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Publisher's version / Version de l'éditeur:
https://doi.org/10.1142/S0219720010004872
Journal of Bioinformatics and Computational Biology, 8, 4, pp. 703-715, 2010-03-01

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Journal of Bioinformatics and Computational Biology
Vol. 8, No. 4 (2010) 703–715
© Imperial College Press
DOI: 10.1142/S0219720010004872

Imperial College Press
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THE IMPORTANCE OF INHIBITORS FOR THE SIMULATION OF METABOLIC PROCESSES: *IN SILICO* Zn^{2+} INHIBITION OF m-ACONITASE FROM ANALYSIS OF GLYCOLYSIS AND KREBS CYCLE KINETIC MODELS

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Received 27 November 2009
Revised 28 January 2010
Accepted 9 February 2010

Metal ions have a major effect on the metabolic processes in cells either as inhibitors or as integral components of enzymes. The inhibition of enzymes can take place either through the inhibition of gene expression or through inhibition of protein function. A particularly interesting example of the effect of a metal ion on metabolism is the observed inhibition of Krebs cycle and alteration of energy metabolism by zinc (II) cations. In this particular case metal ion inhibition of enzyme is linked to one of the major functions of prostate cells of accumulation and excretion of citrate. Experimental results have shown that increase in concentration of zinc (II) in prostate cells effectively blocks progression of a part of the Krebs cycle leading to change in the concentration of several metabolites with largest effect in the accumulation of citrate. Based on previously reported experimental results, several distinct mechanisms for zinc (II) inhibition of Krebs cycle were proposed. In order to determine the precise mechanism of inhibition in this system, a mathematical model of glycolysis and Krebs cycle was constructed. Three different types of inhibition were analyzed, including competitive and uncompetitive inhibition as well as inhibition through the alteration of the expression level of m-aconitase. The effects of different inhibition models on metabolite concentrations were investigated as a time course simulation of the system of reactions. Several kinetic parameters in the model were optimized in order to best resemble experimental measurements. The simulation shows that only competitive inhibition leads to an agreement with experimental data.

Keywords: Inhibition; pathway modeling; Krebs cycle; glycolysis; prostate metabolism; metal inhibition; prostate cancer; cancer metabolism; TCA; energy metabolism.

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1. Introduction

Understanding an organism's metabolism at a system level and obtaining quantitative predictions for the different metabolic variables requires the identification and modelling of the physicochemical as well as regulatory constraints.[1,2] An important regulatory route in metabolism is the inhibition of enzymes with metal ions either as part of normal cellular function or through exposure to toxic metals.[3] The effect of these metal regulators is thus far overlooked in computational and systems biology level studies of cellular pathways and networks.

The effect of zinc (II) ions on Krebs cycle is a very important example of metal ion regulation of metabolism. Human prostate secretory epithelial cells have the uniquely specialized function of accumulating and secreting extremely high levels of citrate. It has been proposed that their ability to accumulate high cellular levels of zinc leads to inhibition of citrate oxidation, leading to the observed high levels of citrate.[4–11] In prostate cancer, the malignant cells undergo a metabolic transformation from zinc accumulating–citrate-producing to citrate-oxidizing cells metabolically more closely related to normal cells of other tissues. In fact, epidemiological studies have demonstrated an association between zinc (II) concentration and prostate cancer development.[12] Based on experimental results, the inhibition of the enzyme m-aconitase (ACO2) by zinc (II) is hypothesized to be the major reason for altered metabolism in prostate cells leading to a higher citrate concentration relative to other normal as well as prostate cancer cells.[11] The malignant process in prostate involves as yet unknown changes that lead to reduced zinc ion concentration and a change in metabolism towards citrate oxidation rather than export. The analysis of Krebs cycle metabolism in mitochondria of various cell lines showed that increased levels of zinc (II) ion lead to an increased level of mitochondrial and cytoplasmic citrate.[4–9] The *in vivo* as well as *ex vivo* analysis of prostate tissues also shows that normal tissues have much higher levels of citrate relative to the prostate cancer tissues.[13] In fact the oxidation of citrate in prostate cancer is used for magnetic resonance spectroscopic diagnosis of prostate cancer as it is recently reviewed in Ref. 14. The specific type of inhibition effect of zinc is still however not understood, with some published experimental work proposing a competitive inhibition route[11] and other published work indicating that zinc is affecting gene expression of m-aconitase and in this way regulating its function.[10] In addition, high levels of substrate present in the system make an uncompetitive inhibition a possible route as well.

