). In Fig6.8a, with the increase in the total amino acid concentration, the SPS stabilization complex AASPS increases and switches from a lower steady state to a higher steady state. The two stable states, in this case, represent the SPS stabilization at higher concentrations of the total amino acid and SPS destabilization at lower concentrations of total amino acid. These states also correspond to the activation and deactivation of the transcription factor STP.

However, in Fig6.8b, The steady state of the activated transcription shows a partial biphasic behavior along with hysteresis. Here, the behavior of the STPa steady state has two phases. In the first phase, there is a rapid rise in the STPa steady-state concentration with an increase in the total amino acid concentration until it reaches the peak, after which, in the second phase, there is a decline in the STPa steady-state concentration with further increase in the total amino acid concentration until it reaches a plateau. Also, hysteresis is seen at the declining phase of the curve.

The rapid rise in the STPa in the first phase can be attributed to the low dissociation rate constants ($KD_i = k_{bi}/k_{fi}$) of the reactions R9 and R6. The second phase of decline can be attributed to the high dissociation rate constant of the reaction R4. From Fig6.7, we can see that, for the activation of STP to STPa, the catalytic S5 (S5C) needs to be generated from the receptor complex SPS. The rapid rise of STPa is because of the quick generation of S5C from SPS via the reactions R9 and R6. SPS can either form AASPS (R3: $KD_3=3.2$), or it can form SPSP (R9: $KD_9=2.5$). Also, SPSP can either form SPSP (R8: $KD_8=2.8$) or AASPS (R6: $KD_6=0.74$). Since the dissociation constants of R9 and R6 are lesser than R3 and

R8, respectively, at low concentrations of AA, the S5C is first rapidly formed by the path SPS-SPSP-AASPSP-AASPSP2-AASPSP2UB-AASPSP2UB2-S5C. However, at higher concentrations of amino acid, when the AASPS concentration increases, the rapid dephosphorylation of AASPSP (R4: $KD_4=14.6$) is dominated as the corresponding dissociation rate constant is very high. This consequently reduces the concentration of S5C, leading to the decline in the partial biphasic curve. Since AA stabilizes and the SPS complex, the reaction R3 is expected to be more favorable. But the parameters generated by CRNT contradict this expectation leading to the unexpected partial biphasic behavior of activated transcription factor (STPa).

6.6 SPS sensing pathway: Alternate networks

6.6.1 Alternate network 1

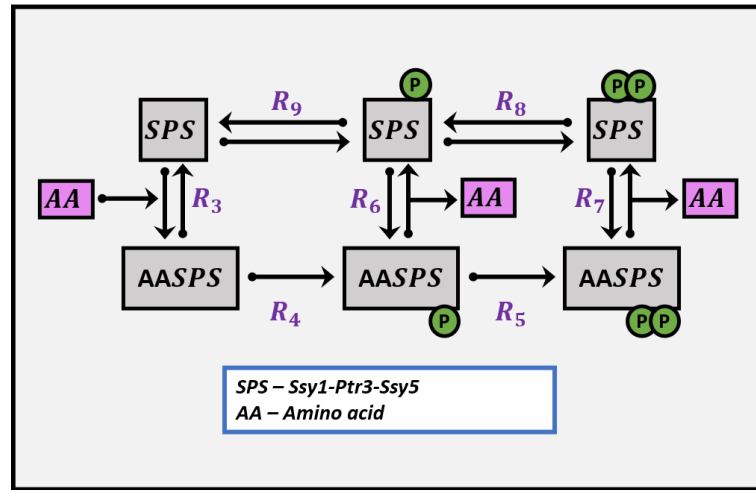


FIGURE 6.10: *Network contributing to bistability in amino acid sensing by SPS. This shows the part of the network that strongly contributes to the bistability. The reactions R3 to R9 can independently generate bistability. Unlike Network 1, the only modification in the network is that the reaction R4 and R5 are taken as irreversible.*

Though Network 1 gave expected results till the stabilization of SPS by the amino acid binding, the steady-state response of the activated transcription factor is unexpectedly biphasic. Hence we explored the network for other possible mechanisms. In the model given in Fig6.7, the bistability is contributed by the reactions R3 to R9. So, to retain bistability, we retained all the reactions but took the reactions R4 and

R5 alone as irreversible, as shown in Fig6.10. The CRNT analysis of the network shows the presence of bistability, and parameters generated by CRNT are used in simulations. The parameters used for this network are given in table6.3.

Fig6.6 shows the bifurcation diagram of the stabilized SPS (AASPS) and the activated transcription factor STPa with total amino acid concentration as the parameter as it is the signal molecule. Here, the activated transcription factor STPa increases with an increase in the amino acid concentration and switches from a lower steady state to a higher steady state. But, with an increase in the amino acid concentration, the steady-state concentration of AASPS first increase and then decreases, showing a biphasic behavior. However, AASPS is also expected to increase with the increase in amino-acid concentration.

