Kinetic Model of Mitochondrial Krebs Cycle: Unraveling the Mechanism of Salicylate Hepatotoxic Effects

Ekaterina Mogilevskaya · Oleg Demin · Igor Goryanin

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Abstract This paper studies the effect of salicylate on the energy metabolism of mitochondria using *in silico* simulations. A kinetic model of the mitochondrial Krebs cycle is constructed using information on the individual enzymes. Model parameters for the rate equations are estimated using *in vitro* experimental data from the literature. Enzyme concentrations are determined from data on respiration in mitochondrial suspensions containing glutamate and malate. It is shown that inhibition in succinate dehydrogenase and α -ketoglutarate dehydrogenase by salicylate contributes substantially to the cumulative inhibition of the Krebs cycle by salicylates. Uncoupling of oxidative phosphorylation has little effect and coenzyme A consumption in salicylates transformation processes has an insignificant effect on the rate of substrate oxidation in the Krebs cycle. It is found that the salicylate-inhibited Krebs cycle flux can be increased by flux redirection through addition of external glutamate and malate, and depletion in external α -ketoglutarate and glycine concentrations.

Key words Krebs cycle · kinetic model · salicylates

Introduction

Understanding of drug side effects is one of the most challenging problems of modern pharmacology [1]. The problem has two aspects. The first is how to reduce adverse effects

E. Mogilevskaya · O. Demin
A.N. Belozersky Institute of Physico-Chemical Biology,
M.V. Lomonosov Moscow State University, Leninskie Gory, Moscow 119992, Russia

E. Mogilevskaya
e-mail: mogilevskaya@insysbio.ru

O. Demin
e-mail: demin@genebee.msu.ru

I. Goryanin (✉)
The University of Edinburgh, Appleton Tower, Crichton Street, Edinburgh EH8 9LE, UK
e-mail: goryanin@inf.ed.ac.uk

of already existing drugs, and the second is how to predict possible side effects of new compounds that are still under development.

The ability to predict drug toxicity for new medicines at an earlier stage of the development could reduce overall costs substantially. The pathways involved in toxic drug effects can be examined using knowledge about the regulatory mechanisms of the intracellular biochemistry. In order to enable this, the strategy of kinetic pathway reconstruction and modeling has been developed [2]. In this paper, we use the kinetic modeling approach as a framework to collect, mine, and analyze data on cellular biochemistry and physiology. The developed kinetic model is used to describe the functioning of the intracellular metabolism and to investigate the consequences of therapeutic interventions.

Usually, drugs have multiple effects on the intracellular metabolism (non-specificity), i.e., more than one enzyme is affected (inhibited or activated) or more than one transporter is involved in utilization and excretion of the drug. Kinetic modeling enables us to study each effect individually. At the same time, we can estimate the synergy of individual impacts to the total adverse effect of the drug. Using this approach, we have studied the mechanisms of hepatotoxic side effects of acetylsalicylic acid by assessing individual contributions to the inhibition of the mitochondrial energy metabolism as a whole.

It is known that, during utilization in the liver, salicylates can inhibit β -oxidation of fatty acids [3], decrease the pool of coenzyme A (CoA) [4], inhibit succinate dehydrogenase and α -ketoglutarate dehydrogenase [5], and increase the permeability of the inner mitochondrial membrane, thereby decreasing the proton motive force [6, 7]. One of the reasons for liver injury during aspirin therapy is the disturbance of the hepatocyte energy metabolism, particularly in the Krebs cycle. We have developed a kinetic model of the Krebs cycle to understand which of the aspirin modes-of-action that are most critical for hepatocytes.

We have estimated the contributions for each mode to changes in the global regulatory properties of mitochondrial energy metabolism. Using our model we have studied the influences of salicylates on the quasi-steady state flux in the Krebs cycle and examined possible ways to prevent such changes.