Quantitative system level models of biological networks provide a method for testing experimentally derived hypotheses. These models generally aim to capture the underlying structure, dynamics and detailed mechanisms of their experimental counterparts in a manner that recapitulates known behaviors, provides means for understanding these behaviors and also predicts previously unmeasured or new behaviors.[1,2,15–18] The potential of mathematical models in describing biological systems is well understood and there are several software tools that allow system level modelling of networks.[2] Different aspects of Krebs cycle where

amongst the first biological processes modeled.[25,26] The network models are now increasingly being utilized for prediction, analysis and parameter determination as well as experiment validation with some recent examples being the description of glucose-stimulated insulin secretion[16] and receptor-tyrosine-kinase–activated MAPK pathway.[17]

Three different avenues for zinc inhibition of the Krebs cycle were investigated in this study. First was the competitive inhibition suggested by the experimental work of Costello *et al.*[11]; second was the model of inhibition through the effect on m-aconitase expression suggested by Tsui *et al.*[10] Finally, we have tried uncompetitive inhibition model which is a form of enzyme inhibition where the inhibitor binds to an already established enzyme and substrate complex. Ordinary differential equation (ODE) models of glycolysis and the Krebs cycle including molecular shuttling across the mitochondrial membrane were developed. Concentration changes of 38 metabolites were calculated at different concentrations of zinc for the three different inhibition models. The simulation results were compared with a range of previously published experimental results leading to a determination of the route for inhibition.

In this paper we present the importance of the inclusion of metals and other cellular inhibitors in kinetic models of biological pathways. Further, the goal of this work was to show the ability of kinetic models of metabolic processes to analyze inhibitor effects. The strategy in this case was based on a kinetic modeling approach which takes into account both *in vitro* data on individual enzymes and *in vivo* data characterizing metabolite concentration. Finally, the developed model was used for the determination of most probable inhibition type. In the future this inhibition mechanism will be further investigated both experimentally and theoretically in order to optimize kinetic parameters and thus to obtain more accurate information about metabolic fluxes. Also, further analysis needs to focus on the investigation of molecular binding mechanism for this inhibition process.

2. Model

The basic components of the model which include glycolysis, Krebs cycle and transport across the mitochondrial membrane are illustrated in Fig. 1.

All metabolic reactions are described in terms of kinetic equations which determine the change of metabolite concentrations as a function of time.[1,2] The components of the model are kinetic parameters and state variables. The model consists of a system of ordinary nonlinear differential equations (ODE) with 21 enzymatic reactions, 39 metabolic state variables and 147 parameters. The rate equations inside the mitochondrion follow complex reaction mechanisms such as Ping-Pong, Bi Bi Ordered Bi Bi, etc. The change in the concentration of a certain metabolite $[m_i]$ over time is determined as the difference between the sum of reaction rates producing m_i and sum of rates of reactions consuming m_i; i.e.,

$$\frac{d[m_i]}{dt} = \sum v_{\text{production}} - \sum v_{\text{consumption}}. \tag{1}$$

