Towards a mechanistic explanation of insulin resistance, which incorporates mTOR, autophagy, and mitochondrial dysfunction

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Type 2 diabetes is a global disease which affects an increasing number of people every year. At the heart of the disease lies insulin resistance in the target tissues, primarily fat and muscle. The insulin resistance is caused by the failure of a complex signalling network, and several mechanistic hypotheses for this failure have been proposed. Herein, we evaluate a hypothesis that revolves around the protein mammalian target of rapamycin (mTOR) and its feedback signals to insulin receptor substrate-1 (IRS1). In particular, we have re-examined this hypothesis and relevant biological data using a mathematical modelling approach.

During the course of modelling we gained several important insights. For instance, the model was unable to reproduce the relation between the EC\(_{50}\)-values in the dose-response curves for IRS1 and its serine residue 312 (Ser-312). This implies that the presented hypothesis, where the phosphorylation of Ser-312 lies downstream of the tyrosine phosphorylation of IRS1, is inconsistent with the provided data, and that the hypothesis or the data might be incorrect. Similarly, we also realized that in order to fully account for the information in the dose-response data, time curves needed to be incorporated into the model.

A preliminary model is presented, which explains most of the data-sets, but still is unable to describe all the details in the data. The originally proposed hypothesis as an explanation to the given data has been revised, and our analysis serves to exemplify that an evaluation of a mechanistic hypothesis by mere biochemical reasoning often misses out on important details, and/or leads to incorrect conclusions. A model-based approach, on the other hand, can efficiently pin-point such weaknesses, and if combined with a comprehensive understanding of biological variation and generation of experimental data, mathematical modelling can prove to be a method of great potential in the search for mechanistic explanations to the cause of insulin resistance in type 2 diabetics.

Keywords
Type 2 diabetes, insulin signalling, insulin resistance, mathematical modelling, ordinary differential equations, mTOR, autophagy, mitochondria
Abstract

Type 2 diabetes is a global disease which affects an increasing number of people every year. At the heart of the disease lies insulin resistance in the target tissues, primarily fat and muscle. The insulin resistance is caused by the failure of a complex signalling network, and several mechanistic hypotheses for this failure have been proposed. Herein, we evaluate a hypothesis that revolves around the protein mammalian target of rapamycin (mTOR) and its feedback signals to insulin receptor substrate-1 (IRS1). In particular, we have re-examined this hypothesis and relevant biological data using a mathematical modelling approach.

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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>DNP</td>
<td>Dinitrophenol</td>
</tr>
<tr>
<td>FFA</td>
<td>Free Fatty Acids</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose Transporter 4</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin Receptor</td>
</tr>
<tr>
<td>IRS1</td>
<td>Insulin Receptor Substrate 1</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian Target of Rapamycin</td>
</tr>
<tr>
<td>mTORC1</td>
<td>Rapamycin-dependent mTOR-raptor Complex</td>
</tr>
<tr>
<td>mTORC2</td>
<td>Rapamycin-independent mTOR-rictor Complex</td>
</tr>
<tr>
<td>ODE</td>
<td>Ordinary Differential Equation</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-Kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>S6K</td>
<td>S6 Kinase</td>
</tr>
<tr>
<td>SBPD</td>
<td>Systems Biology Project Design</td>
</tr>
<tr>
<td>SBT2</td>
<td>Systems Biology Toolbox 2</td>
</tr>
<tr>
<td>SE</td>
<td>Standard Error</td>
</tr>
<tr>
<td>Ser-307</td>
<td>Serine 307 residue on IRS1</td>
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<tr>
<td>Ser-312</td>
<td>Serine 312 residue on IRS1</td>
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Chapter 1

Introduction

1.1 Type 2 Diabetes

Diabetes mellitus is a global disease afflicting an increasing number of people every year. The World Health Organization (WHO) estimated in 2008 that 180 million people worldwide had diabetes, and this number is projected to be doubled by 2030 [1]. Without treatment, the body of a diabetic is unable to control the plasma glucose levels, which under severe conditions can become lethal.

Diabetes is caused by two main dysfunctions in the body, dividing the disease into two categories: type 1 and type 2. Type 1 diabetes is described as a condition where the body develops autoimmunity towards its own $\beta$-cells, causing a disruption in the production of insulin. Type 2 diabetes, accounting for about 90 percent of people with diabetes [1], is described in [2] as a combination of two features. First, the skeletal muscle tissues have developed an impaired responsiveness to insulin, causing the $\beta$-cells to secrete increasingly higher levels of insulin in the circulation in order to keep the blood glucose levels low. Secondly, as the muscle tissues grow more and more resistant to insulin, the $\beta$-cells are eventually unable to keep the production of insulin sufficiently high. This combination of insulin resistance in muscle, liver and fat tissue together with the insufficient production of insulin in the $\beta$-cells are the primary characteristics of type 2 diabetes.

Through a tight control of blood glucose with different pharmacological agents, for example the insulin sensitizing drug metformin, the risk of complications of diabetes can be reduced [3]. Still, diabetes is the cause of many secondary diseases, and a high plasma glucose can over time damage blood vessels, kidneys, heart and nerves, leading to an increased risk for blindness, kidney failure and stroke [1].

Type 2 diabetes has been shown to be strongly correlated to obesity, as reviewed in [2]. The number of people suffering from obesity in the world is expected to increase from 400 million in 2005 to more than 700 million in 2015 [4]. This projected rise is caused mainly by an increase in food intake, combined with a decrease in physical activity [4]. There is also a genetic aspect of the epidemics of obesity and diabetes, and it has been hypothesised that certain genes during starvation have been evolutionary favoured to preserve glucose for the brain by
developing insulin resistance in peripheral tissues, or promoting an increase in size of the adipose tissue [5]. This leads to different populations being more or less susceptible to obesity and insulin resistance.

1.2 Insulin Resistance and the Adipose Tissue

There are two leading mechanistic hypotheses to the cause of insulin resistance in the human tissues: the lipid overload hypothesis and the inflammation hypothesis [6].

The lipid overload hypothesis is derived from the observation that circulating free fatty acids (FFA) are elevated in many insulin-resistant states. The explanation to this, as reviewed in [2], might be that high levels of FFA lead to an accumulation of triglycerides and fatty acid-derived metabolites in muscle and liver, causing an inhibition of early insulin signalling steps. A dysfunctional adipose tissue is unable to fully sequester lipids into adipose tissue stores, which leads to circulating FFA and the accumulation of triglycerides in other tissues in the body.

The other leading hypothesis (the inflammation hypothesis) describes a process in the adipose tissue where excess caloric intake leads to adipocyte enlargement. This causes an increased release of adipokines (cytokines secreted from the adipose tissue), also reviewed in [2]. The increased secretion of adipokines recruits macrophages and creates a chronic inflammatory state which is believed to induce insulin resistance in the muscle tissue. Interestingly, the state of inflammation in the adipose tissue could itself be the cause of the lipid overload scenario described above, connecting the two hypotheses [2].

1.3 The Insulin Signalling Pathway

The insulin signalling pathway is highly complex. Insulin regulates numerous processes in the human metabolism, by for example stimulating glucose uptake, glycogen synthesis and protein synthesis, and by inhibiting lipolysis and glycogenolysis [7] (Figure 1.1).

After a carbohydrate-containing meal, the \( \beta \)-cells in the pancreas release insulin into the blood stream. When insulin reaches the adipose tissues it binds to the insulin receptor (IR), which auto-phosphorylates on tyrosine residues and creates binding sites for several substrates, as reviewed in [8]. One of the substrates that binds to and is phosphorylated by IR is the insulin receptor substrate 1 (IRS1). When phosphorylated, IRS1 triggers a signalling cascade through phosphatidylinositol-3-kinase (PI3K) and protein kinase B (PKB), affecting several metabolic pathways in the cell (Figure 1.1).

A protein downstream of IR that is of particular significance for this thesis is the mammalian target of rapamycin, mTOR, and this protein will be dealt with further in the following section.
1.4 mTOR and Autophagy

The mammalian target of rapamycin, mTOR, is a conserved Ser/Thr kinase that is part of two large multiprotein complexes; mTORC1, formed from mTOR and the protein rictor, and mTORC2, formed from mTOR and the protein raptor [9]. mTORC2 is non-responsive to the inhibition with rapamycin and will not be addressed further in this thesis. mTORC1 on the other hand is selectively inhibited by rapamycin, which is an important property when investigating the role of mTORC1 in the insulin signalling pathway [9]. mTORC1 is regulated by several factors, including nutrients, growth factors, and the energy status of the cell. In response to these factors, mTOR affects protein synthesis, ribosomal biogenesis, and a process in the cell called autophagy [9] (Figure 1.1).

Autophagy in the cell is the formation of a cytosolic double-membrane vesicle, enclosing portions of the cytoplasm in an autophagosome that is eventually degraded in the lysosome. It is the primary intracellular mechanism for degrading and re-cycling damaged and long-lived proteins and organelles, and it is also used by the cell to survive under starvation conditions [10]. Autophagy is under strong regulation by mTORC1, and when nutrient and energy levels in the cell are high, autophagy is inhibited by an active mTORC1. Conversely, when mTORC1 is inhibited with rapamycin, autophagy is activated and cellular degradation increases [11].

It has been determined that the level of autophagy is increased in subjects with diabetes, and that this increase could be due to an attenuated activity of
mTORC1 [7]. It has also been shown that mTORC1 could be responsible for the phosphorylation of the serine 307 residue on IRS1, that induces a positive feedback on the insulin-stimulated tyrosine phosphorylation of IRS1 [12](Figure 1.1). Without the positive feedback from Ser-307, the cell would respond less to the same amount of insulin. In a hypothesis formulated by Anita Öst in [7], the attenuated activity of mTORC1 seen in diabetics could via a lack of the positive feedback signal from Ser-307 be one of the causes to insulin resistance. It is this connection between mTOR, autophagy and diabetes type 2 that is the basis of this thesis.

1.5 Systems Biology

Systems biology is an area of science that is gaining a wide interest in today’s research. Systems biology has been described by [13] to be a coordinated study of biological systems, where investigation of the components of cellular networks and their interactions is performed by integrating experimental and computational methods. Previously, results from biological experimentation were considered sufficient for drawing conclusions about the cell signalling pathways. However, as more proteins and interaction are discovered, the cellular signalling pathways are growing increasingly complex. Therefore, there is a need for a more systematic way of interpreting the data, making systems biology a useful tool.

Another aspect of modelling signalling pathways is that it enables for a more system-level description of the body. Looking at for example insulin signalling, it is important to realise that insulin affects several tissues in the body, giving rise to a number of different signalling cascades. Investigating only skeletal muscle cells merely gives a partial view of the cause to insulin resistance, instead all tissues should be taken into account before conclusions can be drawn.

Since systems biology is a fairly new area of research, not many standards of procedures have been established. One frequent way of modelling biological systems is by ordinary differential equations (ODE:s), but there are several other methods available, such as Boolean networks and Bayesian networks [13]. The model covering the insulin signalling pathway in this thesis will be constructed using ODE:s, as is described in more detail in Section 2.1.

1.6 Aim of the Thesis

During the completion of this thesis, a mathematical model will be used to investigate if the biological relationships described by Anita Öst in [7] are mathematically coherent. The model will cover the main features of the pathways in the cell related to insulin signalling and autophagy, and it will be created using the mathematical software Matlab and Systems Biology Toolbox 2. Possibly, the model could be used to form new hypotheses and predictions regarding the mechanisms of insulin signalling and type 2 diabetes in relation to mTOR and autophagy. In detail, the thesis will include the following parts:
• Creating a mechanistic hypothesis describing insulin signalling and autophagy in adipocytes.

• Translating the mechanistic hypothesis into ordinary differential equations (ODEs).

