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SYSTEMS BIOLOGY FROM MICROORGANISMS TO HUMAN METABOLIC DISEASES – THE ROLE OF DETAILED KINETIC MODELS

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Abstract

Human metabolic diseases are typically network diseases. This holds not only for multifactorial diseases, such as metabolic syndrome or type II diabetes: even when a single gene defect is the primary cause, the adaptive response of the entire network determines the severity of disease. The latter may differ between individuals carrying the same mutation. Understanding the adaptive responses of human metabolism naturally requires a systems biology approach.

Modelling of metabolic pathways in micro-organisms and some mammalian tissues has yielded many insights - qualitative as well as quantitative - into their control and regulation. Yet, even for a well-known pathway like glycolysis precise predictions of metabolite dynamics from experimentally determined enzyme-kinetics have been only moderately successful.

Here, we compare kinetic models of glycolysis in three cell types (African trypanosomes, yeast and skeletal muscle), evaluate their predictive power and identify limitations in our understanding. While each of these models has its own merits and shortcomings, they also share common features. For example, in each case independently measured enzyme kinetic parameters were used as input. Based on these ‘lessons from glycolysis’, we will discuss how to make best use of kinetic computer models to advance our understanding of human metabolic diseases.

Introduction

Recently there has been a growing interest to apply systems biology approaches and, to a lesser extent, kinetic modelling to understand human metabolic diseases [1-3]. Clearly, progress in multifactorial metabolic diseases, like type 2 diabetes, requires an understanding of the control and regulation of the underlying metabolic networks. Besides multifactorial diseases, also single-enzyme deficiencies should be considered as network diseases, as their effects may spread through the entire metabolic network, leading to global adaptations, often distant from the mutated enzyme. For instance, a deficiency of glucose 6-phosphatase, causing glycogen storage disease type I (GSD-I), has pronounced effects on lipid metabolism. Conversely, a deficiency of medium-chain-acyl-CoA dehydrogenase, which is

primarily a fatty-acid-oxidation deficiency, may cause severe problems in blood-glucose homeostasis. In these and many other examples the cross-talk between fat and carbohydrate metabolism is ill understood [4-7]. It is in such cases not self-evident that treatment of the disease should be close to the mutated enzyme, as the network has adapted.

Detailed kinetic modelling of biochemical pathways can yield profound insights into the functioning of metabolic pathways and elucidate how their properties arise from a network of simple biochemical interactions, such as enzyme regulation by reactants and effectors [8]. Metabolic computer models exist in many flavours, ranging from very detailed to extremely simplified descriptions, while some consider small pathways and others genome-scale networks. The purpose of the model, or the scientific question to be answered with the model, largely determines the scope and level of detail of the model.

If the scientific question is related to the precise manner in which known - *i.e.* independently measured - biochemical properties of enzymes give rise to pathway properties, the so-called Silicon Cell approach is a way to go [9]. Such models are based on mechanistic, *in vitro* determined rate equations of enzymes. The purpose of Silicon Cell models is to understand the functional properties of metabolic networks based on the kinetics of the underlying biochemical reactions. To this end, a pure Silicon Cell model does not contain parameters that are fitted from the dynamics of metabolites or fluxes in the cell. Rather, qualitative or quantitative discrepancies between model and experiment will inspire to search for the missing biochemistry required to explain the observed pathway behaviour [10]. The approach may seem idealistic in view of the unprecedented complexity found in metabolic networks (not even taking into account other levels of cellular regulation). Yet, it does provide a mechanistically and biochemically solid methodology through which two levels of complexity can be quantitatively related to each other – the molecular interactions at the lower level and the cell physiology at the higher level. Despite the current impossibility to do this at genome scales, detailed models for subsystems will be instrumental in understanding and predicting the effects of pharmaceutical compounds, which often act on specific enzymes or sets of enzymes in a metabolic network.

In applied settings, such as biotechnology or medicine, it is often not the biochemical basis of a molecular network phenomenon that is being studied. Then, Silicon Cell models are often viewed as being too detailed and too laborious to develop. Accordingly, more coarse-grained models are being made that suit the question being asked. Even then, however, some molecular phenomena may need to be described at great detail. This is almost inevitable, since essentially all perturbations made to cells are by way of molecular interventions that affect the kinetics of specific molecular processes. Predicting the outcome of those interventions, even semi-quantitatively, will require some molecular detail at the perturbation site in the network.