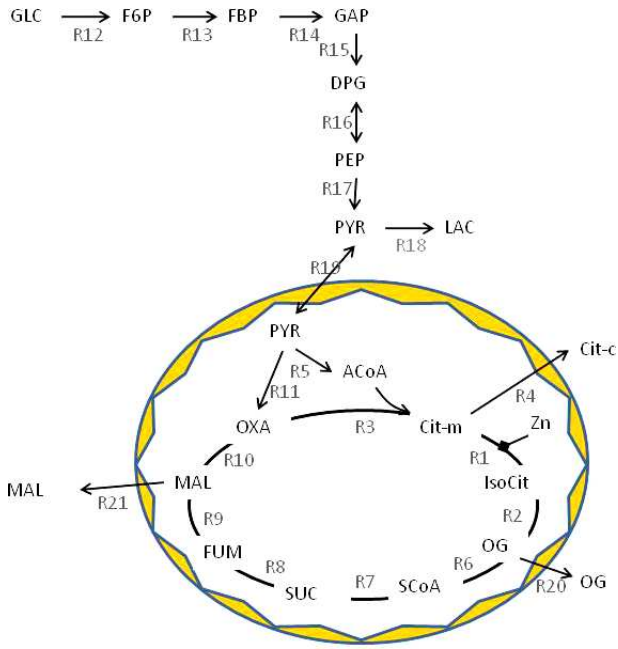


Fig. 1. Schematic representation of pathways included in the model. Individual reactions are labeled R1 to R21. Details of individual reactions, i.e. reaction kinetics as well as kinetic parameters and full names of metabolites, are provided in Supplementary Material.

The simulation is performed by solving the differential equation:

$$\frac{d[m_i]}{dt} = f([m_i], t), m_{t=0} = m_0.$$

Reaction rate equations as well as initial concentration for metabolites, m_0, and kinetic parameters are primarily obtained from the literature[16,18] and are listed in Supplementary Material. The concentrations of glucose and zinc are set to be constant in each calculation. Two compartments included in the model are the mitochondrial matrix and the cytoplasm surrounding the mitochondrion. Pathway Hunter Tool[19] was used for metabolite choke point analysis. Choke points are defined as biochemically essential points in the network and thus it is crucially important to include them in the network model.

The major addition to this model is the inclusion and the analysis of the effect of zinc on the oxidation of citrate to isocitrate (reaction R1 in Fig. 1) through inhibition of the enzyme m-aconitase which is catalyzing this process. Three different types of inhibition were tested.

First, we considered direct reduction of m-aconitase concentration through inhibition of gene expression. In this approach we used previously suggested rate equation for citrate $\rightleftharpoons$ isocitrate reaction[18] with changing concentration of the

enzyme ACO:

$$v = \frac{\text{ACO} \times (k_{cf} k_p [Cit] - k_{cr} k_s [Iso\ Cit])}{(k_s [Iso\ Cit] + k_p [Cit] + k_s k_p)}, \tag{2}$$

where $k_{cf} = 20.47, k_p = 1.1\text{e}{-4}, k_{cr} = 31.44$ and $k_s = 5\text{e}{-4}$ are previously determined kinetic parameters.[16,18] ACO2 is the concentration of m-aconitase estimated in the literature to 3.86e−4 mM. Various microarray data show only minor changes in ACO2 gene expression across different tissues. The prostate normal and cancer measurements provided by Singh *et al.*[20] include measurement of expression level for ACO2 gene. Although there is variation of expression of this gene across different prostate normal and cancer samples, there is an overall trend of higher expression in tumour samples. The overall fold change between average prostate tumour and normal expression levels in this dataset is 1.22. Therefore in the model we have tested the effect of ACO2 concentration change of 1.22 fold as is observed in prostate tumours. The effect of large, 10 times increase and decrease of expression of ACO2 is also investigated and presented.