Several mathematical models of the Krebs cycle with different levels of detail have been developed. Stoichiometric models have been presented in a number of papers [8–10]. These models only account for the stoichiometry of the Krebs cycle reactions and do not consider the kinetic and regulatory properties of mitochondrial energy metabolism. The kinetic properties of the individual reactions have been incorporated in a variety of kinetic models [11–13]. However, these models used values of apparent kinetic parameters to describe the kinetics of the individual enzymes of the Krebs cycle. Detailed kinetic mechanisms for the rate equations of the individual reactions have not been developed, and this is reflected in the limited predictive power of the models developed earlier. The lack of detailed mechanistic understanding is likely to result in distortion of both the rate equations describing the kinetic properties of the individual enzymes, and of the behavior of the overall model describing the kinetic and regulatory properties of the Krebs cycle as a whole. To avoid these drawbacks, we have derived rate equations of the individual reactions from catalytic cycles based on protein structural data of the corresponding enzymes. We have estimated the kinetic parameters of the rate equations by fitting them to *in vitro* data. Furthermore, we have validated our model using experimental data measured on suspensions of mitochondria.

The main aim of the present paper is to describe the strategy which allows us to unravel a mechanistic understanding of drug side effects. The strategy is based on a kinetic modeling approach which takes into account both *in vitro* data on individual enzymes and *in vivo*

data characterizing the kinetic properties of the metabolic system as a whole. The adverse effect of salicylates on mitochondria are used as an example.

Materials and Methods

Kinetic model of Krebs cycle

Our model consists of a system of ordinary nonlinear differential equations which determine the time dependence of the metabolite concentrations [14]. These equations are similar to the chemical kinetics equations and usually are written in the following way:

$$\frac{dX}{dt} = V_{X\text{production}} - V_{X\text{consumption}}$$

where X is a metabolite concentration, and $V_{X\text{production}}$ and $V_{X\text{consumption}}$ are the total rates of production and consumption of the same metabolite.

Traditionally, the Krebs cycle is described as a sequence of nine reactions resulting in the formation of oxaloacetate from citrate through *cis*-aconitate, isocitrate, α -ketoglutarate, succinyl-coenzyme A, succinate, fumarate, and malate. The cycle is closed because of the condensation of oxaloacetate with acetyl-coenzyme A and the formation of citrate. Acetyl-coenzyme A is formed from pyruvate, fatty acid or amino acid oxidation. It was found [15] that under conditions of high energy demand, the Krebs cycle may not work in its full form, but, rather, a shunt exists via transamination of glutamate and oxaloacetate with the subsequent formation of α -ketoglutarate (Figure 1). Such a truncated Krebs cycle is also found in Morris hepatoma 3924A mitochondria [16]. In this case glutamate (Glu) is the carbon atom source, and Glu is transported to the mitochondrial matrix by the aspartate–glutamate carrier (AGC, Figure 1), which exchanges external glutamate Glu_{out} with an internal aspartate (Asp_{in}) carrying a proton from the inter-membrane space of the mitochondria to the matrix. Glutamate donates its amino group to oxaloacetate (OAA) to form α -ketoglutarate (KG_{in}). This reaction (AspAT, Figure 1) is catalyzed by aspartate aminotransferase. The next reaction, an oxidative decarboxylation of KG_{in} to succinyl-coenzyme A (SuccoA), is catalyzed by α -ketoglutarate dehydrogenase (KGDH). KG_{in} may be also exchanged with external malate (Mal_{out}) (KMC). This reaction is catalyzed by the dicarboxylate carrier. SuccoA is broken up to form succinate (Suc). This reaction is associated with phosphorylation of GDP and catalyzed by succinate thiokinase (STK). Oxidation of Suc to fumarate (Fum) is accompanied by reduction of ubiquinone (Q) to ubiquinol (QH_2) and catalyzed by succinate dehydrogenase (SDH). Fum is hydrated to Mal in the reaction catalyzed by fumarase (FUM). Oxidation of Mal to OAA catalyzed by malate dehydrogenase (MDH) closes Krebs cycle. The model also includes a reaction which corresponds to the binding of OAA to the catalytically active state of succinate dehydrogenase (SDH) and results in the formation of a catalytically inactive state of this enzyme, SDH–OAA. Glu_{in} , Asp_{in} , OAA, KG_{in} , SuccoA, CoA, Suc, Fum, Mal_{in} , SDH, SDH–OAA are the model variables, and the concentrations denoted Glu_{out} , Asp_{out} , H_{out} , H_{in} , Mal_{out} , KG_{out} , P, ATP, ADP, Ca^{2+} , GTP, GDP, Q, QH_2 , NAD, NADH are model parameters. The subscripts in and out denote intra- and extra- mitochondrial concentrations, respectively. The constant metabolites appear in Figure 1 in square boxes. Values of the