• Creating a model in Matlab using Systems Biology Toolbox 2.

• Optimizing the parameters of the model to give an acceptable fit to biological data from healthy subjects.

1.7 Delimitations

Some aspects of the thesis have been simplified:

• The amount of biological data used was limited to what was available at the start of the thesis, focusing on the data produced for [7]. There was no further biological experimentation performed during the time of the thesis.

• Several proteins and pathways were disregarded when making the model. Generally, a protein was only included in the model if there was experimental data available for its concentration or activity.

• The time to complete the thesis was limited to 20 weeks.
Chapter 2

Methods

This chapter will cover the basic mathematical theory and methods, which have been used to complete this thesis. It will also contain a brief description over the computer software that were used to create and optimize the mathematical model, the primary settings available to the chosen optimization algorithm, and a discussion about the treatment of biological data.

2.1 Translating an Hypothesis into ODE:s

When an hypothesis of a particular interaction or event has been formulated, it can be tested by using a mathematical model. In order to correctly translate the theoretical biological relationships of the hypothesis into functioning mathematical equations, it is important to be aware of the available tools and methods.

2.1.1 Law of Mass Action

The mechanistic hypothesis created during this thesis is translated into a mathematical equations based on the law of mass action. This law states that the rate of an elementary reaction is proportional to the product of the concentrations of the participating substrates [13]. In Figure 2.1, protein A affects protein B so as to react into protein C. As the reaction proceeds, the amount of B will decrease, and according to the law of mass action, this will result in a decreased reaction rate from B to C.

2.1.2 Formulating Rate Equations

According to the law of mass action, the rate of the forward reaction depicted in Figure 2.1, would be proportional to the concentrations of A and B. The backward rate of the reaction is on the other hand only dependent on the concentration of C.

The rate of a reaction is dependent on several reaction specific properties, such as the number of reacting species and the complexity of the reaction. These
Figure 2.1. An example of a simple reaction involving only three species. As depicted, A stimulates the reaction from B into C. The reaction is reversible, and the forward and the reverse rates depends on the rate constants $k_f$ and $k_b$.

Properties are described by adding a constant to the rate expression. The resulting equations describing the forward and backward rates of the reactions in Figure 2.1 would then be

$$v_f = k_f \cdot [A] \cdot [B] \quad (2.1)$$
$$v_b = k_b \cdot [C] \quad (2.2)$$

2.1.3 Formulating ODE:s

Ordinary differential equations (ODE:s) are used to describe the changes of state variables over time. An ODE can be defined as

$$\frac{dx_i}{dt} = \dot{x}_i = f_i(x_1, \ldots, x_n, p_1, \ldots, p_n) \quad (2.3)$$

where $x$ represents the variables, such as concentration or mass, $p$ represents the parameters, such as kinetic constants, and $t$ represents time.

If applying this nomenclature to the reactions in Figure 2.1, the variables $x_i$ would be $A$, $B$ and $C$, the parameters $p_1 \cdots p_n$ would be $k_f$ and $k_b$, and $\dot{x}$ would refer to the change in concentration over time of proteins A, B and C. The simple system illustrated in Figure 2.1 could then be written as

$$[\dot{A}] = 0 \quad (2.4)$$
$$[\dot{B}] = -v_f + v_b \quad (2.5)$$
$$[\dot{C}] = v_f - v_b \quad (2.6)$$

where $v_f$ and $v_b$ are calculated using Equations 2.1 and 2.2.

To be able to simulate a model built from ODE:s, it is sometimes necessary to state the initial conditions for the state variables in the model. If the simulation starts at $t = 0$, and it is assumed that the concentration of protein C for example
is a tenth that of protein A and B, the initial conditions for the example in Figure 2.1 should be written as
\[\begin{align*}
[A](0) &= 100 \\
[B](0) &= 100 \\
[C](0) &= 10
\end{align*}\] (2.7) (2.8) (2.9)

### 2.1.4 Modifying Rate Equations

It is not likely that all reactions taking place between proteins and molecules in a human cell follows the dynamics described by the law of mass action, but it is often a sufficient approximation to begin with when trying to describe a system with a simplistic model. However, sometimes completely proportional relations prove to be inadequate, and modifications to the reactions are required.

A possible modification to an ODE is to assume that its proportionality is only partial and that the reaction rate reaches saturation at a certain point. A common way of modelling saturations in biological reactions is by Michaelis-Menten kinetics. In the Michaelis-Menten equation, the reaction rate \(v\) is dependent on the substrate concentration \([S]\) according to expression
\[v = \frac{V_{\text{max}} \cdot [S]}{K_M + [S]}\] (2.10)
where \(V_{\text{max}}\) is the maximal rate that can be attained when the reaction has reached complete saturation, and the Michaelis constant \(K_M\) is equal to the substrate concentration that yields the half-maximal reaction rate (Figure 2.2). For additional information about the Michaelis-Menten equation, see [13].

For some types of activations, a more switch-like behaviour is required. An example of a situation where the activation has a switch-like dynamics is when a protein needs to be phosphorylated twice to gain full activity. This type of behaviour can be modelled mathematically with the use of a Hill equation. The Hill equation has its origin in enzymatic equations, where enzymes bind to proteins and reactions occur [13]. The expression can however be written as a modification of the Michaelis-Menten equation, which is how it will be implemented in this thesis (Equation (2.11)). The effect of the modification compared to the Michaelis-Menten equation, is that the protein activation profile gets more rigid. The protein requires a higher stimulation to start responding, but will after that reach its maximum level of activation faster, i.e. it becomes saturated more rapidly.
\[v = \frac{V_{\text{max}} \cdot [S]^n}{K_M^n + [S]^n}\] (2.11)

### 2.2 Matlab and Systems Biology Toolbox 2

The main computer software used during this thesis was Matlab, a technical computing language developed by The MathWorks. Matlab has several applications, for example signal and imaging processing, communications, control systems, and
computeable biology [14]. In this thesis Matlab was used when constructing the optimization script used to optimize the parameters in the designed model.

For model representation and model simulation, the Systems Biology Toolbox 2 for Matlab was used (SBT2). This toolbox was specifically developed to model biological systems and can be implemented with either differential equations or biochemical reaction equations [15]. To improve simulation speed, the add-on package of Systems Biology Project Design (SBPD) for SBT2 was used as well. With SBPD, simulations can be performed by converting the model to C-code, improving simulation performance by a factor between 30 to 150 [16].

2.3 Optimizing a Mechanistic Model

When a mechanistic hypothesis has been translated into a mathematical model, it contains numerous of unknown parameters. To make the model respond to stimuli in a similar way to the real system, the unknown parameters must be assigned reasonable values. A common way of doing so is by searching the literature for approximate values of the constants and add these to the model. The model can then be simulated and compared to experimental data. The downside to this method is the uncertainty of the constants, as many researchers use different model systems and work under different experimental conditions. For example, a rate constant for a certain reaction measured in a particular cell type might differ a lot from the same measurement in another cell type.
2.3 Optimizing a Mechanistic Model

Figure 2.3. An overview of the working process when developing core predictions. In an iterative process between developing possible mechanistic explanations and generating experimental data, mechanistic hypotheses are created. In phase I, mathematical models are constructed and tested against experimental data to see if acceptable parameter-sets can be found. During phase II, the established acceptable models are used to search for all parameter-sets that can describe the biological data. Potentially, the parameter-sets have certain properties in common, forming core predictions that can be experimentally validated. The figure has been adopted from [18].

Another way of solving the problem with the unknown parameters is by fitting the model simulations against experimental data using an optimization algorithm. Usually the aim of the optimization is to minimize the error between the data points and the simulated curves by altering the values of the parameters until the best fit has been achieved. However, since biological systems often are highly over-parametrized, there are usually numerous sets of parameters that all give an acceptable fit to data. With this problem in mind, a good alternative is to reverse the methodology and instead try to find all acceptable parameter-sets. Assuming the possibility that all acceptable parameter-sets can be found, this technique enables the opportunity to find core predictions that can lead to a rejection of the hypotheses instead. Core predictions for a model is when certain characteristics of the behaviour of the model is common to all acceptable parameter-sets. If for example all the acceptable parameter-sets give model simulations which require that a protein is phosphorylated to a certain level, and experiments can contradict that requirement, then the model is incorrect and can be rejected. This could be perceived as a failure, but the rejection of an hypothesis is in fact one of the strongest conclusions that can be drawn in science. The method is summarized in Figure 2.3, and has been described more thoroughly in [17].

In this thesis, we will focus on finding a first parameter-set that gives an acceptable fit to data. When and if that has been successful, the next step will be
to search for all parameter-sets that can adequately describe the data, but this is not included within the framework of this report.

### 2.3.1 The Cost Function

To be able to distinguish between an acceptable model and an inadequate model, a measure stating the quality of models is required. In systems biology, a frequent measure for determining a model’s ability to describe biological data is the cost. Given a fixed set of parameters, the cost of a model is an assessment of how far a model’s simulations deviates from experimental data. Large deviations from data give large costs, while smaller deviations correspond to smaller costs. The magnitude of the cost for a particular model is determined by its cost function. How the cost function is designed may vary, but the basic idea is to calculate the residuals between the simulated curves and every data point and sum them up to a total measurement of the model deviation. A residual is simply the difference between the experimentally determined value and the predicted simulated value. In this thesis the cost function was specified by Equation (2.12).

\[
V(p) = \sqrt{\frac{\sum_{t=1}^{n} (y(t) - \hat{y}(t,p))^2}{SE(t)^2}}
\]  

Equation (2.12)

\( V(p) \) is the cost for the model parameters \( p \), \( y(t) \) represents the data point at time \( t \), and \( \hat{y}(t,p) \) represents the simulated value at time \( t \) using the parameters \( p \). The residuals are normalised by the standard error \( SE \) for each data point. In practice, normalizing the cost function with the magnitude of the error means that the optimization algorithm will put more emphasis on fitting the model to data points with small standard errors rather than data points with large standard errors. For a discussion regarding the standard error of a data-set, see Section 2.4.

### 2.3.2 The Optimization Algorithm

The optimization algorithm used during this thesis was \textit{simannealingSBAOClustering}, a modified version of the algorithm \textit{simannealingSB} which is available in the Systems Biology Toolbox for Matlab. The function \textit{simannealingSBAOClustering} searches for the function minimum by simulated annealing, a method particularly suited for optimization of very large and complex systems with many independent variables [19]. The idea of simulated annealing originated from annealing in metallurgy, where a material is heated followed by controlled cooling. This allows for the atoms in the material to wander through states of higher energy in order to find configurations with lower internal energy. In mathematics, the analogue to the energy of an atom is applied to the minimization of the error of a mathematical model during an optimization. The magnitude of the error is often estimated by the cost function, described in Section 2.3.1. The optimization algorithm searches through the parameter space for a lower cost, i.e. a better parameter set, by temporarily allowing for a higher cost than the currently known optimum. The maximum allowed increase of the cost in each iteration is determined by the
current temperature, which in simulated annealing is a control parameter initially set by the user. In the first stages of the optimization the temperature is high, allowing for large increases in the cost, and then gradually the temperature is lowered stepwise, forcing the optimization algorithm to close in on a local optimal solution.

Algorithm Settings

There are a number of settings available for the algorithm simannealingSBAO-Clustering, some of which are explained below.

- **Starting temperature.** The starting temperature is a measurement of the range of the search. A high starting temperature makes the initial search more global, allowing for the algorithm to step across parameter regions with high costs in order to localise parameter regions with lower costs.

- **Number of iterations.** The maximum number of iterations allowed at every temperature level will determine how thorough the search will be. A model with many variables is likely to require a high number of iterations for every new temperature.

- **Reduction factor.** The reduction factor for the temperature after running through all iterations for the current temperature.