Second, we tested a general competitive inhibition model at the point of enzyme–substrate binding. The equation for competitive inhibition is derived from the reaction path:

$$\text{E} + \text{S} + \text{I} \rightleftharpoons \text{EI} + \text{S} \rightleftharpoons \text{ES} + \text{I} \rightarrow \text{E} + \text{P} + \text{I}.$$

The kinetic equation can be derived following the steady state assumption for the concentration of each enzyme species, E, EI, ES, and is:

$$v = \frac{v_{\max} \times [Cit]}{[Cit] + k_m (1 + \frac{[Zn]}{k_i})}, \tag{3}$$

where $V_{\max} = 1\text{e}{-6}; K_m = 5\text{e}{-5}; K_i = 7\text{e}{-6}$ are the parameters calculated from experimental results.[11] In this model we have tested different concentrations of zinc, $[Zn]$, within the range that was previously obtained experimentally for different cell types. The largest concentration observed in prostate normal cells was approximately 1 mM.

Finally, we tested the uncompetitive inhibition. The kinetic equation for this type of inhibition is derived from the reaction path:

$$\begin{array}{c} \text{E} + \text{S} + \text{I} \rightleftharpoons \text{ES} + \text{I} \rightleftharpoons \text{EP} + \text{I} \rightarrow \text{E} + \text{P} + \text{I} \\ \Updownarrow \\ \text{ESI} \end{array}$$

This inhibition route is often utilized in the presence of a large concentration of substrate as is the case in prostate cells. The kinetic equation derived from this model of inhibition is:

$$v = \frac{v_{\max} \times [Cit]}{k_m + [Cit](1 + \frac{[Zn]}{k_i})}. \tag{4}$$

The values of kinetic parameters were the same as in the competitive inhibition model.

All models were implemented, simulated and analyzed using Matlab (The Matworks, Natick, Massachusets, USA) computing environment. All calculations were performed on desktop PC. Differential equation calculations were performed using Ode15s solver.

3. Results

The model provides time variation information for 38 metabolites in addition to zinc. Previous experimental measurements were primarily focused on the concentration measurements for citrate, isocitrate and lactate. The model determined variations in the concentration of these metabolites and the results are shown in Figs. 2–4. Figure 2 shows the variation and the final concentrations (long-term limit at 10,000s simulation time) for mitochondrial and cytoplasm citrate (Cit-m

