

STUDYING ENZYME KINETICS BY MEANS OF PROGRESS-CURVE ANALYSIS

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ABSTRACT

Almost all chemical reactions and transport processes in a cell are catalysed by specific enzymes and transport proteins, respectively. Kinetic characterization of these auxiliary proteins is a necessary prerequisite for understanding the dynamics and regulation of cellular reactions networks. Progress-curve analysis, i.e. estimation of kinetic parameters by fitting of integrated rate laws to the time-course of a biochemical reaction, allows an efficient kinetic characterization of enzymes. This article outlines the mathematical fundamentals of progress-curve analysis and provides examples for the application of this method in enzyme kinetics and system biology.

INTRODUCTION

The reaction network of a living cell comprises several thousands of chemical reactions and transport processes, most of them catalysed or facilitated by specific enzymes and transport proteins. On one hand, these auxiliary proteins act as catalysts accelerating the rate of the underlying process. On the other hand, and most importantly, they act as regulators in that their activity can be tuned to allow for an optimal functioning of the whole cellular network. Temporal gene expression, binding of allosteric effectors and reversible enzyme phosphorylation and dephosphorylation are prominent types of enzyme regulation.

Computational systems biology is a rapidly growing field of theoretical research aimed at the establishment of computer models that can be used to simulate the dynamics of the complete cellular reaction network.

In my opinion, this ambitious goal can only be reached if the kinetic properties of the participating enzymes - at least of those enzymes that are mainly involved in the regulation of the network - are known. Thus, there is an urgent need for the systematic kinetic characterization of enzymes belonging to a branch of the cellular network subjected to mathematical modelling.

The conventional method used to set up enzyme-kinetic models is to isolate the respective enzyme from the cell and to determine its kinetic properties monitoring the reaction kinetics under well-controlled *in vitro* conditions. This procedure usually ends up in the formulation of a rate law representing a mathematical expression relating the reaction velocity to the concentration of reactants and other metabolites that may function as effectors (activators or inhibitors) of the enzyme. The so called initial-rate method is usually applied to guess the mathematical structure of the rate law and to estimate numerical values of the kinetic parameters (affinity constants for the various ligands, cooperativity indices, maximal velocities etc.) entering it. The initial-rate method consists in measuring the initial rate of the reaction at various concentrations of all relevant ligands [1]. This method has two basic drawbacks. First, it presumes a steady-state regime of the reaction, i.e. the enzyme activity (maximal rate) is constant over the time period of the measurements. For enzymes exhibiting self-activation or inactivation this condition is obviously not met. Second, the initial-rate method is laborious as it requires a series of parallel assays differing in the initial concentrations of the reactants and effectors. A more elegant method is the so-called progress-curve analysis [2-5]. This method extracts information about the kinetic properties of an enzyme from the full time-course of the reaction - and not only its initial part. From the mathematical viewpoint, progress-curve analysis requires the calculation of the time-dependent solution of the kinetic equations governing any enzyme-catalysed reaction and to fit this solution to observed time-courses by an appropriate choice of the model parameters. Thus, progress-curve analysis combines methods for the solution of differential equation systems with non-linear regression methods. With the exception of the Michaelis-Menten equation, for which an explicit integrated form can be derived (expressing the reaction time through the concentration of the substrate) solution of the kinetic equations for more enzymes with complicated rate laws is only possible by using numerical integration methods. This relatively high mathematical effort may explain why progress-curve analysis so far does not enjoy widespread applications.

Studying Enzyme Kinetics by Means of Progress-Curve Analysis

In the following I will briefly outline the mathematical fundamentals of progress-curve analysis and provide some examples from our recent research work demonstrating the usefulness of this method in reducing the experimental effort and in coping with situations where the initial-rate method cannot be applied.

PROGRESS-CURVE ANALYSIS: MATHEMATICAL BACKGROUND

To illustrate the essence of the progress-curve method let us consider the time-courses of substrate consumption and product formation of a monomolecular reaction $S \rightarrow P$ (see Fig. 1). The initial velocity $v_0 = v(S_0; P_0)$ of the reaction is defined by the slope of the quasi-linear phase of the time-course, expressed as amount of product formed or substrate consumed per time. Determining initial rates by varying the initial concentrations S of the substrate at fixed concentration of the product (or of all other ligands in more complex reactions) one arrives at the so-called rate diagram (or v - S -characteristics) showing the relationship between reaction rate and substrate concentration. Similar rate diagrams can be constructed for the other ligands. These rate diagrams are very helpful for guessing the enzyme mechanism and proposing an appropriate mathematical expression for the underlying rate law.