- **Parameter boundaries.** The parameter boundaries limit the parameter space to be searched by the optimization algorithm.

- **Max restart points.** The optimization algorithm is able to restart at the same temperature level the amount of times defined by this option. By increasing the number of iterations points, sub optima can be localized.

Further reading about the algorithm can be found in [20].

2.3.3 Improving the Optimization

With the algorithm simannealingSBAOClustering, the user is required to specify a starting guess of the parameters to be optimized. For a small model it might be trivial to estimate probable values of the parameters, but for large, complicated models it is more difficult. A way of facilitating the selection of a good starting guess is to use the graphical user interface (GUI) available for SBT2, called SBedit. SBedit allows for direct adjustment of each parameter in the model, and with built-in plotting functions the model states and rates can be simulated in a graphical window. Testing different parameter values and viewing the result helps in understanding the dynamics of the system, and reasonable parameter values for the starting guess can more easily be found.

Sometimes the optimization algorithm has difficulties leaving an area of acceptable costs to localize better costs. In these situations, a solution could be to add a penalty to a certain event. For example, if a simulation curve for is unable
to reach a maximum defined by a data point, a conditional statement could be utilized, adding a penalty to the cost if the statement is not fulfilled. An example of a typical penalty is displayed in Equation (2.13), showing a Matlab if construct with a subsequent increase of the cost if the Boolean statement is true.

\[
\text{if } A < 100 \\
\text{cost} = \text{cost} + 1000 \\
\text{end}
\]

(2.13)

The idea of adding penalties is to help the optimization algorithm find another search direction by increasing the cost in the present optimum. However, if several penalties are added to the optimization, the optimization algorithm can have difficulties moving past parameter regions where all penalties are active, since the cost increase there will be too high. This can make the range of the search very limited and restrain the algorithm instead of aiding it. Therefore, depending on the number and magnitude of the penalties added, the starting temperature of the optimization algorithm should be increased. It is also wise to remove the penalties as soon as the algorithm has found acceptable optima, or the penalties might create unwanted restrictions in future optimizations.

### 2.4 Standard Deviation or Standard Error?

The standard deviation is the square root of the variance, which is a measurement of how much each observation deviates from the arithmetic mean of the population [21]. In a sample of \(n\) observations \(x_1, x_2, \ldots, x_n\), the standard deviation \(s\) of the sample is

\[
s = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n - 1}}
\]

(2.14)

The standard error, \(SE\), on the other hand, is a measurement of how much a sample mean deviates from the population mean [21]. Usually there is only one sample available to estimate the mean of the population, and the true standard deviation of a population is rarely known. Therefore, the SE is estimated using the equation

\[
SE = \frac{s}{\sqrt{n}}
\]

(2.15)

where \(s\) is calculated according to Equation (2.14) above, and \(n\) is the number of observations in the sample [21].

So when should the standard deviation be used, and when is the standard error a better measurement? As explained in [21], the standard deviation describes the variation in the data values and should be used when the variability in the data is to be displayed. For example, if experiments are performed on biological systems such as human cells, the standard deviation would display the biological variability between different cells. In contrast, the standard error describes the standard deviation of the sample mean, and should be used if the mean of a sample is of main interest.
In this thesis, the developed mathematical model is only able to calculate and simulate mean values of the existing state variables. If looking at for example the phosphorylation of IR after insulin stimulation, the model is constructed to estimate the mean percentage of phosphorylated IR from all experimental repeats collectively, not different levels of phosphorylated IR in separate repeats. Therefore, due to the nature of the model, the best measurement to use when comparing model simulations to biological data, is the standard error.

2.5 Biological Data

There were no biological experiments performed during the completion of this thesis. All biological data used was provided by the Strålfors research team from the Department of Clinical and Experimental Medicine at Linköping University.

2.5.1 Treatment of Biological Data

In order to make correct comparisons between the mathematical models and the data points, it is important as a systems biologist to understand how biological data is gathered and treated. Some of the common standard procedures of data treatment are explained below.

- **Subtracting the baseline.** If it is of particular interest to study the changes in a system after adding a stimuli or inhibitor, the baseline of what is measured can be subtracted from each data point. The baseline of a system variable is its value at steady-state before it's been subject to any perturbations. Also, if the baseline is very large compared to the stimuli-dependent change, it can be practical to subtract the baseline to increase the readability of the data.

- **Normalization of data.** Many experimental procedures used in cell biology have large experimental variations. This makes it difficult to make comparisons between different experiments, and it is also hard to do replicates of the same experimental procedure. The impact of experimental variation on the data is diminished if the data-sets are normalized, and consequently data from several repeats can be added together. The normalization can be achieved by modifying the data so that the maximum value or the control of the data-set is 100.

- **Logarithmic transformation.** If an exponential relationship exists between what is measured and what is altered, taking the logarithm of the data points can increase the readability of the data-set, particularly if it is graphically represented.

In Figure 2.6 on Page 29, all above mentioned modifications have been used to create a dose-response curve. The baseline has been subtracted so that the lowest value is zero, the maximum value has been normalized to 100, and the x-axis has been transformed logarithmically. For information about how a dose-response curve is generated, see the following section.
Figure 2.4. A fold-over-basal for the insulin-dependent S6K phosphorylation in healthy subjects and in subjects with diabetes. The controls have been normalized to 100 and the phosphorylations after insulin stimulation are measured as a percentages of the controls.

2.5.2 Types of Biological Data

Biological data can be gathered and presented in numerous of ways. The data used for this thesis mainly belongs to three different categories, and they will be described briefly below.

- **Fold-over-basal.** For this data type you have a control measurement and determine the change in the system after a stimulus as a percentage of the control. The control is often normalized to 1 or 100. See Figure 2.4 for an example of a fold-over-basal data-set.

- **Time series.** Time series are used to display changes over time for particular substances or reactions. A time series could be generated by measuring for example the phosphorylation of a protein at a number of subsequent time points after a stimulation has been initiated. Usually the data points are normalised to have a maximum at 100, and sometimes the baseline is subtracted as well. In Figure 2.5 an example of a typical time series can be found.

- **Dose-response curves.** A dose-response curve is generated by taking the steady-state value of several time curves generated at different concentrations of a stimulant or inhibitor, and plotting the values in a graph with response versus concentration. Common modifications applied to dose-response data could be to subtract the baseline, normalize the maximum value to 100, and transform the concentration axis logarithmically. In Figure 2.6, all of the mentioned modifications have been applied.
2.5 Biological Data

Figure 2.5. A graphical representation of the insulin-dependent phosphorylation of IR. The baseline has been subtracted and the data-set is normalized to have its maximum value at 100.

Figure 2.6. A dose-response curve for the insulin-dependent phosphorylation of IR. The baseline has been subtracted, the data-set is normalized to have its maximum value at 100, and the x-axis has been logarithmically transformed.
Chapter 3

Results

3.1 The Initial Model

Creating a mechanistic hypothesis of a cell signalling cascade is an iterative process, requiring a lot of thought and consideration. Pathways are tangled, simplifications are necessary, and there are several unknown aspects of the system, such as kinetic constants and feedback signals. The main purpose of the model created in this thesis was to give a simplistic description of the insulin signalling pathway, and to capture in large the behaviour observed in the biological data. Therefore, the number of proteins included in the mechanistic hypothesis was kept to a minimum, disregarding all features which could not be verified by experimental data.

3.1.1 Constructing an Interaction Graph

An interaction graph shows a mapping of all the proteins and interactions that a particular hypothesis concerns. It serves as an aid for understanding the mechanisms in the system and is very useful in later stages, when the model hypothesis is to be translated into mathematical equations. The interaction graph constructed for the initial hypothesis can be found in Figure 3.1.

As described in Section 1.3, IR is activated as insulin is added to the system. This is depicted in the mechanistic model as two states, one active (IRp) and one inactive (IR), connected by forward and backward rate reaction arrows (Figure 3.1). As IR has been activated, it phosphorylates the tyrosine sites on IRS1, and IRS1 is in turn activated. Downstream of IRS1, the complex mTORC1 is activated via PI3K and PKB (Figure 1.1). However, since the activation is sequential and no data is available for PI3K or PKB, both proteins were be removed from the mechanistic hypothesis without largely affecting the dynamics of the IRS1-dependent mTOR-activation.

Apart from the tyrosine phosphorylation sites present on IRS1, there are two additional phosphorylation sites on the protein that are of importance in this thesis. They are located at the residues serine 307 and serine 312 in IRS1. Since they are activated separately by different proteins, they are depicted in the model.
as individual states, each having its separate effect on the rate of phosphorylation of IRS1 (Figure 3.1). From here on, phosphorylated tyrosine sites will be collectively referred to as phosphorylated IRS1, whereas the serine sites will be referred to by their number, Ser-307 and Ser-312.

To measure the activity of mTORC1 directly is difficult, so instead the phosphorylation of S6 kinase (S6K) was used as an experimental marker of mTORC1 activity. In the same manner, the amount of the protein LC3 and lipofuscin-particles were measured in order to determine the level of autophagy in the cell. LC3 is a protein that is incorporated in the surface of the autophagosomes, and when autophagy increases, the amount of bound LC3 increase [7]. Lipofuscin is in other cell types believed to be a lysosomal remnant that accumulates over time [22], and in adipocytes the amount of lipofuscin is decreased when autophagy is increased [7]. Therefore, to be able to fit the level of activity of mTORC1 and autophagy to data, the states S6K, LC3 and lipofuscin were required in the model (Figure 3.1). Autophagy, LC3 and lipofuscin can in theory increase indefinitely, and this is displayed in the figure by large, black dots to the left of the states.

It has been shown that the amount of functional mitochondrial tubules with cristae is substantially reduced in diabetic cells [7]. Knowing that an important function of autophagy is to degrade damaged organelles in the cell (Section 1.4), a higher level of dysfunctional mitochondria could be the cause of the increased autophagy observed in cells suffering from insulin resistance. Also, if the mitochondria are unable to sustain necessary levels of ATP in the cell, it could be possible that mTOR triggers autophagy as a response to the energy deficiency. The connection between dysfunctional mitochondria and autophagy is one of the theories that has been investigated by Anita Öst in [7], and consequently functional and dysfunctional mitochondria were added to the mechanistic hypothesis. The formation and depletion of functional and dysfunctional mitochondria respectively are displayed using black dots, and the break-down from functional to dysfunctional mitochondria is depicted using two separate states (Figure 3.1). Also, the figure shows how the amount of dysfunctional mitochondria lowers the activity of mTORC1 and thereby increases autophagy, and how the level of autophagy in turn drives the degradation of the dysfunctional mitochondria.

When the experimental data used in the thesis was produced, three substances aside from insulin were added to the system; rapamycin, chloroquine and 2,4-dinitrophenol (DNP). Rapamycin, as was mentioned in Section 1.4, is a very specific inhibitor of mTOR. Chloroquine inhibits the lysosomal breakdown of the autophagosomes, and DNP is an inhibitor of mitochondrial function that acts by uncoupling the proton gradient in the electron transport chain [7]. The input signals of rapamycin and chloroquine are presented in Figure 3.1 as inhibiting, while DNP on the other hand is presented with a normal activating arrow. This is because even though DNP is an inhibitor, the result of adding the substance to the system is an increase in the rate of formation of dysfunctional mitochondria.

Lastly, two feedback-loops to the phosphorylation sites of IRS1 were introduced to the model, one from mTORC1 to Ser-307, and one from S6K to Ser-312 (Figure 3.1). The feedback through the IRS1 Ser-307 residue has been described in [12] as a rapid insulin stimulated phosphorylation that positively regulates IRS1 tyrosine
phosphorylation, and it was determined in [23] that the insulin-stimulated phosphorylation of Ser-307 was sensitive to rapamycin. This indicates that Ser-307 is dependent on the activity of mTORC1, and so this connection was added to the model.