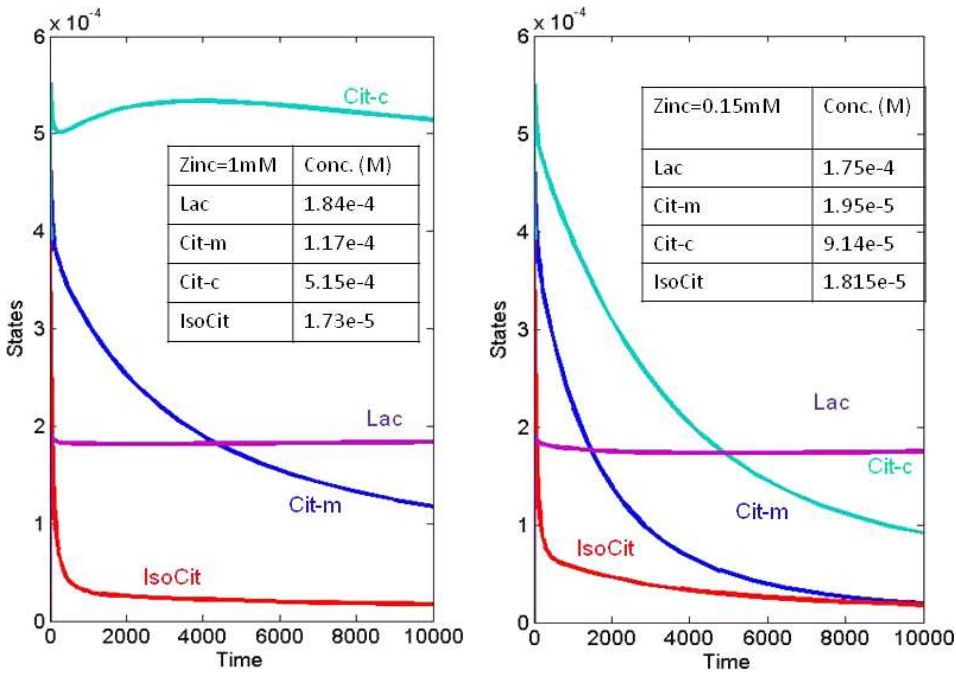


Fig. 2. Concentration changes in citrate, isocitrate and lactate at two different concentrations of Zn in competitive inhibition model. (a) High zinc concentration (1 mM) — normal prostate cell, and (b) low zinc concentration (0.15 mM) — prostate cancer cell. Tables in (a) and (b) provide the concentrations for zinc used in the model as well as final, long-term limit, concentrations for lactate (Lac), mitochondrial citrate (Cit-m), cytoplasmic citrate (Cit-c) and IsoCitrate (IsoCit).

and Cit-c), lactate and isocitrate for two different concentrations of zinc using the competitive inhibition model for the effect of zinc.

The rate constants for the reactions IsoCitrate → Oxoglutarate (R2, Fig. 1) and Pyruvate → Lactate (R8, Fig. 1) were optimized to give, for low concentrations of zinc, better agreement with experimental measurements, i.e. Citrate/IsoCitrate ratio of approximately 10 and comparable concentrations of Lactic acid and Citrate.[5,11]

The effect of zinc on citrate, isocitrate and lactate concentrations with the uncompetitive inhibition model is presented in Fig. 3.

Finally, Fig. 4 shows the same concentration changes of the metabolites as in Figs. 3 and 4 at different concentrations of m-aconitase following the model described in Eq. (2). In this model the effect of zinc is assumed to be on the expression of m-aconitase gene rather than directly on m-aconitase enzymatic function. We have used the originally proposed model for Krebs cycle.[16] The concentration of m-aconitase in this model was 3.86e−4 mM. From microarray measurements[20] we