In this paper we will review three detailed models of glycolysis: in (1) African trypanosomes, (2) yeast and (3) skeletal muscle. Our aim is to evaluate (i) how successful detailed kinetic modelling really is and (ii) if and how it may contribute to the understanding of the large networks implicated in human metabolic diseases.

Modelling glycolysis

Glycolysis is one of the most extensively modelled metabolic pathways. Models of various degrees of complexity have been constructed to explain the different modes in which glycolysis can work. Besides reaching a stable steady state [10-13], glycolysis can enter into sustained limit-cycle oscillations [14, 15] or into a very special ‘turbo’ state [16]. The ‘turbo’ state stems from the autocatalytic design of glycolysis in which 2 molecules of ATP are invested in the first kinase reactions (hexokinase and phosphofructokinase) before there

is a gain of 4 ATP per glucose in the reactions catalysed by phosphoglycerate kinase and pyruvate kinase. If hexokinase is not inhibited – directly or indirectly – by its product glucose 6-phosphate, there is a risk of excessive accumulation of hexose phosphates due to a persistent unbalance between the rates of upper and lower glycolysis. This state has long been ill understood. One might expect that the unbalance should vanish by itself, once the ATP has dropped to a very low level and the high rates of hexokinase and phosphofructokinase cannot be sustained. With a simple model it could be shown, however, that ATP and ADP can enter into a stable steady state, while the production of hexose phosphates is higher than its consumption [16]. When trapped in a stable steady state, the adenine nucleotide cannot adjust the upper and lower part of glycolysis to each other anymore.

Reviewing all models of glycolysis, or even the subset of detailed biochemical models, is beyond the scope of this paper. We limit ourselves to three detailed models of yeast, African trypanosomes and human skeletal muscle, which we have (co-)developed ourselves.

Trypanosome glycolysis

After the classical model of erythrocyte glycolysis [13], which at hindsight was far ahead of its time, the detailed model of trypanosome glycolysis was the next to appear [11]. Glycolysis in the African parasite is special in a number of ways. The first part of the pathway – from hexokinase to phosphoglycerate kinase – is compartmentalized in a peroxisome-like organelle, called the glycosome. Within the glycosome there is an ATP and NADH balance (Fig. 1) and no net ATP synthesis occurs in the glycosome. Furthermore, the extensive allosteric regulation of hexokinase and phosphofructokinase, well known from other organisms, is not found in the trypanosome enzymes. In the differentiation state of the parasite which is dominant in mammalian blood, glycolysis has only a single branch. It involves the production of glycerol and closes the redox balance under anaerobic conditions. A growing cell population converts virtually all consumed glucose to pyruvate, leaving a quantitatively negligible role for branches to biosynthetic pathways [17]. At the time of its first development, the model profited from a unique dataset, since almost all the enzymes of trypanosome glycolysis had been purified and characterized kinetically in both directions, mostly in a single laboratory under identical assay conditions. Since then, the model has had two major updates [18, 19] in which (i) newly characterized enzyme kinetics were implemented and (ii) the enzyme expression levels (the V_{max} values) were adapted from the state of the parasite isolated from rat blood to that of a bloodstream form trypanosome cultivated in liquid medium. The latter cultivation method has become the standard for physiological experiments.

The model of trypanosome glycolysis could predict two important biological phenomena prior to their experimental validation. The first was the high, but not exclusive, flux control exerted by the glucose transporter [20, 21]. This ‘control by supply’ differs from the situation in erythrocytes, where glycolysis is controlled by the demand for ATP [13]. Until then, it had been assumed that glucose transport would be the rate-limiting step of glycolysis. The model however, predicted that the glucose transporter would share its high flux control with other enzymes [20], consistent with an experimental determination of its flux control of 40% [21]. The second phenomenon predicted by the model [22] and experimentally validated much later [23] was that the glycosomal compartmentation protected trypanosome glycolysis from entering into a turbo state. Since trypanosome hexokinase is insensitive to its product glucose 6-phosphate and not otherwise regulated allosterically, the pathway should be prone to entering into a state in which hexokinase works faster than the downstream enzymes. Due to the glycosomal compartmentation, however, the positive feedback by the produced ATP is absent as well (Fig. 1), rendering the

pathway resistant against the turbo state. Although it may be unlikely that this protection is at the evolutionary origin of the glycosome [24], the compartmentation of the pathway made extensive allosteric regulation of upper glycolysis unnecessary [23].