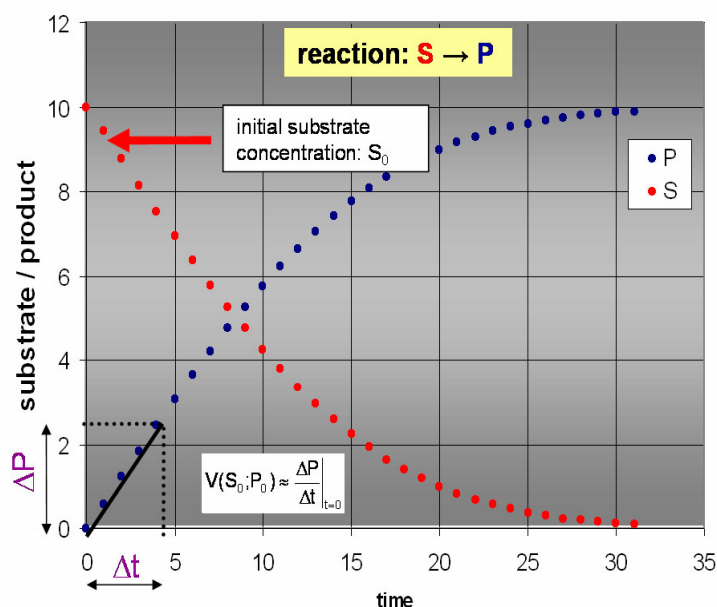


Figure 1. Determination of initial reaction rates. The initial rate is calculated by measuring over a short time period the consumption of substrate (or formation of product) and relating the change of concentration to the time elapsed. Mathematically speaking, this method consists in approximating the slope (= first derivative) of the progress curve at time $t = 0$ by the average slope of the quasi-linear initial part. The initial rate is assigned to the initial concentrations of the ligands.

The construction of rate diagrams does not necessarily require initial-rate measurements. They can be obtained more efficiently by determining reaction velocities from the first derivative of the progress curve. At a given time point of the reaction, the concentrations of the reactants can be assessed from their initial concentrations and the known stoichiometry of the reaction (see Fig. 2).

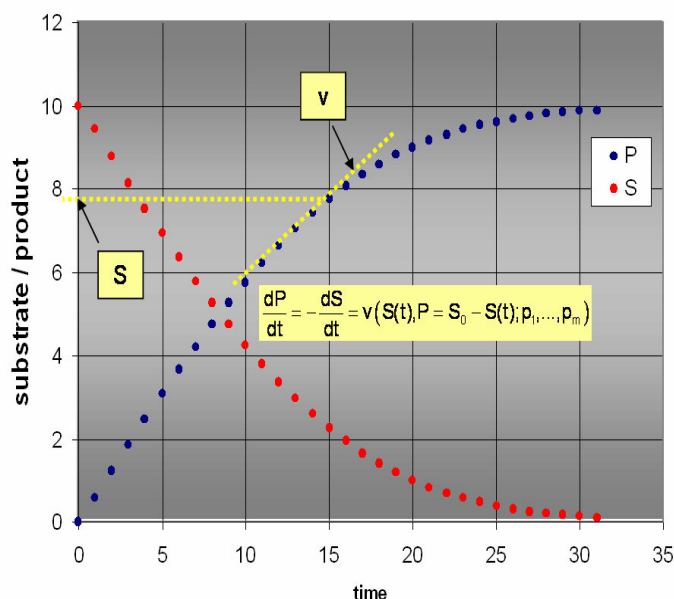


Figure 2. Determination of reaction rates by taking the slope of the progress curve. Reaction rates can be calculated as slopes of the progress curve at any time point of the reaction. The corresponding concentrations of the reactants can be assessed by their initial values and the known stoichiometry of the reaction.

The issue with determining slopes of the progress curve is that the data points typically display some experimental noise which even amplifies in the higher derivatives of the curve (see insert in Fig. 3A). Thus, some smoothing of the progress-curve data is required before taking the first derivative. A multitude of statistical curve-smoothing techniques have been developed [6] all of which are based on a local polynomial approximation of the curve. For the smoothing of polarographically or photometrically monitored progress curves comprising 500 - 5000 data points we have made good experience with the Golay-Savitzky polynomials.