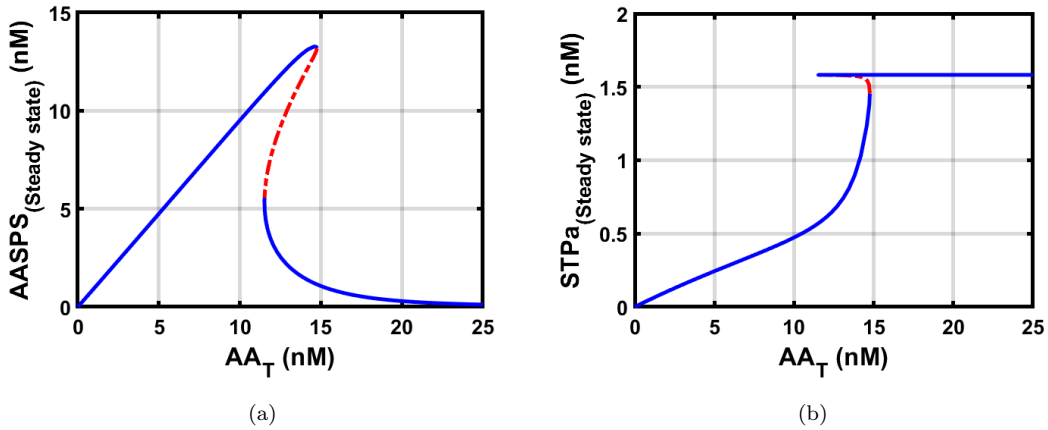


FIGURE 6.11: *Bifurcation diagram: Alternate Network 1*

Bifurcation diagram of the stabilized SPS (AASPS) and the activated transcription factor STPa against the total amino acid concentration for Alternate network 1. The parameters are taken from the CRNT Toolbox as given in table 6.3. The total concentration of amino acid is varied while keeping the total STP, SP, and S5 constant at 1 nM, 17 nM, and 16 nM, respectively.

6.6.2 Alternate network 2

Here, we took the entire network (Fig6.7) along with the autolysis step as shown in Fig6.1. Adding the autolytic step does not disturb the bistability of the system. From the SR graph for the autolytic reactions as given in Fig6.2, we can see that these reactions do not preclude bistability. We could confirm the bistability using CRNT

Toolbox. We used the parameters generated by CRNT Toolbox, for generating the bifurcation diagram of SPS stabilizing complex, AASPS and the activated transcription factor, STPa with total amino acid as the parameter as shown in Fig6.12.

From the Fig6.12 we can see that with the increase in the total amino acid concentration, the complex AASPS increases and switches from a lower steady state to a higher steady state. However, the activated transcription factor STPa shows a partial biphasic response. The behaviour of the model is similar to that of the network shown in Fig6.7. Including the autocatalytic step has not changed the behaviour of the model.

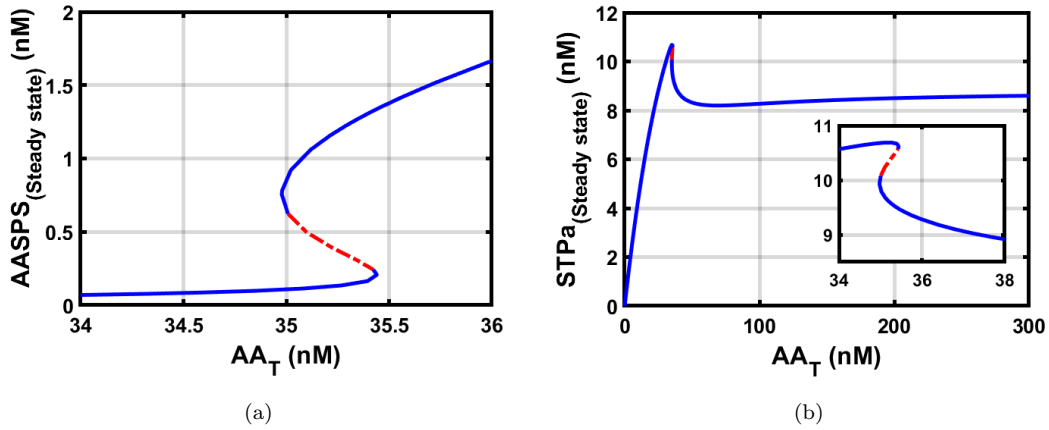


FIGURE 6.12: *Bifurcation diagram: Alternate network 2.*

Bifurcation diagram of the stabilized SPS (AASPS) and the activated transcription factor STPa against the total amino acid concentration for Alternate network 2. The parameters are taken from the CRNT Toolbox as given in table 6.3. The total concentration of amino acid is varied while keeping the total STP, SP, and S5 constant at 20 nM, 80 nM, and 50 nM, respectively.

6.6.3 Alternate network 3

Here, we took the entire network (Fig6.7) along with the autolysis step as shown in Fig6.1 but with reactions R4 and R5 alone as irreversible as shown in Fig6.10. As shown in alternate network 2, adding the autolytic step does not disturb the bistability of the system. The CRNT Toolnox confirmed the presense of bistability in the network. We used the parameters generated by CRNT Toolbox, for generating

the bifurcation diagram of SPS stabilizing complex, AASPS and the activated transcription factor, STPa with total amino acid as the parameter as shown in Fig6.13.

From the Fig6.13 we can see that with the increase in the total amino acid concentration, the complex AASPS shows biphasic curve where there is a initial rise and then the concentration switches to a lower steady state. The activated transcription factor STPa increases with the increase in total amino acid concentration and then switches to a higher steady state following the expected dynamics. The behaviour of the model is similar to that of the alternate network 1. Including the autocatalytic step has not changed the behaviour of the model.