Figure 1 The scheme of the Krebs cycle oxidizing glutamate and malate as substrates showing the influence of salicylates.

parameters are listed in Table I. The model is described by the following system of differential equations:

$$\begin{aligned}
 \frac{d\text{Glu}_{\text{in}}}{dt} &= V_{\text{AGC}} - V_{\text{AspAT}}; \quad \frac{d\text{Asp}_{\text{in}}}{dt} = V_{\text{AspAT}} - V_{\text{AGC}}; \\
 \frac{d\text{OAA}}{dt} &= V_{\text{MDH}} - V_{\text{AspAT}} - V_{\text{ISDH}}; \quad \frac{d\text{KG}_{\text{in}}}{dt} = V_{\text{AspAT}} - V_{\text{KGDH}} - V_{\text{KMC}}; \\
 \frac{d\text{SucCoA}}{dt} &= V_{\text{KGDH}} - V_{\text{STK}}; \quad \frac{d\text{Suc}}{dt} = V_{\text{STK}} - V_{\text{SDH}}; \quad \frac{d\text{Fum}}{dt} = V_{\text{SDH}} - V_{\text{FUM}}; \quad (1) \\
 \frac{d\text{Mal}_{\text{in}}}{dt} &= V_{\text{FUM}} - V_{\text{MDH}} + V_{\text{KMC}}; \quad \frac{d\text{CoA}}{dt} = V_{\text{STK}} - V_{\text{KGDH}}; \\
 \frac{d\text{SDH}}{dt} &= -V_{\text{ISDH}}; \quad \frac{d(\text{SDH} - \text{OAA})}{dt} = V_{\text{ISDH}}.
 \end{aligned}$$

There are four conservation laws in the system: N_{tot} (conservation of amino groups), CoA_{tot} (conservation of CoA), C_{tot} (conservation of four-carbon skeleton) and SDH_{tot} (conservation of succinate dehydrogenase). Pool values are listed in Table I.

Our model does not account for the ability of the aspartate aminotransferase, α -ketoglutarate dehydrogenase and malate dehydrogenase to form metabolon [17] – a complex of enzymes catalyzing consecutive reactions without realizing their intermediates into the bulk phase [18]. This corresponds to the assumption that all enzymes of the Krebs

Table 1 Krebs cycle metabolite concentrations obtained from literature and from the model

Substrates	Concentration (mM)	
	Literature data	Model data
KG _{in}	0.15 [25]; 1.13–1.6 [31]	0.018
KG _{out}	0.54 [25]	0.54
Glu _{in}	7.3 [25]	7.3
Glu _{out}	0.86 [25]	20
OAA	0.002–0.006 [31]	0.0002
Mal _{in}	0.324 [32]; 0.5–2.5 [33]	1.16
Mal _{out}	0.495 [32]	0
Asp _{in}	0.192–0.297 [31]	0.3
SucCoA	0.36–0.91 [24]	0.63
CoA	0.16–0.79 [24]	0.37
N _{tot}	–	7.6
SDH _{tot}	0.05 [34]	0.05
Ca ²⁺	0.001 [35]	0.001
Δψ	139 mV [7]	139 mV
C _{tot}	–	3.801
CoA _{tot}	–	1
Suc	–	0.007
Asp _{out}	–	0
Fum	–	1.94
NAD	2 [36]	2
NADH	1 [36]	1
ATP + ADP	12 [37]	12
ATP/ADP	9.4 [37]	9.4
P	–	5
GDP	–	0.2
GTP	–	1.8
Q	–	19
QH ₂	–	1
H _{in}	5.2e–6 [7]	5.2e–6
H _{out}	–	3.98e–5
Gly	–	1
SDH-OAA	–	0.0458
SDH	–	0.0042