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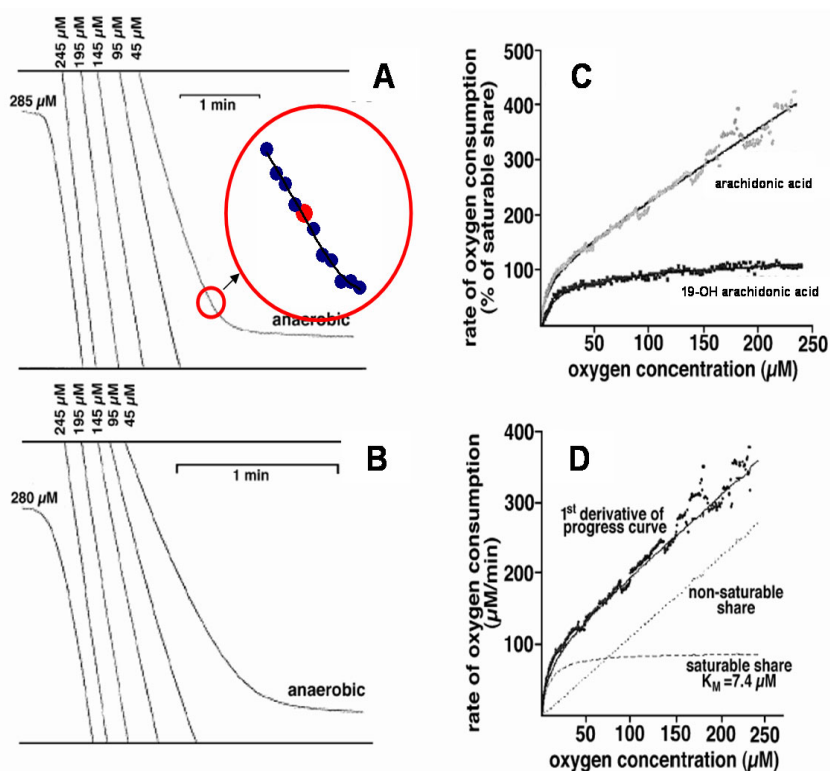


Figure 3. Polarographic progress curves of O_2 -consumption during lipoxygenase reaction. The oxygen concentration during the lipoxygenase-catalyzed dioxygenation of two different fatty acids was monitored by means of a Clark electrode. **Panel A:** dioxygenation of arachidonic acid, **panel B:** dioxygenation of 19-OH arachidonic acid. **Panel C** shows the reaction rates derived from the two progress curves by using a 5th order Golay-Savitzki filtering for the calculation of the first derivatives. **Panel D** illustrates that the rate diagrams in panel C can be well accounted for by splitting the reaction rate into a saturable share (Michaelis-Menten equation) and a non-saturable linear share.

Figures 3A-B depict polarographic progress curves of molecular oxygen monitored in a lipoxygenase assay with two different fatty acid substrates. The red circle enlarges a small part of the progress curve. The solid line represents the slope of the curve calculated by means of a 5th order Golay-Savitzky filter (including 5 data points on both sides of the data point) marked in red. Panels in Fig. 3C, D show the rate diagrams constructed by plotting the slopes of the progress curves versus oxygen concentrations. For both fatty acid substrates the rate exhibits a non-saturable behaviour. As shown in Fig. 3D, the observed rate-versus-oxygen relationships can be well described by a phenomenological rate law consisting of a hyperbolic Michaelis-Menten function and a linear function. One way of determining the unknown kinetic parameters is to fit the empirical rate equation to the velocity data shown in Fig. 3C. However, these numerical estimates may be influenced by the bias of the velocity data that is inevitably associated with the local smoothing of the original progress-curve data.

Hence, a more feasible way to get numerical estimates of the model parameters is to directly fit the solutions of the rate equation to the progress-curve data.

This procedure requires the combination of a numerical integration routine for the solution of differential equations with non-linear regression methods. Figure 4 delineates the typical protocol for progress-curve analysis.