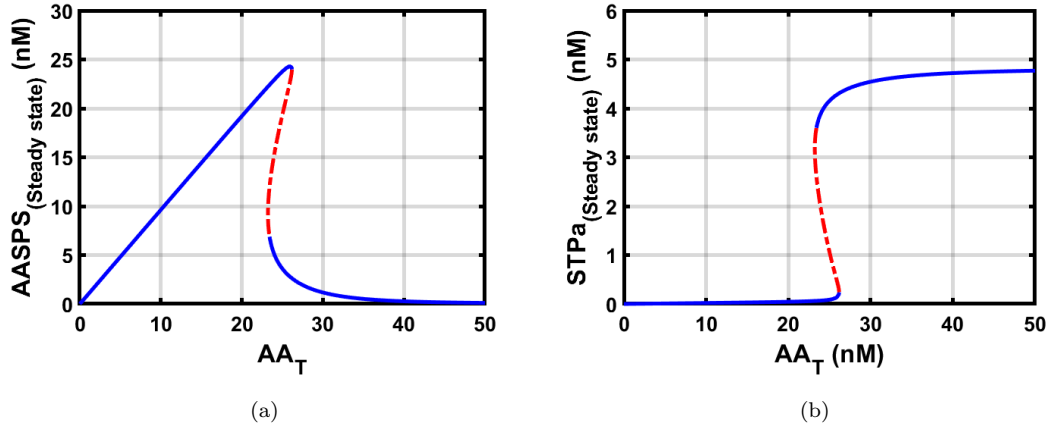


FIGURE 6.13: *Bifurcation diagram: Alternate network 3.*

Bifurcation diagram of the stabilized SPS (AASPS) and the activated transcription factor STPa against the total amino acid concentration for Alternate network 3. The parameters are taken from the CRNT Toolbox as given in table 6.3. The total concentration of amino acid is varied while keeping the total STP, SP, and S5 constant at 5 nM, 31 nM, and 30 nM, respectively.

TABLE 6.3: Kinetic constants of the model from CRNT Toolbox for all the Alternate networks

Reactions	Alternate Network 1		Alternate Network 2		Alternate Network 3	
	kf1	kb1	kf1	kb1	kf1	kb1
R1	11.063738 s^{-1}	11.063738 s^{-1}	35.822711 s^{-1}	38.033327 s^{-1}	9099.3339 s^{-1}	1206.7353 s^{-1}
R1A	-	-	6.235 $nM^{-1}s^{-1}$	-	485625 $nM^{-1}s^{-1}$	-
R2	3.497 $nM^{-1}s^{-1}$	11.070122 s^{-1}	1 $nM^{-1}s^{-1}$	53.466511 s^{-1}	1036.7 $nM^{-1}s^{-1}$	3294.3128 s^{-1}
R3	33111 $nM^{-1}s^{-1}$	1 s^{-1}	2737 $nM^{-1}s^{-1}$	29118.91 s^{-1}	280718 $nM^{-1}s^{-1}$	1 s^{-1}
R4	1.729938 s^{-1}	-	378.49617 s^{-1}	1048.5634 s^{-1}	84.791024 s^{-1}	-
R5	1026.7825 s^{-1}	-	89.37295 s^{-1}	148.4894 s^{-1}	37.045439 s^{-1}	-
R6	593.53717 s^{-1}	10491 $nM^{-1}s^{-1}$	1910.6983 s^{-1}	846 $nM^{-1}s^{-1}$	3208.1104 s^{-1}	132993 $nM^{-1}s^{-1}$
R7	244933.19 s^{-1}	46312 $nM^{-1}s^{-1}$	124.99638 s^{-1}	841 $nM^{-1}s^{-1}$	7657.7204 s^{-1}	937.08391 s^{-1}
R8	199.62542 s^{-1}	28.655432 s^{-1}	84.453912 s^{-1}	322.31626 s^{-1}	32.28205 s^{-1}	52.301301 s^{-1}
R9	45.097507 s^{-1}	32.98794 s^{-1}	42.77425 s^{-1}	627.29306 s^{-1}	4486.9822 s^{-1}	3294.3128 s^{-1}
R10	89721.156 s^{-1}	89721.156 s^{-1}	19.705871 s^{-1}	19.705871 s^{-1}	8.130919 s^{-1}	8.130919 s^{-1}
R11	89721.156 s^{-1}	89721.156 s^{-1}	19.705871 s^{-1}	19.705871 s^{-1}	8.130919 s^{-1}	8.130919 s^{-1}
R12	89721.156 s^{-1}	89721.156 s^{-1}	19.705871 s^{-1}	19.705871 s^{-1}	8.130919 s^{-1}	8.130919 s^{-1}
R13	89691 $nM^{-1}s^{-1}$	19.02797 s^{-1}	2 $nM^{-1}s^{-1}$	10.345172 s^{-1}	4.237 $nM^{-1}s^{-1}$	2.4373492 s^{-1}

6.7 Parameter estimation: Fitting of Time Series using Copasi

Parameter estimation is done in Copasi on the basis of the given experimental dataset. Here, parameter estimation is done for the minimal model as shown in Fig6.4 and the model described in Alternate network 2. The minimal model has 4 reactions and 7 parameters. Alternate Network 2 has 14 reactions and 27 parameters. For both models, the parameters for the reactions R1, R1A, R2, and R13 were fit using the time series data taken from [18] and [19] shown in the Fig6.14. The rest of the parameters for which the data is not available were adjusted in Copasi while fitting the time series data. The fit is performed based on the weighted sum of squares as given in the equation 6.33.