Values of the variables of the model are in bold.

cycle do not interact with each other. This assumption can be partly justified by the fact that binding of these enzymes to each other depends on their concentrations and the availability of their substrates [19] and, consequently, may play an insignificant role under many conditions. Nevertheless, since metabolon formation can contribute substantially to the dynamic and regulatory properties of the Krebs cycle, we will take it into account in our future models and to test whether the modeling results change substantially.

Description of individual enzymes of the Krebs cycle

To describe the kinetics of the individual enzymes we used *in vitro* data available from the literature. When experimental data for hepatocytes were missing, we used kinetic and

structural data for the corresponding heart enzymes. Derivation of the rate equations was accomplished in following stages:

- 1) Construction of enzyme catalytic cycle based on structural and kinetic data (in most cases information on the mechanism of enzyme functioning is available from the literature);
- 2) Derivation of rate equations in terms of the parameters of the catalytic cycle (rate constants and dissociation/equilibrium constants of elementary steps of the catalytic cycle) in accordance with quasi-steady state or rapid equilibration approaches [20];
- 3) Derivation of equations which express parameters of the catalytic cycle in terms of kinetic parameters (Michaelis constants, inhibition constants, catalytic constants);
- 4) Derivation of rate equation in terms of kinetic parameters of the enzyme reaction.

Estimation of the kinetic parameters of the rate equations was accomplished in the following stages:

- a) We have found all *in vitro* experimental data on the kinetics of the selected enzymes available from literature. These data are usually in form of dependences either on the initial rate of substrate/product concentration or on the substrate/product concentration variations with time;
- b) We have described all available *in vitro* experiments quantitatively. To fit initial rate dependences on substrate/product concentration we have used explicit rate equation for each individual enzyme. The rate equation has also been used to find inhibition parameters. Systems of ordinary differential equations have been constructed to describe time series experiments. Such systems included concentrations of measured intermediates as variables from particular experiments. Rate equations and parameters values obtained from fitting against experimental data are listed in [Appendix](#).

Methods of investigation of Krebs cycle model behaviour

All the models presented in this work were studied using the DBSolve 7.0 software package [21]. Time dependences of metabolite concentrations and reaction rates have been calculated by numerical integrators included in the package. Steady-state rate dependences on parameters have been calculated using an original parameter continuation method. This method finds solutions to the system of nonlinear algebraic equations for different values of the parameters. The Hook–Jeeves algorithm [22] has been used to identify parameter values by fitting rate law functions and systems of differential equations to experimental data.

Results and Discussion

Estimation of model parameters from *in vivo* data

As discussed in the previous section, some parameters could not be estimated from *in vitro* experimental data. These parameters were the intramitochondrial concentrations of the enzymes – AGC, AspAT, KGDH, STK, FUM, MDH, KMC – and two kinetic parameters associated with the AGC rate equation – the Michaelis constants for external glutamate and protons. To estimate the values of these parameters we have adjusted the whole model to

Figure 2 Dependence of stationary glutamate consumption rate by mitochondrial suspension on glutamate concentration. Simulation results and experimental points from [23] under the following conditions: $\text{Glu}_{\text{out}} = 0\text{--}20$ mM, $\text{Mal}_{\text{out}} = 3.7$ mM, $\text{pH}_{\text{out}} = 7.4$.

experimental data [23] where the following experiment has been applied: a suspension of mitochondria has been incubated in media containing glutamate and malate, respiration and glutamate consumption rates have been measured. Steady-state flux has been calculated via aspartate–glutamate carrier dependence as a function of concentration of extramitochondrial glutamate. To estimate values of the unknown parameters, we have fitted the observed variation in steady state to experimentally measured dependence on glutamate consumption. Figure 2 demonstrates that experimental data from [23] (symbols) and the theoretical curve generated by the system of differential equations (1) closely coincide. Values of intra-mitochondrial enzyme concentrations and kinetic parameters obtained are listed in Table II (all parameters obtained by fitting to experimental data [23] are marked by an asterisk).