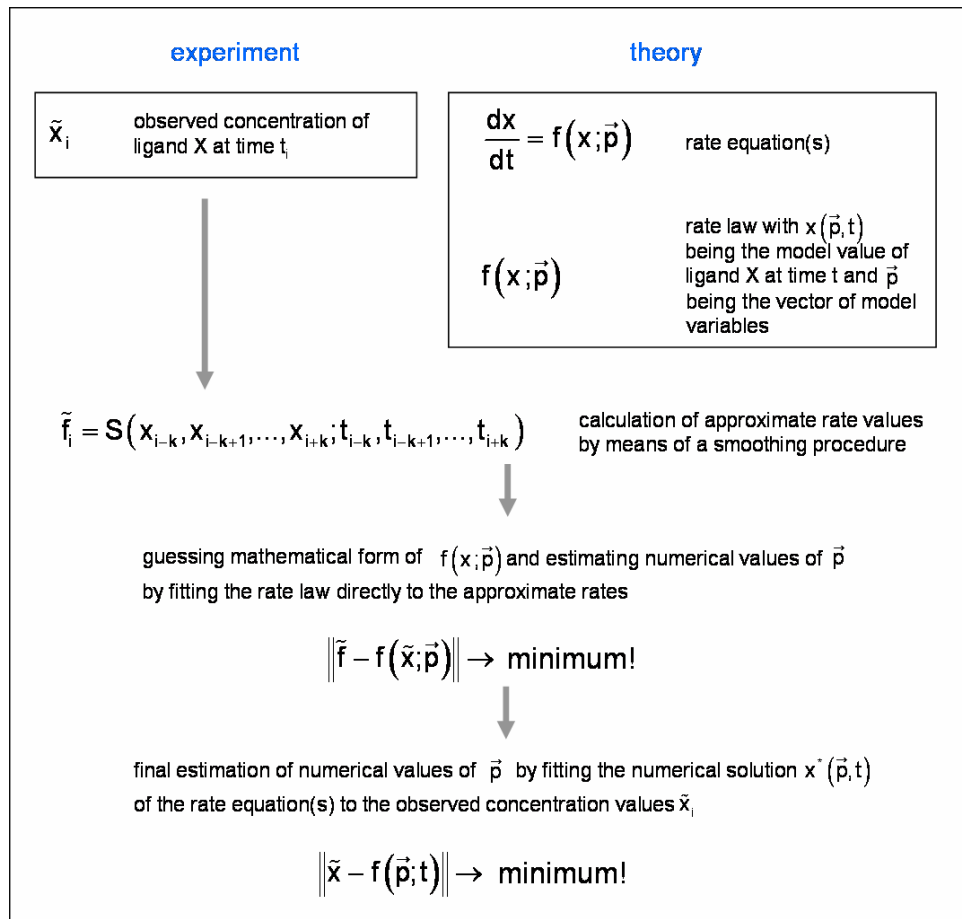


Figure 4. Main steps of progress curve analysis.

PROGRESS-CURVE ANALYSIS OF NON-STATIONARY ENZYME REACTIONS

A striking advantage of progress-curve analysis over the initial-rate method is that it allows the study of enzyme reactions under non-stationary conditions. As an example for such an application, I show here some recent results of kinetic studies with the 15-lipoxygenase (15LOX) from reticulocytes. This enzyme plays an important role in the formation of leucotriens [7].

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Its main function in the living cell seems to be the dioxygenation of arachidonic acid (and of other unsaturated fatty acids) to hydroperoxy products. Intriguingly, the enzyme is also capable of converting the rather unstable hydroperoxy product to a hydroxyl fatty acid. As shown in the reaction scheme (Fig. 5), these two different reactions are bound to different redox states of the catalytic iron atom. Hydrogen abstraction from the fatty acid substrate (with rate k_2) is associated is accompanied by the redox change $\text{Fe}^{(3+)} \rightarrow \text{Fe}^{(2+)}$.

The enzyme-radical intermediate may either react with molecular oxygen under formation of the hydroperoxy product (P) or, alternatively, may leave the oxygenation cycle (marked in yellow in Fig. 5) by decaying into the fatty acid radical and the free $\text{Fe}^{(2+)}$ -enzyme which may re-enter the dioxygenation cycle by transferring one electron to the hydroperoxy product.