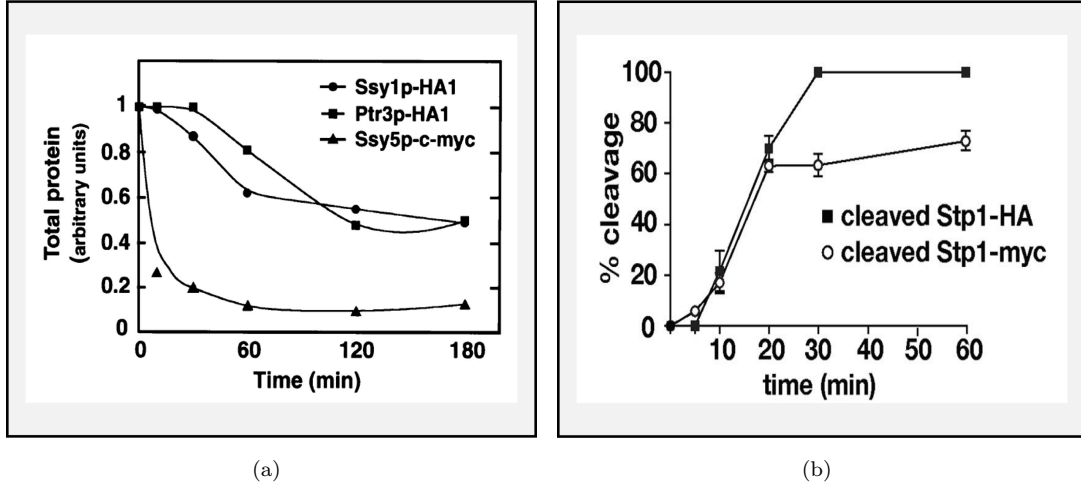
$$E(P) = \sum_{i,j} w_j \cdot (x_{i,j} - y_{i,j}(P))^2 \quad (6.33)$$

In equation 6.33, “P” is the parameter being tested, $x_{i,j}$ is the point from the dataset, and $y_{i,j}(P)$ is the simulated value. Here, i and j denote the rows and columns of the dataset. The weight for each data column is given by w_j . The weight “Mean Square” as given in the equation 6.34 was used. “Evolutionary Programming” was used as the optimization method.

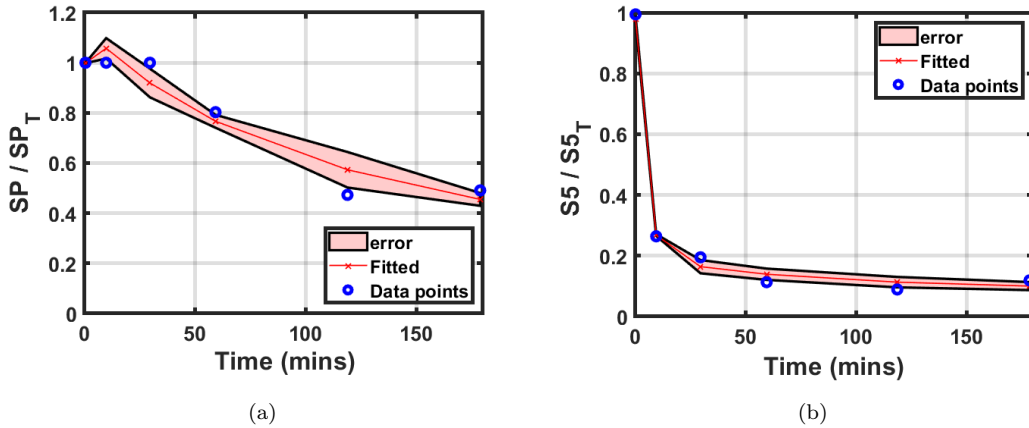
$$w_j = \frac{1}{< x_j^2 >} \quad (6.34)$$

As the Y-axis of the data available is given in arbitrary units, we normalized the Y-axis of the data before performing the fit. The concentration units for the parameters obtained using Copasi are considered as nM, and the units for time are taken in terms of minutes as given in the data. The data of the fit obtained from the Copasi was taken, and the figure was produced with the error band using MATLAB. The fit for the data is shown in Fig6.15 and Fig6.16 for the minimal model and the alternative network 2, respectively.

For the fit of the minimal model, the root mean square value for SP is 0.08, and for S5 is 0.02. The parameters obtained from the fit are given in the table 6.4. For the alternate network 2, the root mean square value for SP is 0.08, S5 is 0.1, and for STPa is 0.15. The parameters obtained from the fit are given in the table 6.4.

FIGURE 6.14: *Experimental time series data.*

(a) Time series is taken from Figure 8 of [18]. The data from the curves *Ssy5p-c-myc* and *Ptr3p-HA1* were used to fit the transient concentrations of the species *Ssy5* (*S5*) and the complex *Ssy1-Ptr3* (*SP*), respectively. (b) The time series from Figure 2 of [19] is taken to fit the transient concentration of the activated transcription factor *STPa*.

FIGURE 6.15: *Fitted time series data for the minimal model given in Fig6.4.*

The time series data for (a) *SP* and (b) *S5* are fitted with the minimal model using *Copasi*. The Y-axis of the data is normalized before fitting. The root mean square value for *SP* is 0.08, and for *S5* is 0.02. The parameters obtained from the fit are given in the table 6.4.