The Ser-312 phosphorylation on the other hand, appears to negatively regulate IRS1 tyrosine phosphorylation, also shown in [12]. The kinase responsible for the Ser-312 phosphorylation in primary human adipocytes has not yet been determined, but in HEK293T cells (cell line derived from human embryonic kidney cells), mTOR and S6K appears to be involved [24]. Therefore, a connection between S6K and Ser-312 was added to the model.

### 3.1.2 Creating a Mathematical Model

The mechanistic hypothesis was translated into a mathematical model using the theory and methods described in Section 2.1. Starting from the top of the model displayed in Figure 3.1, the two first proteins to be translated into state variables and rate reactions would be IR and IRS1. These proteins will serve as a general example for the translation of the whole model.

**IR and IRS1**

The stimulating input of the whole system is the addition of insulin to the medium outside the cell. The presence of insulin was modelled by the use of a parameter called \( \text{ins} \), which was given the value \( \text{ins} = 0 \) when insulin was absent and, depending on the concentration, different values when insulin was present. Following the addition of insulin, IR and IRS1 are activated. The concentrations of the active and inactive proteins IR and IRS1 were defined as state variables by the equations

\[
\begin{align*}
\dot{[IR]} &= -v_{1f} + v_{1b} \quad (3.1) \\
\dot{[IR]}_p &= -v_{1b} + v_{1f} \quad (3.2) \\
\dot{[IRS1]} &= -v_{2f} + v_{2b} \quad (3.3) \\
\dot{[IRS1]}_p &= -v_{2b} + v_{2f} \quad (3.4)
\end{align*}
\]

The initial conditions for the state variables needed to be stated, and since there was no biological data available over the abundance of the different protein states in the cells at the start of the experiments, all the dephosphorylated/inactive states were defined as having the starting value 100, and all phosphorylated/active states was given the value 10, as shown in Equations (3.5)-(3.8) below. The value "100" should not be regarded as "100 insulin receptors", but rather as a relative number describing the proportion of insulin receptors that are inactive in relation to the total amount of insulin receptors.

\[
\begin{align*}
[IR]\,(0) &= 100 \quad (3.5) \\
[IR]_p\,(0) &= 10 \quad (3.6)
\end{align*}
\]
Figure 3.1. A graphical representation of the initial mechanistic hypothesis. The parameters displayed near the reaction arrows are the constants involved in respective rate reaction. mit_functional and mit_dysfunctional stands for functional and dysfunctional mitochondria. For further abbreviations, see Page 9. In short, as insulin binds to IR, IR is phosphorylated and activates IRS1. IRS1 then activates mTORC1, who in turn is responsible for the two feedbacks to IRS1 via the phosphorylation sites Ser-307 and Ser-312. mTORC1 also brakes the process of autophagy in the cell, decreasing the amount of bound LC3 and increasing the amount of lipofuscin. With a high level of autophagy, dysfunctional mitochondria are broken down. For further information about the process, see Section 3.1.1.
3.1 The Initial Model

\[ [IRS1](0) = 100 \]  
\[ [IRS1_p](0) = 10 \]  

The rate reactions were primarily created using the theory of mass action. However, according to provided experimental data, IR has a basal level of phosphorylation even without insulin stimulation. To account for this, the forward rate reaction was split into two parts, one which was dependent on insulin and the rate constant \( k_{1f} \), and one which was constant and independent of insulin (Equation (3.9)). The reverse reaction was dependent only on the rate constant \( k_{1b} \), and was formulated according to Equation (3.10).

\[ v_{1f} = (k_{1f} \cdot ins + IR_{basal}) \cdot IR \]  
\[ v_{1b} = k_{1b} \cdot IR_p \]  

Formulating the rate equations for IRS1 was slightly more complicated, due to the feedback signals from mTOR and S6K via the phosphorylation sites Ser-307 and Ser-312 on IRS1 (Figure 3.1). The feedbacks themselves could not be activating in the same way as insulin was for IR, since the phosphorylation and dephosphorylation of IRS1 requires IRp and IRS1_p. This means that without phosphorylated IR or IRS1, the feedback signals should have no effect. Therefore, the feedback signals were implemented as amplifications of the rates, by a modification of the rate constants with a Michaelis-Menten equation. Looking at Equation (3.11), the constant \( k_{2f} \) in the forward rate reaction was modified by a Michaelis-Menten expression that depends on the level of phosphorylation of Ser-307, ranging in size between zero and one. Thereby, the requirement that the feedback should have no effect if IRp is zero is fulfilled.

\[ v_{2f} = \left( k_{2f} + \frac{V_{max1} \cdot Ser-307_p}{K_{M-307} + Ser-307_p} \right) \cdot IRS1 \cdot IR_p \]  
\[ v_{2b} = \left( k_{2b} + \frac{V_{max2} \cdot Ser-312_p}{K_{M-312} + Ser-312_p} \right) \cdot IRS1_p \]  

The model file

To be able to convert the ODE:s into a model that can be used by SBT2, the equations needed to be inserted into an SBT2 model file. It was also necessary to define all parameters and constants used in the equations. In Example 3.1, the model file with the equations used for modelling IR and IRS1 has been inserted. All parameter values assigned in the example were randomly chosen. Note that the model in Example 3.1 would be impossible to simulate, because in order to account for the feedback signals defined in \( v_{2f} \) and \( v_{2b} \), all the proteins involved in the feedbacks would have to be defined as well.

The equations defining the state variables were inserted below the headline "MODEL STATES", the rate equations were placed below "MODEL REACTIONS", and the parameters defined were placed under "MODEL PARAMETERS". Remaining headlines were unnecessary when building this model.
---Example 3.1: A small model in an SBT2 model file---

********** MODEL NAME
modelExample

********** MODEL NOTES
This is an example of a model containing only four states.

********** MODEL STATES
\[ d/dt(IR) = v_{1b} - v_{1f} \]
\[ d/dt(IRp) = v_{1f} - v_{1b} \]
\[ d/dt(IRS1) = v_{2b} - v_{2f} \]
\[ d/dt(IRS1p) = v_{2f} - v_{2b} \]

IR(0)=100
IRp(0)=10
IRS1(0)=100
IRS1p(0)=10

********** MODEL PARAMETERS
\[ \text{ins} = 0 \]
\[ k_{1f} = 10 \]
\[ k_{1b} = 4 \]
\[ k_{2f} = 0.001 \]
\[ k_{2b} = 20 \]
\[ \text{IR}_{\text{basal}} = 2 \]
\[ \text{Km}_{307} = 2 \]
\[ \text{Km}_{312} = 2 \]
\[ \text{Vmax}_1 = 0.1 \]
\[ \text{Vmax}_2 = 20 \]

********** MODEL VARIABLES

********** MODEL REACTIONS
\[ v_{1f} = (k_{1f} \times \text{ins} + \text{IR}_{\text{basal}}) \times \text{IR} \]
\[ v_{1b} = k_{1b} \times \text{IRp} \]
\[ v_{2f} = (k_{2f} + \text{Vmax}_1 \times \text{Ser}307p/(\text{Km}_{307} + \text{Ser}307p)) \times \text{IRS1} \times \text{IRp} \]
\[ v_{2b} = (k_{2b} + \text{Vmax}_2 \times \text{Ser}312p/(\text{Km}_{312} + \text{Ser}312p)) \times \text{IRS1p} \]

********** MODEL FUNCTIONS

********** MODEL EVENTS
Further modelling details

Working through the translation from the mechanistic hypothesis to the complete mathematical description required some decisions and approximations to be made. For example, the model did not account for how autophagy was activated. To compensate for this, a constant increase of autophagy was introduced, see autophagy$_{\text{basal}}$ in Figure 3.1. This means that the autophagy is controlled only by the activity of mTORC1. If mTORC1 for example has a high activity, the rate of decrease of autophagy will be high, and that will consequently lower the level of autophagy. The same reasoning was applied to the production of lipofuscin and the generation of functional mitochondria, using the basal rates of increase $lipo_{\text{basal}}$ and $mit_{\text{basal}}$ (Figure 3.1).

To model the effect of the different inhibitors added to the system, additional parameters were defined. Looking at mTORC1 for example, the forward rate reaction was defined as

$$v_{5f} = k_{5f} \cdot \text{rapamycin} \cdot mTORC1 \cdot IRS1_p$$

with rapamycin being the parameter that describes the effect of the addition of the mTOR-specific inhibitor rapamycin to the system. The parameter boundaries were in the optimization script defined as $[10^{-10} \cdot 1]$, with rapamycin = 1 indicating the absence of rapamycin from the system. The lower limit was set to $10^{-10}$ instead of 0 to reduce the parameter space to be searched during the optimization. The inhibitor chloroquine was modelled in the same way as rapamycin, while DNP instead received the parameter boundaries $[1 \cdot 10^{10}]$. The explanation to this is that DNP causes an increased breakdown of functional mitochondria, which in the model is solved by assigning the parameter DNP a value higher than 1 when the substance is added.

Lastly, the feedback from dysfunctional mitochondria to mTORC1 is modelled similarly to the feedback signals described in Section 3.1.2, with a modification of the rate constant $k_{5b}$ by a Michaelis-Menten equation.

3.1.3 Incorporating Experimental Data

As described in Section 2.5.1, experimental data-sets are often modified in several ways before being analyzed. To be able to optimize a mathematical model against modified experimental data, it is often necessary make adjustments to the model or the model simulations. To begin with, all experiments performed are initiated when the biological system is at steady-state, i.e. the adipocytes have been resting over night in a nutritious-rich solvent. To accomplish the corresponding conditions in the model, a first simulation without any stimuli or inhibitors was run. The simulation had to be long enough for the system to reach steady-state, i.e. all state variables had reached a stable value. The time required for this varied with the model parameters, but was empirically determined to be sufficiently long at about 1000 minutes.
In this thesis three different types of experimental data has been used; fold-over-basals, time series and dose-response curves, each described in more detail in Section 2.5.2. Each data type required different modifications to the model or the model simulations, and the most important ones will be dealt with below. Note that in the initial model there was no need for time series to be incorporated, but since they were used when completing the final model, the method of incorporating time series will be addressed here as well.

**Fold-over-Basal**

To be able to fit the model simulations to data points from a fold-over-basal, the data from the simulations had to be normalized in the same manner as the data. The first data set that was incorporated into the model was the phosphorylation of S6K, seen to the left in Figure 2.4. For control measurement, which is the level of phosphorylation of S6K without stimuli, the simulated steady-state value of phosphorylated S6K was used. To create a simulation corresponding to the insulin stimulation in the experiment, the parameter \( \text{ins} \) was set to 1. Also, in the experimental procedure the phosphorylation of S6K was measured after 10 minutes post insulin stimulation, and therefore the simulation was set to last for 10 minutes as well. To calculate the normalization, both the control and the value for the stimulation were multiplied by 100 and divided by the steady-state value, according to Equation (3.14) and (3.15).

\[
\frac{\text{simdata1.statevalues}(\text{end},8) \cdot 100}{\text{simdata1.statevalues}(\text{end},8)} \quad (3.14)
\]

\[
\frac{\text{simdata2.statevalues}(\text{end},8) \cdot 100}{\text{simdata1.statevalues}(\text{end},8)} \quad (3.15)
\]

The data structs simdata1 and simdata2 are the simulated steady-state and the simulated insulin stimulation respectively. The first argument within the parentheses refers to the last time point of the simulation (1000 minutes for Equation (3.14) and 10 minutes for Equation (3.15)), and the second argument refers to the state variable of the model (here 8 corresponds to S6K\(_p\)).