Qualitatively the model of trypanosome glycolysis can explain a number of observed biological phenomena (see also [11]). When we carefully compare the model outcome *quantitatively* to experiments, the first version of the model approximated the measured steady-state metabolite concentrations within a factor of 2. This is quite acceptable for a model based on an independent set of *in vitro* parameters that were not fitted to a desired outcome. However, dynamic metabolite measurements that challenge the model more stringently, have been lacking so far. This is due to the glycosomal compartmentation of the pathway as well as to the small amount of cell material available from *in vitro* cultivation. With improved metabolomics tools, however, it is now possible to obtain a precise, quantitative picture of metabolite dynamics in trypanosomes [25]. Such an endeavour is part of the SilicoTryp project which has just started [26].

The overall ambition of the SilicoTryp project is to extend the existing glycolysis model with redox metabolism and the associated signal transduction and regulation of gene expression. Since some 98 % of the glucose flux runs via glycolysis in growing trypanosomes, one would anticipate that adding a small branch to the model should not affect the behaviour of glycolysis in the model drastically. This assumption turns out to be false. Initial attempts to link the oxidative part of the pentose-phosphate pathway to the model failed. This is due to a very special – and possibly artificial – moiety conservation of metabolites with bound phosphates in the glycosome [20]. The oxidative pentose-phosphate pathway drains glucose 6-phosphate from this conserved moiety, without adding anything back. Therefore, the extended model cannot reach a steady state, even if the activity of the pentose-phosphate pathway is very low. Further research has identified a number of ways in which trypanosomes may circumvent this problem (M. Trybilo, manuscript in preparation). Which of these is most plausible, is subject to further experimentation.

Yeast glycolysis

Detailed models of yeast glycolysis, based on enzyme kinetic equations (*e.g.* [10, 14, 27]), have been developed for various purposes. The purpose of the model developed by Teusink and colleagues a decade ago [10] was explicitly to test to which extent *in vitro* enzyme kinetics can predict metabolite concentrations and fluxes *in vivo*. The model covers the anaerobic glycolysis of the yeast *Saccharomyces cerevisiae*, including ethanol formation and the branches to glycogen, trehalose, glycerol and succinate (Fig. 2). Like in the case of trypanosome glycolysis, a coherent set of enzyme kinetic parameters, mostly determined under a single assay condition, was used. Also this model predicted most of the metabolite concentrations within a factor of two. The only exception was pyruvate, for which the model prediction was a factor of five higher than the measured concentration. When the model was subjected to stricter test, however, it turned out that the rates of half of the enzymes could not be predicted reliably. If the experimentally determined metabolite concentrations were substituted into the individual rate equations, the rates of half of the enzymes deviated more than a factor of 2 (in either direction) from their measured rates *in vivo*. The most extreme example was pyruvate decarboxylase, for which the predicted rate was a factor of 6 too low. Specific attention was paid to the reaction catalysed by glyceraldehyde-3-phosphate dehydrogenase. Insufficient parameters were available under physiological conditions, while at the same time the reaction has a low equilibrium constant and may be considered to be a 'thermodynamic hurdle' in glycolysis.

The Teusink model has been used widely to test the performance of various computational algorithms [28-30, 31, 32] and - in a slightly modified form - to study principles underlying metabolic oscillations [33]. It has, however, hardly been used as a starting point for further modelling and exploration of yeast glycolysis under different physiological conditions [34]. This is not too surprising, since the model tends to become readily unstable under other conditions than those for which it had been constructed. One reason is trivial: some branching fluxes have been fixed and will continue to run when their substrate has dropped below zero. This can be easily repaired, either by making the branches sensitive to their substrate concentration, or by fixing them at a new rate measured under the condition of interest [33, 35]. The second reason is more fundamental and was already recognized by Teusink *et al.* [10]. As the original model does not contain the negative feedback on hexokinase by trehalose 6-phosphate, it is prone to develop a turbo phenotype [16].