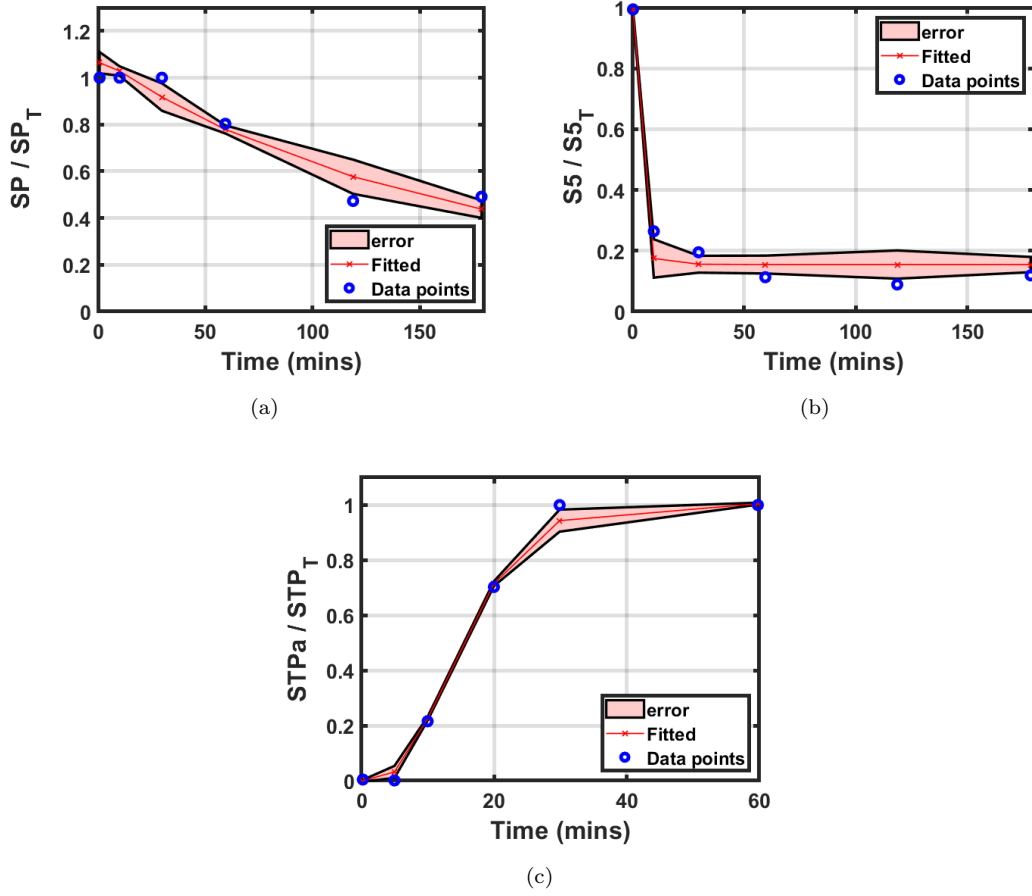


FIGURE 6.16: *Fitted time series data for the Alternate Network 2.* The time series data for (a) SP , (b) $S5$, and (c) $STPa$ are fitted with the Alternate Network 2 using *Copasi*. The Y-axis of the data is normalized before fitting. The root mean square value for SP is 0.08, $S5$ is 0.1, and for $STPa$ is 0.15. The parameters obtained from the fit are given in the table 6.4.

6.7.1 Simulations

The parameters obtained from fitting the time series data as shown in Fig6.15 and Fig6.16 are used to simulate the bifurcation diagram for the minimal model and the model described in the alternated network 2. The parameters used for both the models are given in tables 6.4 and 6.5, respectively. The bifurcation diagram generated using the parameters obtained from the fitting of the experimental data is compared to the bifurcation diagrams generated using the parameters used in the earlier sections.

TABLE 6.4: Kinetic constants of the minimal model from Copasi

Reactions	kf1	kb1
R1	0.0416795 min^{-1}	0.144778 min^{-1}
R1A	0.664751 $nM^{-1}\text{min}^{-1}$	-
R2	0.0104934 $nM^{-1}\text{min}^{-1}$	0.104269 min^{-1}
R3	487.201 min^{-1}	0.00128645 min^{-1}

TABLE 6.5: Kinetic constants of the Alternate Network 2 from Copasi

Reactions	kf1	kb1
R1	42.9317 min^{-1}	209.632 min^{-1}
R1A	14.2456 $nM^{-1}\text{min}^{-1}$	-
R2	0.0291455 $nM^{-1}\text{min}^{-1}$	96.2421 min^{-1}
R3	6471.55 $nM^{-1}\text{min}^{-1}$	20696.7 min^{-1}
R4	2217.94 min^{-1}	346.992 min^{-1}
R5	0.259052 min^{-1}	619.809 min^{-1}
R6	360.317 min^{-1}	1001.36 $nM^{-1}\text{min}^{-1}$
R7	43.2433 min^{-1}	133663 $nM^{-1}\text{min}^{-1}$
R8	265.061 min^{-1}	55.1568 min^{-1}
R9	0.324708 min^{-1}	9961.15 min^{-1}
R10	39.777 min^{-1}	13.7342 min^{-1}
R11	58.6424 min^{-1}	0.0477934 min^{-1}
R12	376.023 min^{-1}	104.471 min^{-1}
R13	0.217533 $nM^{-1}\text{min}^{-1}$	2.16e-5 min^{-1}

In the minimal model, the presence of imperfect transcritical bifurcation is seen due to the autolytic step represented by the reaction R1A. The imperfect transcritical bifurcation is shown in Fig6.6a. While using the parameters obtained from fitting the time series data, the imperfect bifurcation is still retained. Also, there is a presence of perfect transcritical bifurcation. Fig6.4 shows the steady state of S5S plotted with varying total concentrations of S5 ($S5_T$). Though the perfect bifurcation is seen at $S5_T = 1$, it occurs when S5S is slightly negative, which is not in the biological range. In the biological range of concentrations, only imperfect bifurcation is seen.