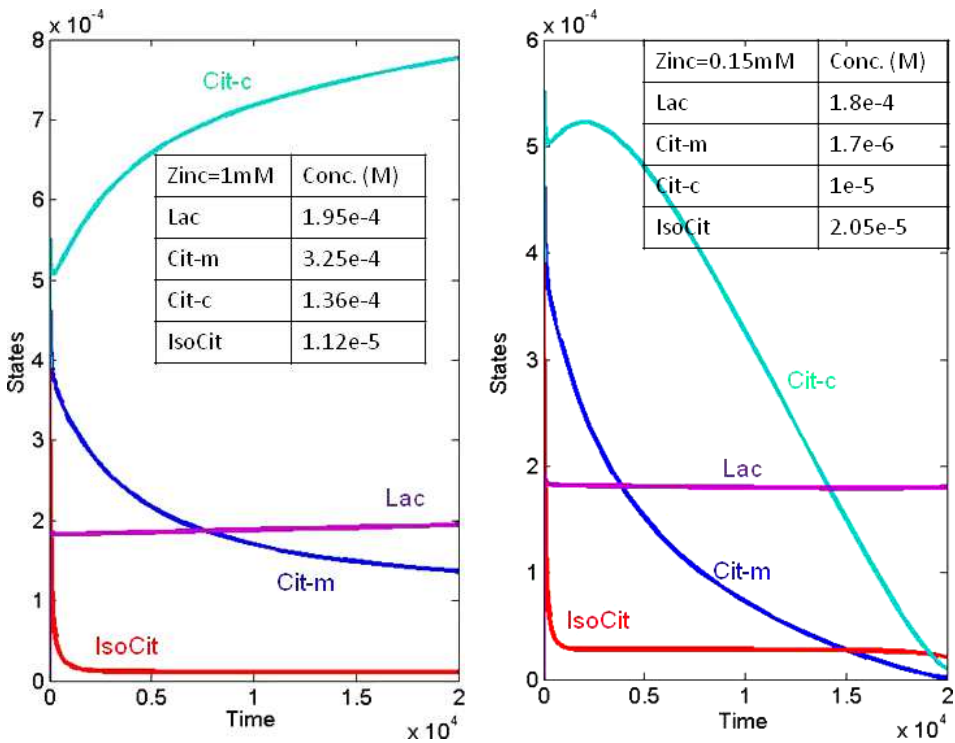
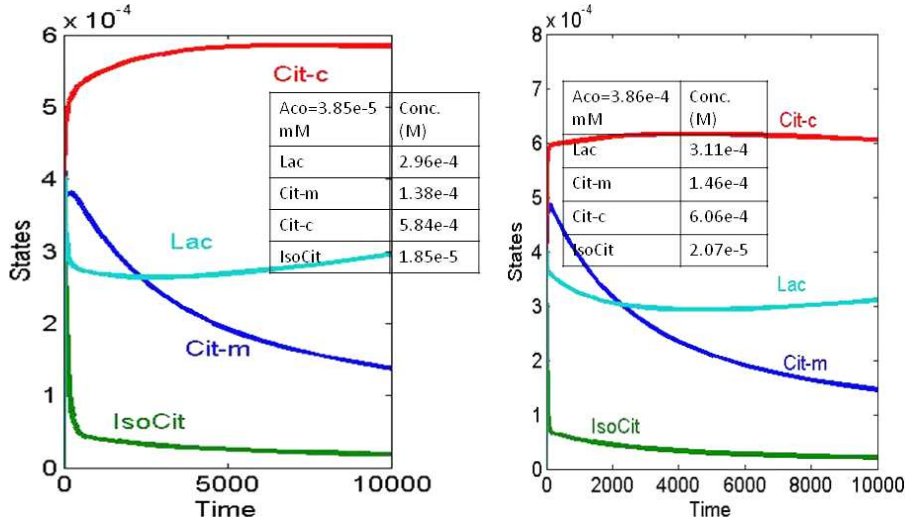


Fig. 3. Concentration changes in citrate, isocitrate and lactate at two different concentrations of zinc. (a) High zinc concentration (1 mM) — normal prostate cell, and (b) low zinc concentration (0.15 mM) — prostate cancer cell. The inhibition of m-aconitase with zinc is modeled here as uncompetitive.



(a) (b)

Fig. 4. Concentration changes in citrate, lactate and isocitrate at two different concentrations of m-aconitase. Although only minor expression changes in ACO2 gene are observed in this example, we have modeled metabolite concentration changes at different ACO2 concentrations. (a) 10 fold decreased expression of ACO2; (b) concentration of ACO2 observed in nonprostate cells.

have determined that in normal prostate cells relative to tumour cells there is a 1.22-fold decrease in concentration of m-aconitase mRNA. The effect of this change is negligible on the concentrations of metabolites and even 10-fold decrease in ACO2 enzyme causes only minor concentration change. With the 10-fold concentration reduction, the Citrate/Isocitrate ratio changes from 36 to 39 (Fig. 4).

For the competitive model concentrations were determined for citrate, isocitrate and lactate at 14 different concentrations of zinc within the range of 0 to 1.95 mM. The results are presented in Fig. 5.

4. Discussion

The models presented here demonstrate the effects of the inclusion of one inhibitor of one reaction step on the whole metabolic system. As could be expected, different avenues for inhibition lead to distinct overall effects on the modeled system as well as different concentration changes of specific metabolites. All three kinetic models resulted in a change in citrate concentration following the increase of zinc concentration as well as in the change in the citrate/isocitrate ratio. The comparisons of the citrate/isocitrate ratios obtained from three computational models of inhibition with the experimental results allowed determination of the most probable route for aconitase inhibition.