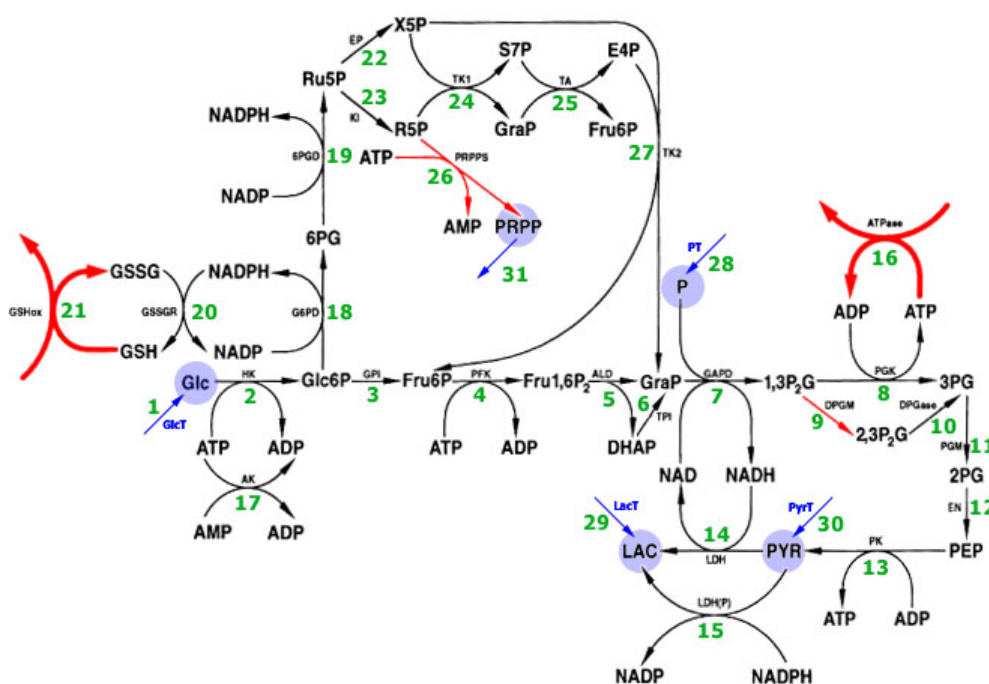


Figure 5. Metabolic reactions in red cells taken into account by the kinetic model [9] used for the analysis of systemic progress curves. *Enzymes:* HK - hexokinase [2.7.1.1]; PGI - phosphohexose isomerase [5.3.1.9]; PFK - phosphofructokinase [2.7.1.11]; ALD - aldolase [4.1.2.13]; TIM - triosephosphate isomerase [5.3.1.1]; GAPD - glyceraldehyde-3-phosphatedehydrogenase [1.2.1.12]; PGK - phosphoglycerate kinase [2.7.2.3]; DPGM - bisphosphoglycerate mutase [5.4.2.4]; DPGase - bisphosphoglycerate phosphatase [3.1.3.13]; PGM - phosphoglycerate mutase [5.4.2.1]; EN - enolase [4.2.1.11]; PK - pyruvate kinase [2.7.1.40]; LDH -lactate dehydrogenase [1.1.1.28]; AK -adenylate kinase [2.7.4.3]; G6PD - glucose 6-phosphate dehydrogenase [1.1.1.49]; 6PGD - phosphogluconate dehydrogenase [1.1.1.44]; GSSGR - glutathione reductase [1.8.1.7]; EP - phosphoribulose epimerase [5.1.3.1]; KI - ribose phosphate isomerase [5.3.1.6]; TK - transketolase [2.2.1.1]; TA - transaldolase [2.2.1.2]; PRPPS - phosphoribosylpyrophosphate synthetase [2.7.6.1].

Recently, we observed an unusual oxygen dependence of the LOX reaction when we used a hydroxylated fatty acid (19-OH arachidonic acid, 19-OH-AA) as substrate (see Fig. 6A). Up to oxygen concentrations of about 550 μM we could not see a saturation level of the initial rates. Moreover, the progress curves clearly showed biphasic behaviour. Our interpretation of the latter feature was that starting the reaction with all enzyme resident in the active $\text{Fe}^{(2+)}$ -form, a time-dependent transition into the inactive $\text{Fe}^{(3+)}$ -form occurs when an equilibrium has been established between inactivation (rate constant k_4) and re-activation (rate constant k_1).

To test this hypothesis and to determine the kinetic parameters of the LOX for this special substrate we applied the kinetic model [8] governing the reaction scheme shown in Fig. 5. Treating the reversible binding of the fatty acid substrate (S) and the hydroperoxy fatty acid product (P) as fast equilibrium reactions one may introduce the equilibrium pools

$$(X_1) = (E) + (ES) + (EP)$$

$$(X_2) = (E^*) + (E^*S) + (E^*P)$$

$$(X_3) = (ES\bullet)$$

which add up to the total enzyme concentration $(X) = (X_1) + (X_2) + (X_3)$.

The kinetic equations read

$$\frac{d(S)}{dt} = \angle f_2(X_2)$$

$$\frac{d(O_2)}{dt} = \angle f_3(X_3)$$

$$\frac{d(P)}{dt} = f_3(X_3) \angle f_1(X_1)$$

$$\frac{d(X_1)}{dt} = f_4(X_3) \angle f_1(X_1)$$

$$\frac{d(X_2)}{dt} = f_1(X_1) + f_3(X_3) \angle f_2(X_2)$$

$$\frac{d(X_3)}{dt} = f_2(X_2) \angle f_3(X_3) \angle f_4(X_3)$$

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where the functions f_1 , f_1^* , f_2 , f_3 and f_4 have the following meaning:

$$f_1 = \frac{k_{1P}(P)}{K_{mP}(1 + (S)/K_{iS}) + (P)} \quad f_2 = \frac{k_2(S)}{K_{mS}(1 + (P)/K_{iP}) + (S)}$$

$$f_3 = k_3(O_2)$$

$$f_4 = k_4$$

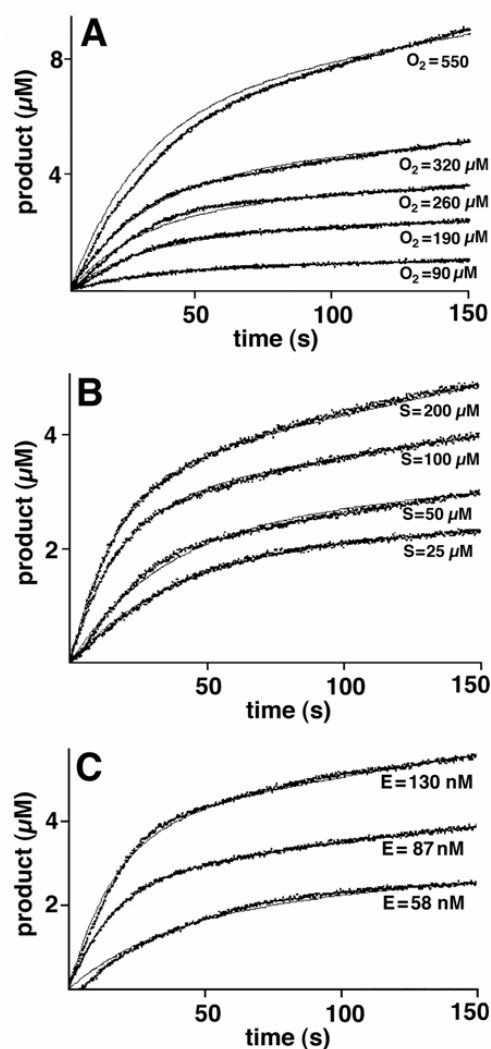


Figure 6. Photometric progress curves of the 15-lipoxygenase reaction using 19-OH-arachidonic acid as substrate. Bold (discontinuous) curves = experimental data. Thin (continuous) curves = model simulations.