In the alternate network 2, the entire network, as given in Fig6.7, is taken along with the autolytic reaction R1A. CRNT showed bistability for this network. But the bifurcation diagram generated using CRNT parameters for the activated transcription factor STPa was partially biphasic (Fig6.12b). However, with the estimated parameters (Fig6.16), we can see that with the increase in the total amino acid concentration, the complex AASPS increases and switches from a lower steady state

to a higher steady state. Also, the activated transcription factor STPa increases with the increase in total amino acid concentration, as expected. With the estimated parameters, the behavior of the model is close to the biological interpretations.

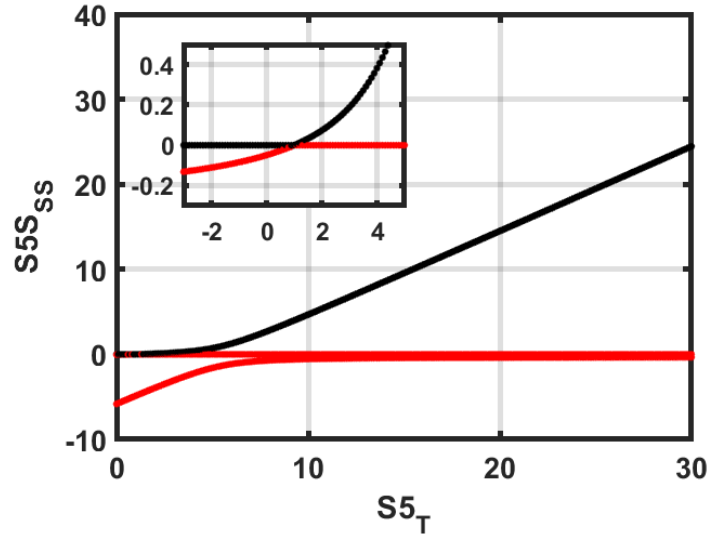


FIGURE 6.17: *The bifurcation diagram for the minimal model as given in Fig6.4. The steady state of S5S is plotted with varying total concentrations of S5 ($S5_T$). The parameters obtained from Copasi by fitting the time series data were used. While using the fitted parameters, the existence of both perfect and imperfect transcritical bifurcation is seen. The total concentration of S5 is varied while keeping the total STP a and AA constant at 1 nM and 25 nM, respectively.*

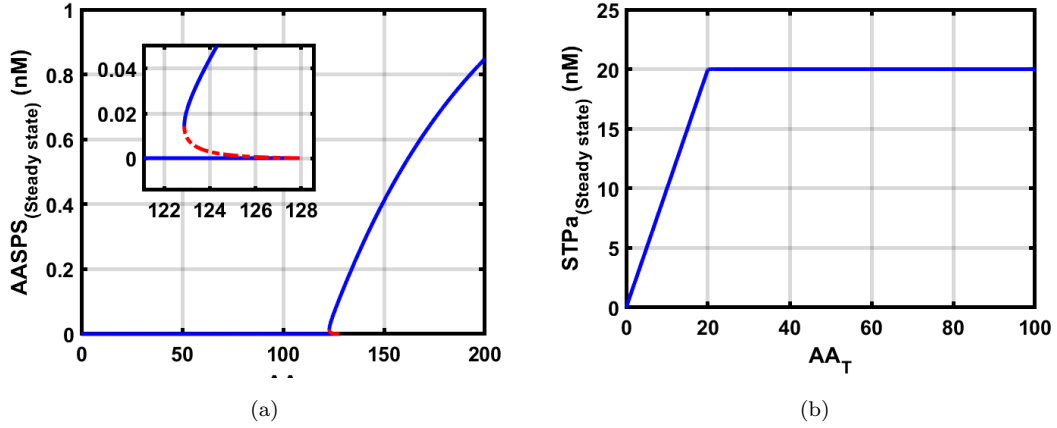


FIGURE 6.18: *Bifurcation diagram with fitted parameters: Alternate network 2.* Bifurcation diagram of the stabilized SPS (AASPS) and the activated transcription factor STPa against the total amino acid concentration for Alternate network 2. The parameters are taken from Copasi by fitting the time series data as given in table 6.5. The total concentration of amino acid is varied while keeping the total STP, SP, and S5 constant at 20 nM, 130 nM, and 130 nM, respectively.