Recently, we have updated the model to describe the fermentative capacity of bakers' yeast upon nitrogen starvation. Fermentative capacity is the production of carbon dioxide upon transfer of the yeast to a sugar-rich, anaerobic environment. It is a measure of the performance of the yeast in dough. Since the anaerobic carbon-dioxide production coincides largely with ethanol production from anaerobic glycolysis, the latter is usually monitored. The most important changes to the model were: (i) to include the positive feedforward regulation of pyruvate kinase by fructose 1,6-bisphosphate [27]; (ii) to include the negative feedback regulation of hexokinase by trehalose 6-phosphate [16]; (iii) to measure the V_{max} values for the model under conditions that resemble the environment of the yeast cytosol [36]; (iv) to redetermine the kinetic parameters of glyceraldehyde-3-phosphate dehydrogenase under the same *in-vivo*-like conditions as the V_{max} values. Under four different conditions (two specific growth rates, with and without subsequent nitrogen starvation), these modifications were absolutely essential to reach a steady state (K. van Eunen, unpublished). Under one condition (high specific growth rate, non-starved) the concentrations and fluxes predicted by the model agreed reasonably well with the measurements [35]. Under the other three conditions subtle fitting of the kinetics of glyceraldehyde-3-phosphate dehydrogenase and hexokinase was sufficient for the model to approach the experimental results. All these modifications relate to different extents to the delicate balance between upper and lower glycolysis.

We learnt two important lessons from this study. First, the use of *in-vivo*-like assay conditions to measure enzyme activities [36] was crucial to obtain a realistic model output. Second, the allosteric regulation loops are of key importance. Obviously, the latter should not be surprising. Yet, one can easily become tempted to omit some of the allosteric regulators, as this simplifies the model. Moreover, enzymes are often insensitive to their regulators when they are added to *in vitro* assays at the concentrations that are found *in vivo*. When fitting simplified (lin-log) models over a large dynamic range, however, the need for allosteric regulation becomes immediately apparent [37]. It will be interesting to redetermine the whole set of kinetic parameters for the allosterically regulated enzymes under *in-vivo*-like assay conditions.

Skeletal-muscle glycolysis

Energy balance in fast-twitch skeletal muscle cells (FT myofibers) depends heavily on a high activity of glycolysis, starting from the breakdown of stored glycogen. In contrast to oxidative slow-twitch myofibers, FT myofibers convert a large part of the produced pyruvate anaerobically into lactate. A particular challenge is the exceptionally large dynamic range of ATP turnover between the resting and maximally active state in this particular myofiber type (100-fold [38] in comparison to some 10-fold in yeast [39]). Moreover, neurally controlled muscle activity - and thereby ATP turnover - can switch between rest

and maximal activity in a period of seconds. Both of these special features pose a formidable challenge to metabolic regulation.

Lambeth and Kushmerick [12] developed a kinetic model of glycogenolysis in mammalian FT skeletal muscle (Fig. 3). They parametrized the model using *in vitro* enzyme kinetic parameters taken from various mammalian species. The model was partly validated on the basis of measurements in murine FT muscle [12]. Recently, this model was used by Schmitz *et al.* to uncover the sites of regulation that cause the rapid inactivation of glycolysis immediately after exercise in human skeletal muscle [40]. Unlike in the previous two glycolysis models, here the impact of biological and statistical variation of the parameters was explicitly taken into account via a Monte Carlo approach.