After these modifications have been done, it is possible to use the model simulations in an optimization to find parameters that minimize the error between the simulations and the data points. The normalized data point is however useless in this sense, since the control always is 100 regardless of the model (Equation (3.14)). The optimization can therefore only try to find a model that mimics the relative changes in the system, not the absolute.

**Time Series**

The modifications required on the model simulations when comparing to time series were slightly more complicated. Since the baseline was subtracted from the data, the same had to be done with the simulated values. The baseline is the system at its steady-state, which meant that the steady-state simulation \( \text{simdata1} \)
3.1 The Initial Model

could be used for the baseline-subtraction (Equation (3.16)). In this example, the
time course data for the phosphorylation of IR has been used.

\[ A = \text{simdata2.statevalues(:, 2)} - \text{simdata1.statevalues(end, 2)} \]  
(3.16)

\[ A_{\text{norm}} = \frac{A * 100}{\max(A)} \]  
(3.17)

In Equation (3.16), \( A \) is a vector containing all the simulated response values for
the phosphorylation of IR, with the baseline subtracted (here the state variable 2
 corresponds to IR). In Equation (3.17), the maximum of the vector \( A \) is extracted
 with the function \( \max \), and the whole vector is normalized to have its maximum
 at 100.

Dose-response curves

The simulations used to create the dose-response curves were modified similarly
to the time series simulations. In the case of IR phosphorylation, all the response
curves for the different concentrations of insulin needed to be created first. This
was solved by giving the parameter \( \text{ins} \) different values, corresponding to the
increase in insulin concentration in the experiments. This generated several new
insulin-stimulated time curves, and for each curve the steady-state value (i.e. the
last value of each series) was extracted. As in Equation (3.16), the baseline was
then subtracted from each steady-state, and lastly, the data points were normalized
to have its maximum at 100 for the highest concentration of insulin.

No data modifications

A few data-sets used in this thesis had not been modified at all by the experimen-
talist. In for example the data used for LC3, the average fluorescent activity of
bound LC3 in cells from different subjects were registered, before and after the
addition of rapamycin and chloroquine. To be able to translate how the addition
of a substance in the model affects the fluorescent activity in a cell, a new pa-
rameter had to be added to the model. This new parameter serves as a scaling
factor, that translates the actual value of how much bound LC3 the model has at
a certain time point, into a measure of fluorescence that is compatible with the
experimental data.

The scaling with the new parameter was implemented by creating a new vari-
able under the headline MODEL VARIABLES in the model file (Appendix A). As
can be seen in Equation (3.18), the variable \( \text{LC3}_{\text{scaled}} \) depends on the state vari-
able \( \text{LC3} \) and the scaling parameter \( k \). During the optimization, it is the variable
\( \text{LC3}_{\text{scaled}} \) that the optimization algorithm will fit to the experimental data.

\[ \text{LC3}_{\text{scaled}} = \text{LC3} \ast k \]  
(3.18)

3.1.4 Optimizing the First Hypothesis

After the interaction graph shown in Figure 3.1 had been completely translated
into mathematical equations and all relevant modifications had been done to make
the model compatible with experimental data, it was time to optimize the model parameters against all data. At that point, three files had been constructed to be able to perform the optimization: the \textit{SBT2 model file} (Appendix A), containing all the equations that formed the model, the \textit{optimization script}, where all data was loaded into the workspace and all optimization settings were defined, and the \textit{cost function}, where the simulations were performed and the model cost was calculated.

The aim so far was to try to find a parameter-set that would make the model capture the dynamics of the experimental data in large. To acquire an adequate fit, numerous of subsequent optimizations were required, and it was an iterative process between modifying the model or adding new data, and making more optimizations. In Figure 3.2 and 3.3, the resulting simulations for an optimized parameter-set is displayed. As can be seen in the graphs, many of the simulated curves manage to fall within the estimated SE. There were however a few data points which the model fails to describe. The first problem is in Figure 3.2 E, showing the phosphorylation of S6K with and without the addition of the mitochondrial inhibitor DNP. According to the experimental data, the phosphorylation of S6K is supposed to drastically decrease when DNP is added. This should be made possible in our model with the feedback from the dysfunctional mitochondria to mTOR, seen in Figure 3.1, but still the optimization fails to find a parameter-set which produces a difference between the simulations.

Another data-set which the model is unable to fully reproduce is the reduction of lipofuscin after the addition of DNP, seen in Figure 3.2 F. The optimization algorithm had difficulties in general in optimizing the lipofuscin state, something that could happen if the model is lacking some states or equations which are required to fully describe the dynamics of lipofuscin. However, since the data points for lipofuscin with rapamycin and chloroquine in Figure 3.2 C are well fitted, it is more plausible that the problem lies with modelling the addition of DNP instead.

The last difficulty for the model was to render simulations that correctly describe the dose-response data, see Figure 3.3 A-C. The dose-response curve for IRS1 ends up slightly below many of the data points, while the dose-response curve for Ser-312 ascends too rapidly. Both dose-responses also fail to describe the last point in the data-set, but there is nothing in the model that could allow for such a decrease, so this error was disregarded.

### 3.2 Towards a Final Model

As described in the previous section there were some aspects of the experimental data that the model was unable to capture, i.e. the dynamics of S6K with DNP, the decrease of lipofuscin with DNP, and also the IRS1 and Ser-312 dose-responses. However, since the most important signal in the system, the insulin stimulation, activates IR and propagates downwards, it was better to focus on describing the experimental data for the early proteins in the chain first, such as IRS1 and its phosphorylation sites.
Figure 3.2. The resulting simulations in comparison to data for fold-over-basals, after having optimized the initial model hypothesis. The model equations can be viewed in Appendix A. For 3.2 D-E, only the changes after insulin has been added are shown, i.e. the 18 hours of inhibition with DNP are not displayed. The experimental data is represented by stars, each point with a vertical error bar showing the standard error for the point. The simulations are represented by continuous or dashed lines, see individual graph labels for more information.
Figure 3.3. The resulting simulations in comparison to the dose-response data, after having optimized the initial model hypothesis. The model equations can be viewed in Appendix A. The experimental data is represented by stars, each point with a vertical error bar showing the standard error for the point. The simulations are represented by continuous lines.
3.2 Towards a Final Model

Figure 3.4. The insulin-dependent dose-response curves for IRS1, Ser-307 and Ser-312 from 3.2, plotted in the same window.

3.2.1 Removal of Ser-312

To elucidate why the current model failed to describe the experimental data, the results needed to be analyzed further. To gain more knowledge about the different dose-response curves, they were all plotted in the same graph (Figure 3.4). As can be seen in the graph, the simulated curves had very similar responses to insulin, while the biological data indicate that the $EC_{50}$ (the half maximal effective concentration) of Ser-312 should be higher, i.e. half of the maximal response should occur at a higher insulin concentration for Ser-312 compared to Ser-307 and IRS1.

To interpret what modifications to the model that would correspond to changes in the $EC_{50}$ was difficult. Also, it was necessary to establish the most suitable site where to insert the modifications. In Figure 3.1, both Ser-307 and Ser-312 are directly or indirectly phosphorylated by mTORC1a through similar kinetic equations. Therefore, if a state or rate equation was to be altered prior to mTOR, both Ser-307 and Ser-312 would be very similarly affected. Hence, it became evident that in order to separate the dose-response curves of Ser-307 and Ser-312, an alteration in the rate equations downstream of mTORC1 was necessary.

The first simple modification that was tested was the addition of a Michaelis-Menten equation on the activation of Ser-307 by mTORC1a, and on the activation of Ser-312 by S6Kp, see Equations (3.19) and (3.20) respectively. The previous rate constants $k_{3f}$ and $k_{4f}$ were incorporated into $V_{max4}$ and $V_{max5}$ and were
therefore removed from the model.

\[ v_{3f} = \frac{V_{\text{max}4} \cdot m\text{TORC1}_a}{K_{M-m\text{TOR}} + m\text{TORC1}_a} \cdot \text{Ser-307} \]  

(3.19)

\[ v_{4f} = \frac{V_{\text{max}5} \cdot S6K_p}{K_{M-S6K} + S6K_p} \cdot \text{Ser-312} \]  

(3.20)

After the addition of the Michaelis-Menten equations, a new optimization was run, but the modifications turned out to have no effect on the outcome; the dose-response curves were still very close to each other. The explanation to this was that even though the optimization algorithm had the opportunity to insert a saturation in the activation of Ser-307 or Ser-312, the parameters were optimized so as to keep the same dynamics as before, i.e. the value of \( K_{M-m\text{TOR}} \) and \( K_{M-S6K} \) were chosen to be so large that the activation was still proportional to the concentration of mTORC1a and S6Kp. To increase the probability of finding a parameter-set where the activation of Ser-312 or Ser-307 would become saturated, different penalties on \( K_{M-S6K} \) and \( K_{M-m\text{TOR}} \) were added to the cost function. The penalties were formulated to increase the cost a lot if the values of \( K_{M-m\text{TOR}} \) or \( K_{M-S6K} \) were smaller than half the maximum concentration of active mTORC1 or S6K respectively, assuring that the saturating property of the Michaelis-Menten would be effective.

The addition of penalties did change the \( EC_{50} \) of Ser-307 and Ser-312 as expected, but shifted the curves to the left instead of to the right. See Figure 3.5 for an example where a penalty to \( K_{M-S6K} \) had been added before optimizing the parameters. Hypothetically, if the dose-response for Ser-307 would be forced by penalties to shift to the left, the optimization algorithm could compensate by choosing parameters that keeps the dose-response for Ser-307 in the same place and instead shifts the dose-responses for Ser-312 to the right, giving a better fit to the biological data. However, after testing this hypothesis through several optimizations it became evident that whatever penalty was added to the cost function, the dose-response for Ser-312 was never placed to the right of the IRS1-curve (Figure 3.6). Potentially this could mean that a downstream protein is bound to have a lower \( EC_{50} \) than the upstream protein. The idea is interesting and could be very useful when determining the activation profile amongst several proteins, but it would need mathematical and experimental validation to be fully proven.

Despite the efforts taken above, the current model seemed unable to describe the provided biological data. One obvious reason for this, as has already been partly dealt with, is that the model was insufficient and that there were for example more proteins and activations necessary to account for the particular behaviour seen in the dose-response curve of Ser-312. There is however always a slight risk that the data is flawed, and to assess the data points more thoroughly, the different repeats from the experiment were plotted separately in a graph (Figure 3.7). As can be seen in the graph, the different responses are very varied, and most importantly, the \( EC_{50} \) differs a lot between the curves. Also, as was mentioned in Section 3.1.1, there is no clear evidence that Ser-312 is actually phosphorylated by mTOR in primary human adipocytes. Together, the inability of the model to shift
3.2 Towards a Final Model

**Figure 3.5.** The insulin-dependent dose-response curves for IRS1, Ser-307 and Ser-312, after a penalty to $K_{M,S6K}$ has been added to the cost function, implementing a saturation on the activation of Ser-312 from S6K.

**Figure 3.6.** The insulin-dependent dose-response curves for IRS1, Ser-307 and Ser-312, after a penalty to prevent IRS1 from having a higher $EC_{50}$ than Ser-312 has been added to the cost function. The optimization algorithm found a parameter-set where they completely overlap, but nowhere along the curve is Ser-312 to the right of IRS1.
the $EC_{50}$ of Ser-312, the uncertainty of the data and the only hypothetical connection between S6K and Ser-312, makes it uncalled for to keep Ser-312 in the model. Therefore, the state variable of Ser-312 and its related rate equations were removed from the SBT2 model file. Also, since the purpose of the model was to describe a normal cell minimalistically, and the only aim of adding the Michaelis-Menten between mTORC1 and Ser-307 was to change the dose-response for Ser-312, the Michaelis-Menten modification was removed. The resulting interaction graph can be viewed in Figure 3.8. Since S6K$_p$ serves as a measurement of the activity of mTORC1a, both S6K and S6K$_p$ are kept in the model, even though they no longer affect any other states.