The published experimental analysis of prostate tissues has shown that zinc (II) ion concentration in prostate cells is 2–20 folds higher than in other cell. At the

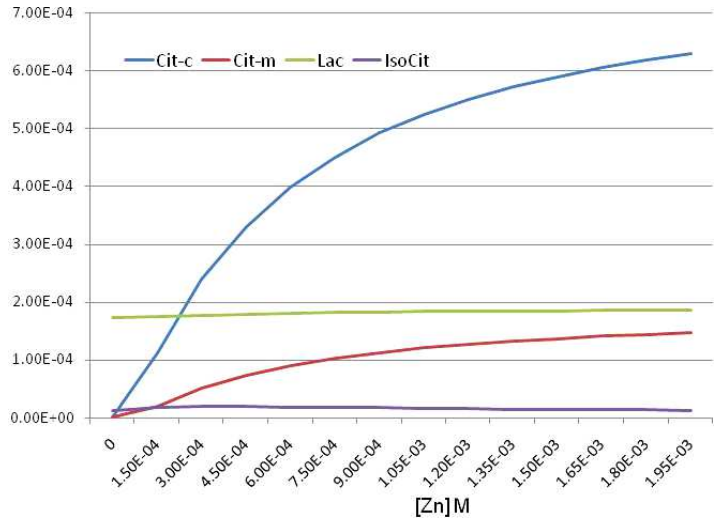


Fig. 5. Variation of metabolites cytoplasmic citrate (Cit-c), mitochondrial citrate (Cit-m), lactate (Lac) and IsoCitrate (IsoCit) with zinc in a competitive inhibition model.

same time the citrate/isocitrate ratio in prostate cells is determined to be of the order of 30/1 in comparison to 10/1 in other cells. The *in vivo* measurements of prostate normal and prostate cancer cells have further shown a significant change in the concentration of citrate and only small change of concentration of lactate in two prostate cell phenotypes.[13] Furthermore, we have determined from microarray analysis of prostate normal and cancer tissues that the concentration of m-aconitase gene changes up to approximately 1.22 times between cancer and normal tissues.[20]

The experimentally observed changes in zinc or aconitase concentrations were included in three different models for aconitase inhibition, namely competitive, uncompetitive and gene expression inhibition. Kinetic parameters for reactions of isocitrate and lactate (reactions R2 and R18 in Fig. 1) were optimized from the values published for *Homo sapiens* cell lines in order to get experimentally observed values for the citrate-to-isocitrate ratio. Competitive inhibition model led to a change in citrate/isocitrate ratio from 36 for a zinc concentration of 1 mM to ratio of approximately 6 for an approximately 7-fold reduction in zinc concentration. These values correspond very well with the experimentally determined citrate/isocitrate ratio level changes with variation of zinc concentration.[5,11] Additionally, the competitive inhibition model shows only minor changes in lactate concentrations. This is once again in agreement with experimental observations obtained by *in vivo* magnetic resonance spectroscopy. In the uncompetitive model, the large concentration of zinc leads to the citrate/isocitrate ratio of 41, which is comparable to the experimentally observed values of about 30. However, the reduction of zinc concentration leads to the dramatic increase of citrate oxidation. The 7-fold decrease