Schmitz *et al.* measured the concentrations of ATP, phosphocreatine, inorganic phosphate and hexose monophosphates in human upper leg muscle at rest, during incremental exercise to exhaustion and recovery by *in vivo* ^{31}P -NMR [40]. The concentration of hexose monophosphates increased during maximal exercise and attained a peak concentration of 8 mM within 60 s after exercise was halted [40]. When the original Lambeth and Kushmerick model was used to simulate the subsequent recovery phase, the modelled concentration of glucose 6-phosphate decreased 100-fold more rapidly than observed in reality. At the same time, the resting-state fructose 1,6-bisphosphate was dramatically underestimated by the model as compared to muscle biopsy data [40]. Metabolic Control Analysis identified phosphofructokinase and pyruvate kinase as the key sites of metabolic control. The only way to reproduce the measured dynamics of glycolytic intermediates was by inactivating both enzymes simultaneously upon entry into the recovery phase. Interestingly, the biochemistry underlying this rapid inactivation is not known. It has, however, been found previously that reversible and pH-dependent association of phosphofructokinase to the cytoskeleton in vertebrate skeletal muscle results in a large change of enzyme activity [41]. In addition, allosteric regulation of pyruvate kinase by fructose 1,6-bisphosphate may be involved [40]. Although the pyruvate kinase isoform that prevails in skeletal muscle is insensitive to fructose 1,6-bisphosphate, it becomes sensitive by a single point mutation [40]. One may hypothesize that the interaction with other metabolites and / or small ions *in vivo* may restore the sensitivity of the enzyme to fructose 1,6-bisphosphate.

This particular modelling study illustrates three important points. First, it illustrates – together with the other two models above – the limitations of modelling based on *in vitro* kinetics. At the same time, it shows how the use of parameter distributions rather than single values may in part overcome the former limitation. But foremost, it illustrates how modelling may advance our understanding by identifying the enzymes that are the most likely targets for simultaneous inactivation after termination of muscle contraction. The important role of allosteric regulators and the possible discrepancies between *in vitro* and *in vivo* parameters for these regulators, have been discussed above. New elements to the discussion are the intracellular pH and the rapid association – dissociation equilibria of enzymes with other proteins or cell structures that may affect their activity. Rapid dynamics of intracellular pH can be monitored quantitatively by GFP fluorescence [42] and its effect can in principle be implemented into kinetic models. The role of rapid intracellular association and dissociation of enzymes and its effect on the enzyme kinetics, is with the current analytical techniques more difficult to capture with sufficient precision.

The future of kinetic models based on detailed biochemistry

When planning new, large-scale systems biology endeavours to understand metabolic networks and apply this knowledge to understand and combat human metabolic disease, we have to consider how well a detailed kinetic and mechanistic modelling approach (“The

Silicon Cell”) performs in answering our scientific questions as compared to other modelling strategies. Clearly, this particular approach will be the most direct one if we are aiming at a dynamic view on the relation between biochemical kinetics and metabolic function. This is not only scientifically appealing. It will also be instrumental to understand how the effects of pharmaceuticals, which interact with specific enzymes, spread through the metabolic network. Nevertheless, even today, constructing detailed and realistic models is a slow process. It yields small-scale models (typically 20 to 30 reactions), which - when taken out of their original context - do not perform extraordinarily well. How would we be able to improve on this situation?

From the above evaluation of three models of glycolysis, we came to the following conclusions:

1. Realistic kinetic models of biochemical pathways require kinetic parameters that are measured under physiological conditions, rather than under optimized conditions.
2. Allosteric regulation is crucial and must not easily be discarded, even if the *in vitro* parameters may suggest that they are unimportant under physiological conditions.
3. The omission or incorporation of side branches may have a dramatic impact, even if their flux seems almost negligible. We tend to delineate pathways according to their textbook descriptions. And often, parameters are lacking to build large networks at once. An alternative to the current modelling practice in which the network surrounding the pathway of interest is simply omitted, would be to model it with phenomenological kinetics, such as lin-log kinetics [37]. Such hybrid models give surprisingly good predictions [43] and the simplified surrounding network will give the pathways of interest a much more realistic context. Of particular importance is the fact that pathway delineations may introduce artificial moiety conservations, illustrated here for bound phosphate in the trypanosome model, but recently also demonstrated experimentally for the ATP paradox in yeast [44]. The measured drop of total AXP upon glucose addition is impossible in the current versions of glycolysis models in which the sum of ATP, ADP and AMP is conserved. In reality, however, there is a drain of AXP via an AMP shunt into the inosine salvage pathway which occurs at the same timescale as the initial events in glycolysis.
4. Since the dynamics of intracellular pH are experimentally tractable by a variety of techniques, we should consider to incorporate the effects of pH into our models. This is a major effort, obviously, as pH is likely to affect many, if not all, kinetic parameters.
5. Futile cycling has not been discussed in this paper, but it may drastically affect the network behaviour. As it is often experimentally tractable via stable isotope methods [45-48], it will be possible to include it into our models.
6. When all the above has been implemented, the properties of enzymes *in vivo* may still depend on rapid interactions with other macromolecules and/or posttranslational modifications. The current challenge is to obtain the experimental data that are required to incorporate its impact realistically into our models.