3.2.2 Adjusting the Model for Time Curves

Normally, when constructing a dose-response curve, the response of a particular state is not measured until a steady-state level has been reached. However, after discussing with the experimentalists, we came to the realization that the response measurements taken for Ser-307 and IRS1 were not taken at steady-state. For Ser-307, the reason for this was that only a short period after insulin stimulation, Ser-307 starts to dephosphorylate, returning back to its baseline level of phosphorylation. To be able to read insulin-dependent variations between different systems or experimental set-ups, the Ser-307 dose-response is constructed by measuring the peak value instead, which is believed to occur about ten minutes after insulin stimulation. For IRS1, the dephosphorylation halts temporarily after about
3.2 Towards a Final Model

Figure 3.8. The reduced model, where the states Ser-312 and Ser-312p have been removed from the interaction graph.
ten minutes, creating a small but stable plateau where the response measurements are taken.

By constructing the dose-response curves in this manner, important information could be lost or incorrect. If for example the peak value of Ser-307 for some reason occurs later than after ten minutes, the dose-response curve could receive a different shape because the response measurement is taken before the peak has occurred. This makes the response measurements very sensitive to changes in the system, and since it is in fact often the changes that are to be detected, this causes a potential problem with the data. However, by incorporating time curves for the phosphorylation of IR, IRS1 and Ser-307 into our mathematical model, the problem with the sensitivity can be overcome. The biological data would contain important dynamics about how the proteins’ phosphorylations vary with time, putting constraints on the model behaviour. The time curves would then be more likely to have proper shapes, strengthening the accuracy of the dose-response curves and making them more interpretable.

To put the decision of adding time series into practice, some changes needed to be made to the mathematical model. The experimental data for phosphorylated IR over time show a distinct overshoot-behaviour, i.e. the insulin-stimulated phosphorylation of IR is followed by a rapid dephosphorylation. It has been shown both experimentally and in situ that in order to describe the rapid dephosphorylation using a simplistic model such as the one designed here, a mechanism called internalization is necessary [17]. Internalization, or endocytosis, occurs when the cell absorbs the receptor into the cell by sequestering a small section of the cell membrane to where the IR is attached. However, internalization alone is not sufficient to describe the overshoot behaviour, and in [17] it has been shown that one possible mechanism, which together with internalization fully accounts for the dynamics of the overshoot, is a feedback from IRS1 that increases the dephosphorylation of the internalized phosphorylated IR.

The implementation of internalization required according to [17] the addition of five new states to the model (Figure 3.9). First of all, a receptor intermediary was added, where IR in the membrane is bound to insulin but has not yet been phosphorylated (IR_{ins}). Secondly two states of internalized IR were added, one where IR is internalized and phosphorylated (IR_{ip}) and one where IR is internalized and dephosphorylated (IR_i). Both the membrane-bound IR and the internalized IR can activate IRS1 as long as they are phosphorylated. Finally, to represent the unknown protein or proteins responsible for the feedback signal from IRS1, a protein X was added to the model. After IRS1 has been activated it phosphorylates and activates X into X_p, and thereby X_p can start to dephosphorylate IR_{ip}.

The additional rate equations that needed to be added as a consequence of the new state variables were adopted from [17]. Apart from the X_p-dependent activation of the dephosphorylation of IR_{ip} that contained a Michaelis-Menten equation, all new rate equations were based on simple mass action theory. The activation of IRS1 however, turned out slightly more complicated than in the initial model, as it was now dependent on all four states of IR_{p}, IR_{ip}, Ser-307_p and IRS1.
### Figure 3.9.
The modified interaction graph describing the process of internalization of IR. As insulin binds to IR it becomes phosphorylated. When phosphorylated, IR can become internalized by sequestration of the cell membrane. Both IR\textsubscript{p} and IR\textsubscript{ip} phosphorylates IRS1, who in turn activates the feedback that dephosphorylates the internalized IR. Otherwise, the downstream effects of IRS1 remain unchanged and are the same as in Figure 3.8.
By evaluating the expression, it might be more easily understood. The first part, \( k_{2f_1} \cdot IR_p \cdot IRS1 \), represents the activating effect on \( IRS1 \) from the membrane-bound phosphorylated \( IR \). The second part, \( k_{2f_1} \cdot k_{2f_2} \cdot IR_{ip} \cdot IRS1 \) represents the activating effect from the internalized phosphorylated \( IR \). The extra constant \( k_{2f_2} \) symbolizes a potential modification to the activation of \( IRS1 \) that could occur if \( IR \) is inside the cell. The last part, \( k_{2f_1} \cdot (IR_p + k_{2f_2} \cdot IR_{ip}) \cdot V_{max1} \cdot \frac{Ser-307_p}{K_{M-307} + Ser-307_p} \cdot IRS1 \), represents the modifying effect on \( IRS1 \) due to the phosphorylation of \( Ser-307 \), with a Michaelis-Menten expression. Since an active \( Ser-307 \) is regarded only as an amplification of the \( IR \)-dependent activation of \( IRS1 \), the last term is multiplied by the sum of \( IR_p \) and \( IR_{ip} \). By doing so, \( Ser-307 \) cannot phosphorylate \( IRS1 \) without \( IR \) being activated.

After the addition of all modifications required to describe the process of internalization, the time curves could be incorporated into the model. The experimental data for the time curve for phosphorylated \( IR \) contained both \( IR_p \) and \( IR_{ip} \), and therefore both the cost function and the graphs over the simulations were constructed to reflect the experimental data.

### 3.2.3 Separation of the S6K-simulations

After the addition of internalization and time curves to the model, and a few more optimizations had been run, most simulations fitted well to the data. A clear exception to this, however, was the simulation for S6K with and without the addition of the mitochondrial inhibitor DNP. After studying the graphs slightly (Figure 3.10), it became evident that the same simulation was fitted twice against two different data points. This might be regarded as unnecessary, but should however not pose a problem if the data is valid. Unfortunately, the two data points differed by more than 1000, which was a lot when comparing to the standard errors for the data points: \( \pm 68 \) and \( \pm 426 \). This made the optimization algorithm unable to find a parameter-set that fitted both data points, and instead optimized against the point with the smallest standard error (Figure 3.10 A).

To find a cause to the large difference in the data, it was important to be fully aware of the experimental conditions under which the experiments were performed. The difference was explained by the experimentalists to be caused by a change in batch of the antibody used when detecting phosphorylated S6K. A change in batch means that the antibody has been produced under the same conditions, but at a different time period, making variations in its properties possible. The new batch appeared to be less stable, indicated by the much larger standard error for the data point. We discussed the option of removing the data for the new batch, i.e. the data where the effect of DNP was studied, but decided for an approach where we tried to compensate for the batch-change by modifying the model instead. To generate simulations that allowed for variations in antibody effect, a new variable
was created according to Equation (3.22). During the optimization, the new variable $S6K_{p2}$ was fitted against the data point where the new batch of antibody had been used. The parameter $k_3$ was limited to be larger than the baseline of phosphorylated S6K, to avoid creating a negative variable.

$$S6K_{p2} = S6K_p - k_3$$  \hspace{1cm} (3.22)

After running an optimization with the new variable, the simulated curves for phosphorylated S6K after insulin stimulated had separated slightly. The separation was however not large enough to make the simulations fall within the limits of the standard errors. A probable holdback is the data point for phosphorylated S6K with insulin and DNP (Figure 3.10 B, depicted as a dot), that was described well even before the modification was added (dashed line). It is possible that if the optimization algorithm could find a parameter-set that described the insulin stimulations well, the insulin/DNP would be poorly fitted instead.

Based on these results, we concluded that the current model was unable to fully describe all the available data for S6K. One reason could be that the new batch of antibody was too unreliable to produce trustworthy data, but it is also possible that a more complex modification of the model was required to account for the new experimental set-up. However, even if the model failed to entirely describe the insulin/DNP data-set, the data still displayed important dynamics that we wanted to maintain in the model, i.e. that the phosphorylation of S6K is affected by DNP. Therefore, we decided to keep optimizing against the insulin/DNP data-set, but for the moment without putting further efforts into finding a better fit to the data.
3.2.4 The Hill Equation

By incorporating time curves into the model, new properties that the model was unable to reproduce were discovered. According to the experimental data for IR and IRS1, the time curves were supposed to reach a steady-state higher than the baseline after stimulation with insulin. However, due to the very low steady-state of Ser-307, both IR and IRS1 were forced to keep low steady-states as well. For the model to be able to lower the level of phosphorylation of Ser-307 without affecting the proteins upstream of Ser-307, the activation of Ser-307 needed to have a more switch-like behaviour. As described in Section 2.1.4, a switch-like behaviour could be achieved with the use of a Hill equation. Subsequently, the forward rate reaction \( v_{3f} \), which determines the rate of phosphorylation of Ser-307, was modified according to Equation (3.23).

\[
\begin{align*}
v_{3f} &= \frac{V_{\text{max}3} \cdot m\text{TORC1}_a^n}{K_{M-m\text{TOR}}^n + m\text{TORC1}_a^n} \cdot \text{Ser-307} \\
&= (3.23)
\end{align*}
\]

After running an optimization with the modified model, the steady-states of IR and IRS1 did in fact increase to some degree. The steady-state of Ser-307 did however also increase and after studying the parameter-set that the optimization algorithm located, we realized that the current optimum had a Hill coefficient of \( n = 1.009 \). With a Hill coefficient close to one, the Hill equation in this form functions more as a Michaelis-Menten, i.e. the modification has a saturating effect on the activation rather than inducing the switch-like behaviour that was initially sought for. To investigate if there was a better fit available by implementing the Hill dynamics, the parameter corresponding to the Hill coefficient was given a lower limit of \( n = 2 \), and a new optimization was run. The result was an improvement when looking at the steady-states of the time curves, but the other simulations for Ser-307 deviated greatly from their data points (Figure 3.11 D). It appeared as if the optimization algorithm was for this model incapable of finding a parameter-set that lowered the steady-state of Ser-307 (C) without at the same time destroying the fold increase for Ser-307 (D).

Due to the complications of enforcing Hill dynamics on the model, we decided to return to the previously optimized parameter-set, where the steady-states of IR and IRS1 are close to data, while the steady-state of Ser-307 is higher than the experimental data depicts. However, since the activities of IR and IRS1 are essential in the insulin signalling pathway and also in the process of autophagy, it is of greater importance to focus on describing the IR and IRS1 data, rather than that of Ser-307. This view was strengthened by the experimentalists, who based on previous experiments felt confident that the IR steady-state is heightened for a long period of time after insulin stimulation.

3.2.5 The Final Model

After the addition of time curves and after several modifications, the model was able to give an acceptable fit to most of the experimental data. The complete interaction graph for the final model and the corresponding SBT2 model file can
Figure 3.11. The simulated curves after Hill dynamics has been enforced upon the activation of Ser-307. IR and IRS1 have acceptable steady-state levels. At the same time the steady-state of the Ser-307 time curve (C) has managed to maintain a low level, but at the cost of a poor fit to the fold increase of Ser-307 (D).
Results

be found in Figure 3.12 and Appendix B respectively. The interaction graph shows all the activations and reactions taking place as insulin is added to the system, from the activation and internalization of IR to the activation of mTOR and its effects on autophagy. The resulting simulations can be viewed in Figure 3.13 and 3.14. As can be seen in the graphs, most simulations manage to describe the data properly. Except for the issue with S6K that was addressed in Section 3.2.3, the model successfully describes all fold-over-basals (Figure 3.13). The dose-responses were slightly more complicated for the model to describe (Figure 3.14 A-C), and still there could be some improvement to the IR dose-response, but the simulated curves of IRS1 and Ser-307 follow the data adequately. As for the data points from the time curves (Figure 3.14 D-F), they were the most difficult to fit. However, since the steady-states of IR and IRS1 no longer decline to immediately reach the basal steady-state after insulin stimulation, the model still manages to account for most of the important dynamics of the time curves.