6.8 Summary

1. All the nonlinear ordinary differential equations written from the network is based on mass action kinetics.
2. The modeling of the SPS sensing pathway and its structural analysis using CRNT and SR graph properties shows that the network, as provided in Fig6.7, has the capacity to exhibit bistability.
3. Adding the autolytic step to the network does not disturb the capacity for bistability, but the possibility of transcritical bifurcation is also seen. This sensing pathway involves the stabilization of the SPS (Ssy1-Ptr3-Ssy5) complex by the external amino acid, which triggers the signaling pathway for the activation of the transcription factor STP by releasing the catalytic Ssy5 (S5C).
4. By using the parameters generated by CRNT, the results for the stabilization of the SPS complex is as expected (Fig6.8a). However, the behavior of the activated transcription factor (STPa) (Fig6.8b) contradicts the theory. This is because the parameter taken from CRNT is not close to the biological parameters. By performing parameter estimation using the existing time series

data and using the parameter set close to the experimental data, we saw that the behavior of the model is close to biological interpretation.

5. This is still an ongoing study. Exploring more alternate networks and using other experimental data, such as the steady-state data, will help in better assessment of the dynamics of the network.

6.9 Codes

6.9.1 Code: XPPAUT file for Ssy5 autolysis

XPPAUT

```
# XPPAUT FILE FOR Ssy5 autolytic reactions
# The file name is SPS_auto_xppaut.txt
# R1: S5 --> S5S : v1 = kf1[S5]
# R2: S5 + S5S ---> 2S5S : v2 = kf2 [S5][S5S]
# R3: S5S --> S5 : v3 = kf3[S5S]

par S5T =1
#par S5T =5

par kf1 = 0.0001
par kf2 = 0.01
par kf3 = 0.01

a = (kf2/kf1)*S5T
r = a - (1 + kf3/kf1)
par h=1
x'=-a*(x2) + r*x + h

#numerical settings @ TOTAL=10,Dt=0.001,METH=cvode,atol=3e-
16,toler=3e-16,newt_tol=.0001
@ XHI=15,YLO=0,YHI=15,BOUND=1000,maxstorage=100000
```

done

Instructions:

For AUTO, keep S5T=-1 and the range of par:-10 to 10

6.9.2 Code: Rate balance Plot

MATLAB

```
clear
close all
clc
pl = 1;
h=1;
x = -5:0.001:5;

figure(2)
h=1;
for S5T = 0.8:0.1:1.3
kf1=0.0001; kf2 = 0.01; kf3=0.01;
a = S5T*kf2/kf1;
r = a - (1 + kf3/kf1);
y1 = r.*x - a.*(x.^2) + h;
axis([-0.4 0.4 -1 2])
plot(x,y1,'LineWidth',2)
grid on
hold on
yintercept = -1:length(y1);
plot(zeros(size(yintercept)),yintercept,'k','LineWidth',2)
plot(x,zeros(size(x)), 'LineWidth',2)
axis([-0.4 0.4 -1 3])
xeq1 = (r + sqrt(r^2 + 4*a))/(2*a);
xeq2 = (r - sqrt(r^2 + 4*a))/(2*a);
plot(xeq1,0,'or','MarkerSize',7,'MarkerFaceColor','r')
plot(xeq2,0,'om','MarkerSize',7,'MarkerFaceColor','r')
hold on
end

xlabel('\it x','fontweight','bold','fontsize',20)
ylabel('\it dx/dt','fontweight','bold','fontsize',20);
ax=gca;
ax.XAxis.LineWidth = 3;
ax.XAxis.Color = 'k';
```

```

ax.YAxis.LineWidth = 3;
ax.YAxis.Color = 'k';
ax.FontSize = 10;
ax.FontWeight = 'bold';
grid on
iplot=1;
alphabet = char(iplot+64);
title(alphabet,'fontsize',15,'horizontalAlignment','right')

```

6.9.3 Code: Bifurcation plot for autolysis of Ssy5

MATLAB

```

clear
clc
syms x la

for S = 0:0.001:10
    S5T=S;
    a = (kf2/kf1)*S5T;
    r = a - (1+ (kf3/kf1));
    fun = -a*(x^2) + r*x + h;
    assume(x,'real')
    sol = vpasolve(fun==0,x);
    len = length(sol);
    jac = jacobian(fun,x)-la*eye(1,1);
    for q = 1:len
        CE = subs(jac,x,[sol(q)]);
        eqn = det(CE);
        soleig=vpasolve(eqn==0,la);

        if (soleig(1) < 0 )
            plot(S,sol(q),'.k','MarkerSize',12)
            hold on
        else
            plot(S,sol(q),'.r','MarkerSize',12)
            hold on
        end
    end
end

xlabel('\it S5T','fontweight','bold','fontsize',20)
ylabel('\it x_{ss}','fontweight','bold','fontsize',20);
ax=gca;
ax.XAxis.LineWidth = 3;
ax.XAxis.Color = 'k';

```

```

ax.YAxis.LineWidth = 3;
ax.YAxis.Color = 'k';
ax.FontSize = 10;
ax.FontWeight = 'bold';
grid on
iplot=1;
alphabet = char(iplot+64);
title(alphabet,'fontsize',15,'horizontalAlignment','right')
axis([0 10 -0.1 1])