The numerical values of the kinetic parameters entering the kinetic equations are shown in Table 1. They were obtained by progress-curve analysis, i.e. fitting the numerical solutions of the above equation system to the time-courses of product formation monitored at varying concentrations of oxygen, substrate and enzyme shown in Fig. 6.

Table 1. Numerical values of the model parameters.

Parameter	Meaning	Value	Unit
k_1	activation (product)	1.86E-02	s^{-1}
k_2	H-abstraction	1.45E+01	s^{-1}
k_3	O ₂ -insertion	9.58E-03	$s^{-1}\mu M^{-1}$
k_4	inactivation	5.79E-02	s^{-1}
K_{mp}	P-binding to inactive enzyme	37	μM
K_{ms}	S-binding to active enzyme	87	μM
K_{is}	S-binding to inactive enzyme	101	μM
K_{ip}	P-binding to active enzyme	n.d.	μM

The excellent correspondence between experimental and simulated progress curves demonstrates that the kinetic model is able to capture all those unusual features observed with 19-OH AA. In particular, the very low oxygen affinity can be accounted for by the high value of the inactivation rate k_4 causing the enzyme to permanently drop off from the catalytic cycle to the inactive Fe²⁺-form. With increasing concentration molecular oxygen is capable of out competing this inactivation step at the level of the enzyme-radical complex ES·.

ANALYSIS OF SYSTEMIC PROGRESS-CURVES: ESTIMATION OF ENZYME PARAMETERS *IN VIVO*

In the previous example progress-curve analysis was applied to a single enzyme comprising a complex kinetics. Here I present an even more advanced application of this technique aimed at determining the temperature coefficients of enzymes under *in vivo* conditions. This study was performed with human erythrocytes. The objective was to determine the (maximal) activities of a larger group of erythrocyte enzymes at low temperature (4°C) in order to better understand the metabolic control of the red cells under blood preservation conditions. To this end, we have monitored the time-dependent metabolic changes in human erythrocytes induced by a drop of the incubation temperature from 27°C to 4°C.

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To prevent acidification of the medium by accumulating lactate, the pH value was buffered at constant value (7.0). Figure 7 shows some typical time-courses of red cell metabolite