in zinc concentration leads to the citrate/isocitrate ratio of 0.57 which is significantly lower than the ratio of about 10 that was observed in prostate cancer as well as non-prostate normal cells. The method for zinc inhibition of citrate oxidation proposed by Tsui[10] through the effect on the m-aconitase gene expression was also explored. In this model the reaction kinetic for citrate oxidation previously utilized for other cell types[16,18] is employed and the effect of zinc is explored by changing concentrations of the enzyme. The initial concentration of m-aconitase of 3.86e−4 mM was previously published for normal, nonprostate cells that have low zinc ion concentrations. In this case the citrate-to-isocitrate ratio at low concentrations of zinc is approximately 36 — significantly higher than ratio measured in the conditions of low concentration of zinc. The concentration of m-aconitase is lower in normal prostate cells but is only 1.22 times lower as determined from microarray data. This small change in m-aconitase in the model does not lead to any significant change in the citrate-to-isocitrate ration. In fact, even the tenfold reduction of m-aconitase concentration leads to the citrate-to-isocitrate ratio of 39 which is once again higher than observed in normal prostate cells and only insignificantly different than the ratio determined for higher concentrations of ACO2. The exact citrate/isocitrate ratio for high or low levels of ACO2 can possibly be obtained by optimization of kinetic parameters, in order to fit the experimental values better. However, the insensitivity of this model to large concentration changes of ACO2, in fact much larger concentration changes than observed experimentally, shows the inability of this inhibition route to explain the effect of zinc (II) on Krebs cycle.

From these simulations, it can be suggested that the competitive inhibition model mirrors the behavior observed experimentally in terms of relative citrate-to-isocitrate concentration as well as concentration changes for different metabolites. The dependence of citrate, lactate and isocitrate on zinc, as obtained from the model with competitive inhibition (Fig. 5), matches very well the values observed experimentally. Absolute value differences between this simulation and the experimental results are expected and clearly show that further optimization of parameters is necessary through a combination of computational and focused experimental measurements.

There is a growing understanding of the relevance of metabolism in cancer development.[22] The glycolysis-citrate-lipogenesis pathway is understood to be a major source of synthetic and bioenergetic requirements that are essential for growth and proliferation of tumour cells.[23] In the case of prostate tissue and prostate cancer development, specific changes to the glycolysis and tricarboxyl acid (TCA) cycle are crucially important.[9] Previous analyses of glycolysis and TCA pathways resulted in highly detailed models.[16,18,25,26] However, the effect of zinc inhibition on one of the TCA cycle enzymes was never included in computational models. The inhibition of m-aconitase by high concentration of zinc is a major feature in normal prostate cells. In cancer cells, zinc concentration is reduced to the level of non-prostate cells and the m-acotinase function is restored. The presented model of TCA cycle and glycolysis pathway which includes competitive inhibition of

zinc (II) ion on m-aconitase simulates the behavior of prostate and normal cells correctly.

5. Conclusions and Future Prospects

This study compares three different routes for Krebs cycle inhibition by zinc ion. The results obtained from these models indicate competitive inhibition as the only possible route for Krebs cycle inhibition through the inhibition of m-aconitase. The accumulation of citrate caused by the inhibition of m-aconitase is one of the major features of normal prostate cells function. At the same time, one of the characteristics of prostate cancer cells is the reduction in the concentration of citrate. Therefore, detailed description of Krebs cycle including this major regulatory mechanism is essential for understanding of the metabolism of prostate normal as well as cancer cells. The determination of the type of enzyme inhibition caused by high concentration of zinc ion allows further molecular mechanics analysis of the interaction between zinc ions and m-aconitase. Future work of molecular models will allow development of specific m-aconitase inhibitors. In addition, the study presented here shows the importance of inclusion of the effect of metal ions on enzymes in pathway and network models, as without this step it would not have been possible to get a true presentation of cellular metabolism.

Zinc ions are known to have regulatory effects on other targets in metabolism and energy production pathways as well as in other cellular process.[24] At the same time other factors, such as other metal ions and small molecules, are likely to have a regulatory role on the metabolic processes. Therefore, a complete prediction of concentration changes of metabolites requires testing of the inhibitory effect of zinc ion on other reactions as well as careful consideration of the effects of other major regulators. Future work will aim to expand the system in order to include other related pathways and interactions. In addition, we will work on improving the accuracy of the model by focused experimental measurement of metabolite concentrations and kinetic factors in the prostate cellular system.

Acknowledgment

The author would like to thank Dr. R. B. Franklin from University of Maryland for providing kinetic parameter information.

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