This work has by no means been a comprehensive review of glycolysis models, nor of model development methods. The results suggest that the development of realistic and precise kinetic models of small parts of metabolism is almost within reach. However, speeding up the process of model development to meet our needs, will need careful planning and strategic decisions. Eventually, it is always the biological question that should inspire

the type of model to be made. Looking back at the biological insights that were obtained, some indeed required a detailed model (*e.g.* to predict the distribution of flux control in trypanosomes or to pinpoint the enzymes that undergo inactivation in skeletal muscle), while others could have been obtained with a more coarse-grained model (*e.g.* the function of compartmentation in trypanosome glycolysis). The above recommendations may help to avoid false side tracks in research. At the same time, however, to obtain the required experimental data will imply a major effort. We believe therefore, that the key to efficient progress is the development of hybrid models in which different levels of detail are combined. Moreover, in view of the many levels of regulation within the cell as well as in the entire organism, the parallel development of models of widely different levels of complexity will be insightful. Sensitivity analysis of fitted models comprising a larger part of the network, but including less detail, may suggest for which subsets of enzymes the more detailed biochemical approach may be rewarding.

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Figure 1.

A schematic representation of the reactions included in the computer model of glycolysis in *Trypanosoma brucei* [18]. Reaction numbers indicate: 1. glucose transport; 2. hexokinase; 3. phosphoglucose isomerase; 4. phosphofructokinase; 5. aldolase; 6. triose-phosphate isomerase; 7. glyceraldehyde-3-phosphate dehydrogenase; 8. phosphoglycerate kinase; 9. phosphoglycerate mutase; 10. enolase; 11. pyruvate kinase; 12. pyruvate transport; 13. glycerol-3-phosphate dehydrogenase; 14. glycerol-3-phosphate oxidase (a combined process of mitochondrial glycerol-3-phosphate dehydrogenase and trypanosome alternative oxidase); 15. glycerol kinase; 16. combined ATP utilization; 17. glycosomal adenylate kinase; 18. cytosolic adenylate kinase. Question marks indicate uncharacterized transport processes. Abbreviations of metabolite names: Glc-6-P: glucose 6-phosphate; Fru-6-P: fructose 6-

phosphate; Fru-1,6-BP: fructose 1,6-bisphosphate; DHAP: dihydroxyacetone phosphate; Gly-3-P: glycerol 3-phosphate; GA-3-P: glyceraldehyde 3-phosphate; 1,3-BPGA: 1,3-bisphosphoglycerate; 3-PGA: 3-phosphoglycerate; 2-PGA: 2-phosphoglycerate; PEP: phospho-*enol*pyruvate.

Figure 2.

A schematic representation of the reactions included in the computer model of glycolysis in *Saccharomyces cerevisiae* [10]. Reaction numbers and metabolite abbreviations have the same meaning as in Fig. 1. Additional reactions included are pyruvate decarboxylase (19), alcohol dehydrogenase (20) and branches consisting of multiple enzymes towards trehalose (21), glycogen (22), glycerol (23) and succinate (24). AcAld denotes acetaldehyde.

Figure 3.

A schematic representation of the reactions included in the computer model of glycolysis in skeletal muscle in the original version by Lambeth and Kushmerick [12]. Reaction numbers and metabolite abbreviations have the same meaning as in Fig. 1. Additional reactions included are glycogen phosphorylase (25), phosphoglucomutase (26), lactate dehydrogenase (27) and creatine kinase (28).