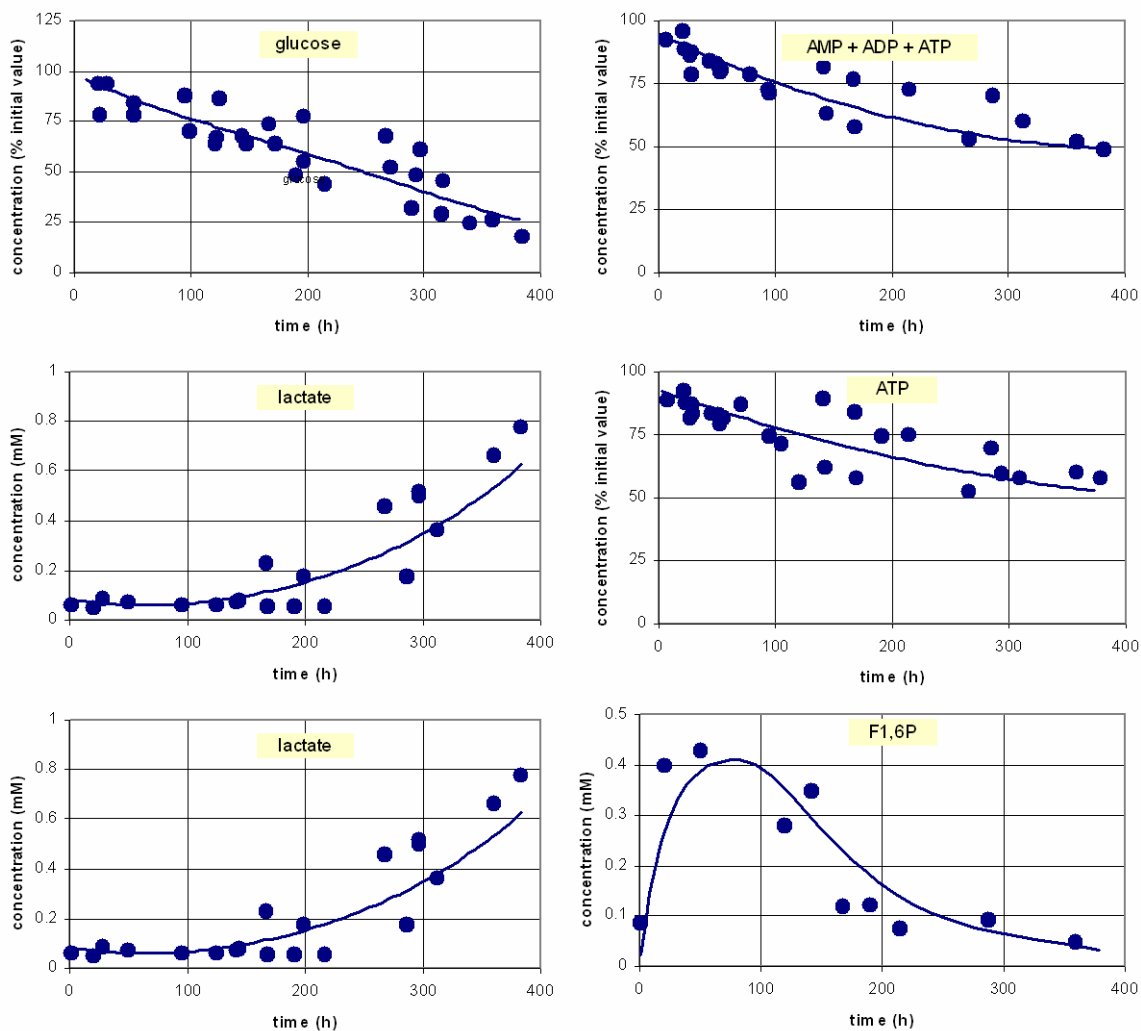


Figure 7. Time courses of selected red cell metabolites observed during a 15 day incubation at $T = 4^{\circ}\text{C}$ at $\text{pH} = 7.0$

From this information we tried to estimate the activity of red cell enzymes at 4°C ($v_{max}^{4^{\circ}}$) by simulating the observed time-courses by a comprehensive kinetic model of the red cell metabolism [9].

The reaction scheme of this model is shown in Fig. 8. It comprises two major pathways of the cell: (i) glycolysis, degrading glucose to lactate and pyruvate and representing the only source of ATP and (ii) the hexose monophosphate shunt, responsible for the inter-conversion of hexoses and riboses and the production of NADPH₂ needed for various reductive processes of the cell. The system of algebro-differential equations describing the time-evolution of this system is given in [9].