The resulting parameters from the optimization of the final model can be found in Table 3.1. Even though the model is final within the borders of this thesis, it is still a working model and will be continuously improved in the future. Therefore, we have not regarded the optimized parameters to be of any great importance at this point, and we have therefore not made any particular efforts in elucidating whether they are likely or not. Also, as discussed in Section 2.3, a better approach when analyzing the accuracy of a model is to create core predictions by searching for all acceptable parameter-sets instead.

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Figure 3.12. The complete interaction graph over the final model, including insulin stimulation, activation of IRS1 and mTORC1, and the subsequent attenuation of autophagy.
Figure 3.13. The model simulations for the fold-over-basals produced when using the final model and the optimized parameter-set displayed in Table 3.1. The experimental data is represented by stars, each with a vertical error bar showing the standard error for the data point. The simulations are represented by continuous or dashed lines, see individual graph labels for more information.
3.2 Towards a Final Model

Figure 3.14. The model simulations for the dose-responses and the time curves produced when using the final model and an optimized parameter-set displayed in Table 3.1. The experimental data is represented by stars, each with a vertical error bar showing the standard error for the data point. The simulations are represented by continuous lines.
Table 3.1. The optimized parameters for the final model.

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Chapter 4

Discussion and Conclusions

4.1 Discussion

To create a mathematical model that mimics the properties of a cellular signalling pathway is not a simple task. However, to draw valid conclusions based on experimental data without mathematical evaluation can be even more complicated, and carries the risk of making inaccurate judgements. During the work of this thesis, it has become clear that even with the approach of building a simplistic model that only describes the experimental data in large, careful consideration and re-evaluation of both the hypothesis and the data has been necessary.

The hypothesis to be evaluated states that an attenuated activity of the protein complex mTORC1 stands as one of the causes to insulin resistance and the heightened level of autophagy that has been observed in subjects with diabetes. As a first step in outlining the biological hypothesis, the interaction graph seen in Figure 3.1 on page 34 was constructed. The aim was to find a model that adequately managed to describe all the data-sets related to the hypothesis, serving as a first step towards mathematically elucidating how mTOR and autophagy play a part in the insulin signalling pathway.

After the interaction graph had been constructed and the model had been translated into mathematical equations, the model was to be fitted against biological data. One of the issues discovered when trying to implement the developed interaction graph as a mathematical model, was the model’s inability to fully describe the dynamics of the dose-response for phosphorylation of Ser-312. As was mentioned in Section 3.2.1, the model was incapable of mimicking the difference in $EC_{50}$ between Ser-312 phosphorylation and IRS1 phosphorylation that the data expressed. It appeared from the efforts taken to force the model to shift the $EC_{50}$ of Ser-312, that a protein cannot have an $EC_{50}$ higher than the upstream proteins in the signalling pathway. Biologically, this would make sense, since it would be unexpected of a protein to continue to increasingly activate other proteins after it has reached its point of saturation. Therefore, we started to suspect that other proteins apart from S6K needed to be involved in the activation of Ser-312, or that the data of Ser-312 were not to be relied upon. To fully investigate these
suspicions, further experimental evaluation is necessary, and that falls outside the framework of this thesis.

One aspect which has not been fully addressed in this report is how and in what way the phosphorylation of Ser-307 affects the phosphorylation of IRS1 at the tyrosine residues. It has been shown in [12] that the phosphorylation of Ser-307 positively regulates the phosphorylation of IR. The mechanism underlying the positive feedback has however not been elucidated. The experimental data seen in Figure 3.14 potentially speak against a positive correlation: The phosphorylation of Ser-307 peaks after ten minutes, when the level of phosphorylated IR has already started to decline. There is however a suggestion that Ser-307 is responsible for the heightened steady-state of IR seen after insulin stimulation, but this is contradicted by the experimental data which show that the steady-state of phosphorylated Ser-307 has returned to baseline long before IR has. With our current model, there appears to be no way of maintaining a high steady-state of IR if the phosphorylation of Ser-307 returns to its basal level of activity, suggesting that the model needs additional features to gain proper dynamics. An interesting theory that could be experimentally investigated is if the phosphorylation of Ser-307 together with IR triggers any transcriptional signals in the cell. That would result in long-term alterations of the cell signalling and could give rise to the heightened steady-state of IR and IRS1 observed in the experimental data.

In all cases where no acceptable fit to data have been found during the optimizations, we have either drawn the conclusion that the data were flawed or that the model structure was incorrect or insufficient. However, there is always a small risk that there are acceptable parameter-sets which the optimization algorithm has been unable to locate, for example due to bad starting guesses or incorrect settings of the optimization algorithm. To minimize that possibility, we have for each set of model equations run several optimizations using different starting guesses and different maximum temperatures. Also, where no adequate solution has been found, the cost function has been modified with penalties to help the optimization algorithm to search in a direction which we believe would give a better fit to data. Although, to be assured that the optimization algorithm can localize parameter-sets that are good enough to draw conclusions from, an assessment of the qualities of the optimization algorithm could be beneficial.

During the development of the model, several problems were encountered which could have been prevented if the experimental data had been more carefully studied, and all aspects of how the experimental data were generated had been made clear. First of all, there was the issue with the dose-response curves addressed in Section 3.2.2. We were initially unaware of the fact that the standard way of producing dose-response curves was not applicable on the phosphorylation of Ser-307 and IRS1. Therefore the model and the model simulations were at first constructed using incorrect assumptions, creating potential flaws in the comparison between model simulations and the experimental data.

Another example is the issue with S6K discovered in Section 3.2.3, where we wanted the model to describe two very different data points using the same simulation. Unfortunately, many of the methods used when measuring protein levels in cells, such as immunoblotting, carry large experimental uncertainties. As in
4.2 Conclusions

the case with S6K, the model could not account for the method variation that occurred when the antibody used for the immunoblotting was changed. We tried to compensate in our model for the change in batch of antibody and managed to get a minor improvement, but either the antibody was too unstable to produce reliable results, or the model needed a more complex modification to give an adequate fit to both data points.

Also, when experimentalists construct biological experiments where something is added to the system and an effect is being measured, they always take control measurements. This is because the experimental and biological variation often makes comparisons between experiments unreliable. Still, in systems biology a model is often fitted against several data-sets simultaneously, and the model is required to account for all data points regardless of when and how they were generated. This naturally poses a problem in the interpretation of the model simulations, and being aware of this issue is essential when constructing the mathematical relationships in the model.

Biological and experimental variations are two large problems that need to be fully addressed when building mathematical models, something which the above mentioned issues are all evidence of. This has been one of the main discoveries during the development of the model, and it is evident that as a systems biologist it is important not only to be theoretically skilled in mathematical modelling, but also to have a comprehensive understanding of biological experimentation and generation of data.

4.2 Conclusions

To gain better knowledge about the mechanisms that lead to type 2 diabetes, it is important to understand all aspects of the insulin signalling pathway. It is however difficult to get a full grasp over all details, considering the numerous activations, feedback signals and cross-talks that exist among signalling cascades. Therefore, mathematical modelling has started to become an increasingly useful tool when studying the complexity of cell signalling, and works as a means to test various hypotheses, but also as an aid when developing new hypotheses and experimental try-outs.

In this thesis, a mechanistic hypothesis and related biological data have been mathematically evaluated using a model-based approach. The original hypothesis, which states that a decreased activity of mTOR could be a cause to the insulin resistance and heightened level of autophagy observed in type 2 diabetics, is summarized in an interaction graph in Figure 3.1.

Using the theory of mass action and the Michaelis-Menten equation, the interaction graph was translated into ODE:s. To be able to adequately describe the available experimental data through optimizations, the model required a couple of modifications. First of all, phosphorylation at Ser-312 was removed from the model, since the model was unable to describe the \( EC_{50} \) of its dose-response, indicating that either the data were unreliable or that the hypothesis was inadequate. Secondly, time curves were added to the model, which we discovered to
be necessary if all the important dynamics in the dose-response data were to be accounted for (Section 3.2.2). Due to the addition of time curves, the process of internalization had to be added to the mechanistic hypothesis, as described in [18]. Lastly, a new variable was introduced to make it possible for the model to compensate for the different experimental set-ups discovered for S6K, and a Hill equation was added to improve the dynamics of the Ser-307 phosphorylation. The concluding interaction graph can be viewed in Figure 3.12, and the corresponding model equations are available in Appendix B. With the chosen parameter set (Table 3.1), the model simulations manage to describe most of the data-sets, but are still unable to fully account for some of the details seen in the data (Figures 3.13 and 3.14). All the fold-over-basals were successfully described, except one of the data-sets with S6K, as has already been mentioned. The dose-responses are also adequately described, even though there could be some improvement to the IR dose-response. The time curves were the most difficult to fit, and even though IR and IRS1 manage to keep the heightened level of steady-state that the data depict, Ser-307 does not return fully to its basal level of phosphorylation after insulin stimulation as it should. However, the procedure of model-based hypothesis testing is an iterative process between improving and rejecting hypotheses, and by developing our preliminary model we have gone one step closer to either rejecting the current hypothesis, or finding a modification that makes the model able to describe the data.

During the course of modelling, we have gained several important insights about model-based hypothesis testing. By evaluating a mechanistic hypothesis through mathematical modelling, weaknesses in the hypothesis or the data can be detected. Partly, this is due to the forced re-examination of the hypothesis and the relevant data when the model equations are constructed, and partly it is as a result of the mathematical optimizations, i.e. if the model is unable to describe the experimental data. However, for a model-based approach to be at its full potential, a comprehensive understanding of the biological system and the generation of biological data is crucial.
Chapter 5

Recommendations

The task of elucidating the mechanisms of type 2 diabetes is an ongoing process, and so is determining what role mTOR and autophagy play in the cause of insulin resistance. To create a mathematical model that manages to capture the main features of the limited amount of biological data available so far was only a beginning, and there are several further steps that can be taken to follow up the work that has been presented in this report.

- **Modification of the model to describe data from diabetics.** As a way of analyzing the current model, the model simulations could be compared to biological data from subjects with diabetes. It would be very interesting to see how the model simulations behave if for example the forward rate reaction of the activation of mTOR was modified to be half as fast. If the model simulations could describe the data from diabetics by down-tuning the activity of mTOR or IRS1, then our hypothesis that mTOR can cause insulin resistance would be supported by the model.

- **The \( EC_{50} \) of the dose-response curves.** An interesting discovery during this thesis was the indication that a protein cannot have a larger \( EC_{50} \) than its upstream activators. However, to be able to fully use this property as a tool to figure out connections and pathways in cell biology, the theory must first be mathematically and experimentally verified.

- **Generation of new biological data.** The more reliable data that can put constraints on the model behaviour, the more the probability of finding a model that can be either rejected or be used to form core predictions increase. Suggestions of possible data-sets to generate next could be time curves for S6K and IRS1 with insulin, and DNP plus insulin, to be able to closer study the effects of dysfunctional mitochondria on the insulin signalling pathway. Also, due to the problems of modelling the dose-response of Ser-312 and the insulin-dependent steady-state of IR and IRS1, another suggestion could be to verify the existing data-sets of the phosphorylation of Ser-312 and Ser-307 respectively, by generating new data-sets.
• **Investigate how Ser-307 affects IRS1.** As was described in Section 4.1, the exact mechanistic explanation to how Ser-307 affects the phosphorylations of IRS1 has never been established. Since this positive feedback appears to be important as a partial cause to insulin resistance, the model-based evaluation of the current hypothesis would greatly benefit from an investigation of how the Ser-307 phosphorylation affects IRS1.