```

6.9.4 Code: XPPAUT file for minimal model

XPPAUT

```

# XPPAUT FILE FOR minimal model
# The file name is SPS_2_xppaut.txt
# R1: S5 --> S5S : v1 = kf1[S5]
# R2: S5 + S5S ---> 2S5S : v2 = kf2 [S5][S5S]
# R3: S5S --> S5 : v3 = kf3[S5S]
# R4: S5S + SP <---> SPS : v4 = kf4 [S5S][SP] - kb4[SPS]
# R5: SPS + AA <---> AASPS : v5 = kf5 [SPS][AA] - kb5[AASPS]

par S5T =10,SPT=1, AA=1

par kf1=0.0001, kf2=0.01
par kf3=0.01
par kf4=0.5,kb4=0.1
par kf5=0.5,kb5=0.1

S5 = S5T-(S5S +SPS + AASPS)
SP = SPT-(SPS + AASPS)

v1 = kf1*S5
v2 = kf2*S5*S5S
v3 = kf3*S5S
v4 = kf4*S5S*SP - kb4*SPS
v5 = kf5*SPS*AA - kb5*AASPS

```

```

S5S' = v1+v2-v3-v4
SPS' = v4-v5
AASPS' = v5

#numerical settings @ TOTAL=500,Dt=0.001,METH=cvode,atol=3e-
16,toler=3e-16,newt_tol=.0001
@ XHI=500,YLO=0,YHI=15,BOUND=1000,maxstorage=100000
done

```

6.9.5 Code: Bifurcation plot for minimal model

MATLAB

```

clear
close all
clc

syms S5 S5S SPT SPS AASPS AA S la S5T

kf1=0.0001;
kf2 = 0.01;
kf3=0.01;
kf4=0.5;kb4=0.1;
kf5=0.5;kb5=0.1;
S5T =10; h=1;
SPT=1; AA=1;

for S = -1:0.01:5
    S5T=S;
    S5 = S5T-(S5S + SPS + AASPS);
    SP = SPT-(SPS + AASPS);

    v1 = kf1*S5;
    v2 = kf2*S5*S5S;
    v3 = kf3*S5S;
    v4 = kf4*S5S*SP-kb4*SPS;
    v5 = kf5*SPS*AA-kb5*AASPS;

    fun = [v1+v2-v3-v4...
           v4-v5...
           v5];
    assume([S5S SPS AASPS],'real')
    sol = solve(fun==0,[S5S SPS AASPS]);

```

```

sol.S5S = vpa(sol.S5S);
sol.SPS = vpa(sol.SPS);
sol.AASPS = vpa(sol.AASPS);
len = length(sol.S5S);
jac = jacobian(fun,[S5S SPS AASPS])-la*eye(3,3);
for q = 1:len
    CE = subs(jac,[S5S SPS AASPS S],...
        [sol.S5S(q),sol.SPS(q),sol.AASPS(q),S]);
    eqn = det(CE);
    soleig=vpasolve(eqn==0,la);
    if (soleig(1) < 0 && soleig(2)<0 && soleig(3)<0)
        plot(S,sol.S5S(q),'k','MarkerSize',12)
        hold on
    else
        plot(S,sol.S5S(q),'r','MarkerSize',12)
        hold on
    end
end
end
end
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%% The following is to get a good figure %%%%
xlabel('S5T','fontweight','bold','fontsize',20)
ylabel('x_{ss}','fontweight','bold','fontsize',20);
ax=gca;
ax.XAxis.LineWidth = 3;
ax.XAxis.Color = 'k';
ax.YAxis.LineWidth = 3;
ax.YAxis.Color = 'k';
ax.FontSize = 10;
ax.FontWeight = 'bold';
grid on
iplot=1;
alphabet = char(iplot+64);
title(alphabet,'fontsize',15,'horizontalAlignment','right')

```

Chapter 7

Conclusion

Developing a mathematical understanding of biological processes helps in understanding the behavior of the system. Bistable dynamics play an important role in acting as a checkpoint in most biological networks. In this thesis, we show the existence of a checkpoint in receptor-activated proteolysis (RAP) of *C. albicans*. We have built a network for RAP and also proposed alternate mechanisms for the pathway. RAP is the amino acid-induced activation of the transcription factor Stp1/2 in the SPS sensing pathway of yeast cells. We have divided the network into four major steps.

1. Autolysis of Ssy5.
2. The complex SPS (Ssy1-Ptr3-Ssy5) is stabilized by binding with the amino acid.
3. The binding of the amino acid triggers the release of the catalytic domain of Ssy5.
4. Finally, the released catalytic Ssy5 cleaves the transcription factor Stp1/2 facilitating its translocation to the nucleus, where it can regulate the amino acid permease gene for the uptake of extracellular amino acid.

Positive feedback is a necessary but not a sufficient condition for bistability. However, the positive feedback in this network is implicit. To detect the bistability, we performed structural analysis using CRNT and SR graphs. The CRNT analysis and the properties of the SR graph indicate that the network could exhibit bistability

for the choice of kinetic parameters. Even though the network exhibits bistability, the estimated kinetic parameters need not give rise to bistability. In that case, the parameters need to be recalibrated, or a new mechanism should be proposed.

We have proposed four networks for RAP and also discussed two subnetworks, autolysis of Ssy5 and stabilization of SPS. For the RAP network, we estimated the parameters with the available experimental time series data, using COPASI. Using the estimated parameters, the network still showed bistability. Also, the subnetwork, when studied independently, exhibited transcritical bifurcation. Based on the results, all the mechanism seems plausible. For clarity on the right mechanism, the networks must be explored with more experimental data. As part of future work, the stochastic analysis will be carried out for the receptor-activated proteolysis by converting the ordinary differential equations into Gillespie's algorithm or stochastic differential equations (SDEs).

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