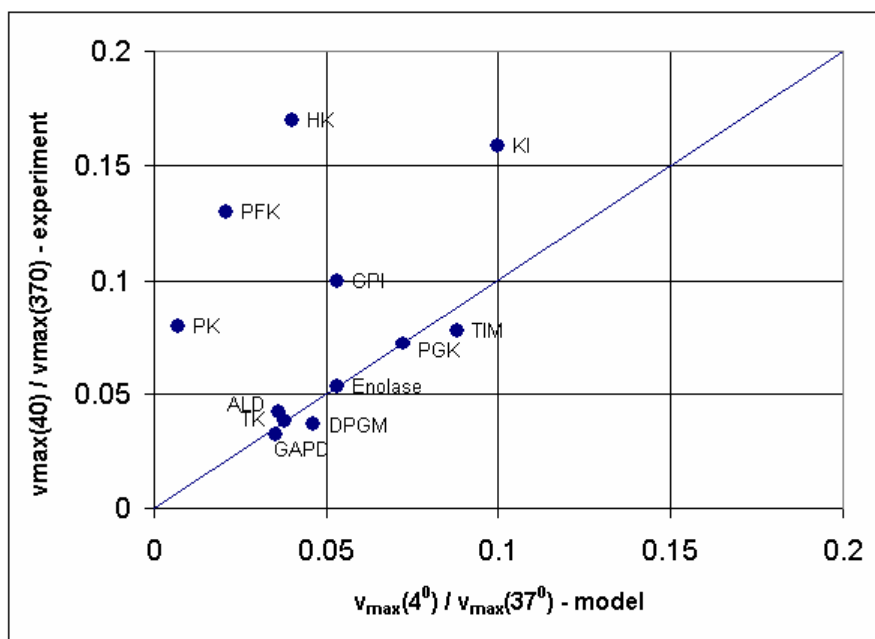


Figure 8. Metabolic reactions in red cells taken into account by the kinetic model [9] used for the analysis of systemic progress curves. Enzymes:HK - hexokinase [2.7.1.1]; PGI - phosphohexose isomerase [5.3.1.9]; PFK - phosphofructokinase [2.7.1.11]; ALD -aldolase [4.1.2.13]; TIM - triosephosphate isomerase [5.3.1.1]; GAPD - glyceraldehyde-3-phosphatedehydrogenase [1.2.1.12]; PGK - phosphoglycerate kinase [2.7.2.3]; DPGM - bisphosphoglycerate mutase [5.4.2.4]; DPGase - bisphosphoglycerate phosphatase [3.1.3.13]; PGM - phosphoglycerate mutase [5.4.2.1]; EN - enolase [4.2.1.11]; PK - pyruvate kinase [2.7.1.40]; LDH -lactate dehydrogenase [1.1.1.28]; AK -adenylate kinase [2.7.4.3]; G6PD - glucose 6-phosphate dehydrogenase [1.1.1.49]; 6PGD - phosphogluconate dehydrogenase [1.1.1.44]; GSSGR - glutathione reductase [1.8.1.7]; EP - phosphoribulose epimerase [5.1.3.1]; KI - ribose phosphate isomerase [5.3.1.6]; TK - transketolase [2.2.1.1]; TA - transaldolase [2.2.1.2]; PRPPS - phosphoribosylpyrophosphate synthetase [2.7.6.1].

Progress-curve analysis was performed by fitting the time-dependent solution of the kinetic model to the observed time-courses of red cell metabolites by treating the maximal activities of the enzymes as the only temperature-sensitive parameters of the model, i.e., neglecting temperature effects on other kinetic parameters as, for example, affinity constants.

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This simplification seems to be justified in the light of numerous enzymatic studies demonstrating that the catalytic rate constant of an enzymatic reaction exhibits a much more pronounced temperature dependence than other kinetic parameters. Figure 9 compares the relative temperature coefficients $v_{max}^{4^{\circ}} / v_{max}^{37^{\circ}}$ calculated by means of systemic progress-curve analysis with experimental values taken from the literature. Larger discrepancies result for the glycolytic enzymes hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK), catalysing irreversible reaction steps and exerting the largest control over red cell glycolysis.

The temperature sensitivity of these enzymes as measured with the isolated enzyme under *in vitro* conditions is significantly lower than that predicted by progress-curve analysis. The most likely explanation for the overestimation of the temperature dependence predicted by systemic progress-curve analysis is due to the fact that important effectors down-regulating the activity of these enzymes at lower temperatures *in vivo* are lacking in the kinetic model. Hence, the drop in the activity of these enzymes needed to account for the observed time courses of these regulatory enzymes is attributed to the time-dependence of their v_{max} values.

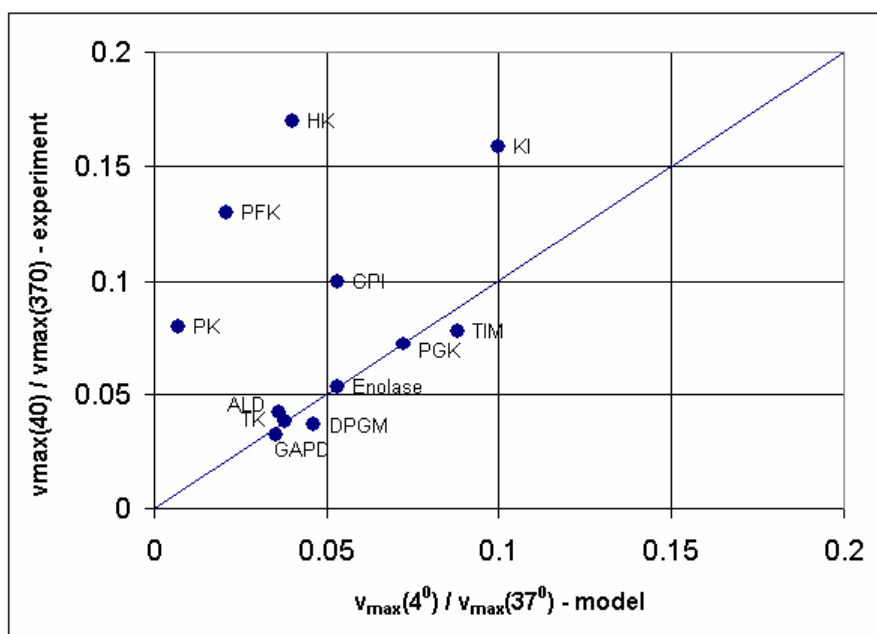


Figure 9. Relative temperature coefficients $v_{max}(4^{\circ}) / v_{max}(37^{\circ})$ of selected red cell enzymes. x-Coordinate: values obtained by fitting a comprehensive kinetic model to time-courses of metabolite concentrations (systemic progress-curve analysis). y-Coordinate: *in vitro* experimental values measured with the isolated enzyme (data taken from references [10-22]).

However, it cannot be excluded that the *in vitro* data for these enzymes are wrong because some physiological effectors, making them more susceptible for changes in temperature, have not been considered in the *in vitro* assay. This points to a general problem arising when building enzyme-kinetic models on *in vitro* data and using them in the context of systems biology. The final decision to rely on mathematical models of complex cellular networks based on kinetic parameters obtained either from *in vitro* measurements or from systemic progress-curve analysis will ultimately depend on the goodness of testable predictions made with either types of models.

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