• **Making core predictions.** If the model developed in this thesis can be modified to give a satisfactory description of all relevant biological data, the next step would be to find all acceptable parameter-sets and form core predictions about the model’s properties. By the formation of core predictions, new insights about the biological mechanisms underlying the experimental data can be found, and possibly the current model can be rejected to develop a new and improved hypothesis to be tested.
Bibliography


Appendix A

The Initial Model Equations

********** MODEL NAME
Initial hypothesis

********** MODEL NOTES

********** MODEL STATES
\[ \frac{d}{dt}(IR) = v_1b - v_1f \]
\[ \frac{d}{dt}(IRp) = v_1f - v_1b \]
\[ \frac{d}{dt}(IRS1) = v_2b - v_2f \]
\[ \frac{d}{dt}(IRS1p) = v_2f - v_2b \]
\[ \frac{d}{dt}(Ser307) = v_3b - v_3f \]
\[ \frac{d}{dt}(Ser307p) = v_3f - v_3b \]
\[ \frac{d}{dt}(Ser312) = v_4b - v_4f \]
\[ \frac{d}{dt}(Ser312p) = v_4f - v_4b \]
\[ \frac{d}{dt}(mTORC1) = v_5b - v_5f \]
\[ \frac{d}{dt}(mTORC1a) = v_5f - v_5b \]
\[ \frac{d}{dt}(S6K) = v_6b - v_6f \]
\[ \frac{d}{dt}(S6Kp) = v_6f - v_6b \]
\[ \frac{d}{dt}(autophagy) = v_7f - v_7b \]
\[ \frac{d}{dt}(LC3) = v_8f - v_8b \]
\[ \frac{d}{dt}(mit\_functional) = v_9f - v_9\_degraded \]
\[ \frac{d}{dt}(mit\_dysfunctional) = v_9\_degraded - v_9a \]
\[ \frac{d}{dt}(lipofuscin) = v_{10f} - v_{10b} \]

IR(0) = 100
IRp(0) = 10
IRS1(0) = 100
IRS1p(0) = 10
Ser307(0) = 100
Ser307p(0) = 10
Ser312(0) = 100
Ser312p(0)=10
mTORC1(0)=100
mTORC1a(0)=10
S6K(0)=100
S6Kp(0)=10
mit_functional(0)=100
mit_dysfunctional(0)=10
autophagy(0)=50
LC3(0)=50
lipofuscin(0)=50

********** MODEL PARAMETERS
k1f = 10
k1b = 4
k2f = 0.001
k2b = 20
k3f = 0.1
k3b = 2
k4f = 0.1
k4b = 2
k5f = 0.05
k5b = 2
k6f = 0.01
k6b = 2
auto_basal = 50
k7b = 0.01
k8f = 0.5
k8b = 2
k9_degraded = 0.5
k9a = 2
lipo_basal=0.01
k10b = 0.0001
Vmax1= 0.1
Vmax2 = 20
Vmax3 = 2
Km_307 = 2
Km_312 = 2
Km_mit = 2
mit_basal = 40
IR_basal = 2
k=2
k2=2
DNP = 1
chloroquine = 1
rapamycin = 1
ins = 0
********** MODEL VARIABLES

LC3_scaled = LC3 * k
lipofuscin_scaled = lipofuscin * k2

********** MODEL REACTIONS

v1f = (k1f * ins + IR_basal) * IR
v1b = k1b * IRp
v2f = (k2f + Vmax1 * Ser307p / (Km_307 + Ser307p)) * IRS1 * IRp
v2b = (k2b + Vmax2 * Ser312p / (Km_312 + Ser312p)) * IRS1p
v3f = k3f * mTORC1a * Ser307
v3b = k3b * Ser307p
v4f = k4f * Ser312p * S6Kp
v4b = k4b * Ser312p
v5f = k5f * rapamycin * mTORC1 * IRS1p
v5b = (k5b + kfeed3 * mit_dysfunctional / (Km_mit + mit_dysfunctional)) * mTORC1a
v6f = k6f * S6K * mTORC1a
v6b = k6b * S6Kp
v7f = auto_basal
v7b = k7b * mTORC1a * autophagy
v8f = k8f * autophagy
v8b = k8b * chloroquine * LC3
v9f = mit_basal
v9.degraded = k9_degraded * mit_functional * DNP
v9a = k9a * mit_dysfunctional * autophagy
v10f = lipo.basal
v10b = k10b * chloroquine * autophagy * lipofuscin

********** MODEL FUNCTIONS

********** MODEL EVENTS

********** MODEL MATLAB FUNCTIONS
Appendix B

The Final Model Equations

********** MODEL NAME
The final model

********** MODEL NOTES

********** MODEL STATES
\[
\begin{align*}
\frac{d}{dt}(IR) &= -v_1a + v_1b + v_1h + v_1g \\
\frac{d}{dt}(IR_{ins}) &= v_1a - v_1b - v_1c \\
\frac{d}{dt}(IR_p) &= v_1c - v_1d - v_1g \\
\frac{d}{dt}(IR_i) &= v_1d - v_1e \\
\frac{d}{dt}(IRS_1) &= v_2b - v_2f \\
\frac{d}{dt}(IRS_{1p}) &= v_2f - v_2b \\
\frac{d}{dt}(\text{Ser307}) &= v_3b - v_3f \\
\frac{d}{dt}(\text{Ser307}_{p}) &= v_3f - v_3b \\
\frac{d}{dt}(X) &= v_4b - v_4f \\
\frac{d}{dt}(X_{p}) &= v_4f - v_4b \\
\frac{d}{dt}(\text{mTORC1}) &= v_5b - v_5f \\
\frac{d}{dt}(\text{mTORC1}_{a}) &= v_5f - v_5b \\
\frac{d}{dt}(\text{S6K}) &= v_6b - v_6f \\
\frac{d}{dt}(\text{S6K}_{p}) &= v_6f - v_6b \\
\frac{d}{dt}(\text{autophagy}) &= v_7f - v_7b \\
\frac{d}{dt}(\text{LC3}) &= v_8f - v_8b \\
\frac{d}{dt}(\text{mit\_functional}) &= v_9f - v_9_{\text{degraded}} \\
\frac{d}{dt}(\text{mit\_dysfunctional}) &= v_9_{\text{degraded}} - v_9a \\
\frac{d}{dt}(\text{lipofuscin}) &= v_{10f} - v_{10b}
\end{align*}
\]

IR(0)=100
IR_{ins}(0) = 2
IRp(0)=10
IRip(0) = 10
IRi(0) = 10
IRS1(0)=100
IRS1p(0)=10
Ser307(0)=100
Ser307p(0)=10
X(0) = 100
Xp(0) = 10
mTORC1(0)=100
mTORC1a(0)=10
S6K(0)=100
S6Kp(0)=10
autophagy(0)=50
LC3(0)=50
mit_functional(0)=100
mit_dysfunctional(0)=10
lipofuscin(0)=50

********** MODEL PARAMETERS

IR_basal = 25964,6263075138
k1a = 152528,675332224
k1b = 563242,209104686
k1c = 0,0848632751153629
k1d = 626925,262108390
k1e = 1,48290658206133
k1f = 16078,0745593720
k1g = 0,00729029886700206
k1h = 0,0129561409975261
k2f1 = 0,0184252163902909
k2f2 = 224950,477189589
k2b = 4,13907321328606
k3b = 878951,82793818
k4f = 93164,7160305780
k4b =4,54210619490228
k5f = 57719,6038205653
k5b = 4,3014359091252
k6f = 31,8617431768879
k6b = 527050,974021439
auto_basal = 0,306459205102978
k7b = 81,3361426821254
k8f = 0,00220701374825227
k8b = 4,59892228868945
mit_basal = 970897,536868777
k9_degraded = 115,735279552773
The Final Model Equations

\[ k_{9a} = 0.964931478787304 \]
\[ \text{lipo}_\text{basal} = 63787.4232098859 \]
\[ k_{10b} = 923.506405760202 \]
\[ \text{Vmax1} = 1.35018165218074 \]
\[ \text{Vmax2} = 0.0159473753600085 \]
\[ \text{Vmax3} = 111.117937060706 \]
\[ \text{Km}_{307} = 0.249681523274938 \]
\[ \text{Km}_\text{mit} = 0.0934162508182784 \]
\[ \text{Km}_\text{mTOR} = 2001.2084735395 \]
\[ k = 0.355497005018693 \]
\[ k_2 = 16042.1498545280 \]
\[ k_3 = 0.0206652805123902 \]
\[ n = 1.009 \]

\[ \text{mit}_\text{inhibitor} = 388819.190744595 \]
\[ \text{chloroquine} = 0.628834120214538 \]
\[ \text{rapamycin} = 1.59499740618535 \times 10^{-08} \]
\[ \text{ins} = 0 \]

********** MODEL VARIABLES

\[ \text{LC3}_\text{scaled} = \text{LC3} \times k \]
\[ \text{lipofuscin}_\text{scaled} = \text{lipofuscin} \times k_2 \]
\[ \text{S6Kp2} = \text{S6Kp} - k_3 \]

********** MODEL REACTIONS

\[ \text{v1a} = k_{1a} \times \text{ins} \times \text{IR} + \text{IR}_\text{basal} \times \text{IR} \]
\[ \text{v1b} = k_{1b} \times \text{IRins} \]
\[ \text{v1c} = k_{1c} \times \text{IRins} \]
\[ \text{v1d} = k_{1d} \times \text{IRp} \]
\[ \text{v1e} = \text{IRip} \times (k_{1e} + k_{1f} \times \text{Xp} / (1 + \text{Xp})) \]
\[ \text{v1g} = k_{1g} \times \text{IRp} \]
\[ \text{v1h} = k_{1h} \times \text{IRi} \]
\[ \text{v2f} = k_{2f1} \times (\text{IRp} + k_{2f2} \times \text{IRip}) \times \text{Vmax1} \times \text{Ser307p} / (\text{Km}_307 + \text{Ser307p}) \times \text{IRS1} \]
\[ \text{v2b} = k_{2b} \times \text{IRS1p} \]
\[ \text{v3f} = (\text{Vmax3} \times \text{mTORC1a} \times n / (\text{Km}_\text{mTOR} \times n + \text{mTORC1a} \times n)) \times \text{Ser307} \]
\[ \text{v3b} = k_{3b} \times \text{Ser307p} \]
\[ \text{v4f} = k_{4f} \times \text{X} \times \text{IRS1p} \]
\[ \text{v4b} = k_{4b} \times \text{Xp} \]
\[ \text{v5f} = k_{5f} \times \text{rapamycin} \times \text{mTORC1} \times \text{IRS1p} \]
\[ \text{v5b} = (k_{5b} + \text{Vmax2} \times \text{mit_dysfunctional} / (\text{Km}_\text{mit} + \text{mit_dysfunctional})) \times \text{mTORC1a} \]
\[ \text{v6f} = k_{6f} \times \text{S6K} \times \text{mTORC1a} \]
\[ \text{v6b} = k_{6b} \times \text{S6Kp} \]
\[ \text{v7f} = \text{auto}_\text{basal} \]
\[ \text{v7b} = k_{7b} \times \text{mTORC1a} \times \text{autophagy} \]
\[ \text{v8f} = k_{8f} \times \text{autophagy} \]
v8b=k8b*chloroquine*LC3
v9f=mit_basal
v9_degraded=k9_degraded*mit_functional*mit_inhibitor
v9a=k9a*mit_dysfunctional*autophagy
v10f=lipo_basal
v10b=k10b*chloroquine*autophagy*lipofuscin

******* MODEL FUNCTIONS

******* MODEL EVENTS

******* MODEL MATLAB FUNCTIONS