Combining all of these approaches has allowed us to find all parameter values in the kinetic model. To test how the model describes the kinetic and regulatory properties of the Krebs cycle, we have compared calculated values of steady-state metabolite concentrations with those available in the literature measured in mitochondria oxidizing glutamate (see Table I). Electric potential difference, intramitochondrial concentrations of ubiquinone, ubiquinol, GTP, GDP, ATP, ADP, phosphate and protons, assigned with values available from the literature and steady-state concentrations of intermediates of the Krebs cycle, have been calculated. As demonstrated in Table I, the calculated values of the steady-state concentrations of aspartate, glutamate, malate, coenzyme A and succinyl-CoA are quite close to those appearing in the literature. The concentration of α -ketoglutarate differs from its literature value. This could be due to different experimental conditions in [25] where α -ketoglutarate concentration has been determined.

Kinetic description of the influence of salicylates on the Krebs cycle

Salicylates have multiple influences on the Krebs cycle (see Figure 1). These are:

- (A) sequestration of coenzyme A,
- (B) uncoupling of oxidative phosphorylation,
- (C) inhibition of α -ketoglutarate and succinate dehydrogenases.

To account for all these impacts in the kinetic model, we have assumed the intra-mitochondrial salicylate concentration to be constant, i.e., the concentration of salicylates

Table II Kinetic parameters values of the Krebs cycle enzymes known from the literature and estimated via the fitting of rate equations to literature experimental data (Michaelis constants, dissociation constants and enzymes concentrations are in millimolars, rate constants are in 1/min)

Enzyme designation	Literature values of kinetic parameters taken from [ref.]	Kinetic parameters values estimated via fitting to experimental data taken from [ref.]
AGC	$K_m^{\text{Glu}_{\text{out}}} = 0.25$; $K_m^{\text{Glu}_{\text{in}}} = 3$; $K_m^{\text{ASP}_{\text{out}}} = 0.12$ [42]; $K_m^{\text{ASP}_{\text{in}}} = 0.0435$ [23]	*AGC = 2; $k_{1,0} = 99,800$; $k_{-1,0} = 9,940$; $k_2 = 100,000$; $k_{-2} = 9,940$; $K_m^{\text{H}_{\text{in}}} = 0.00004$; $K_m^{\text{H}_{\text{out}}} = 0.01$; * $K_m^{\text{H}_{\text{out}}} = 0.1$; $K_m^{\text{Glu}_{\text{out}}} = 9.3$; [23]
AspAT	AspAT = 0.14 [38]; $k_r = 51,870$; $K_m^{\text{KG}_{\text{in}}} = 6.9$; $K_m^{\text{ASP}_{\text{in}}} = 1.9$ [47]; $K_m^{\text{OAA}} = 0.088$ [32]; $K_{\text{eq}} = 6.6$ [31]	*AspAT = 1.5; $k_1 = 5e7$; $k_f = 10,000$ [23]; $K_m^{\text{Glu}_{\text{in}}} = 0.55$; $k_{-1} = 51,999$ [47]
KGDH	KGDH = 0.002 [38]; $k_f = 83,110$ [39]; $K_m^{\text{CoA}} = 0.0027$ [40]; $K_m^{\text{NAD}} = 0.05$ [41]; $K_m^{\text{KG}_{\text{in}}} = 0.2$; $K_i^{\text{ATP}} = 0.1$; $K_i^{\text{ADP}} = 0.1$; $K_i^{\text{a}} = 0.0012$ [35]	*KGDH = 1; * $K_i^{\text{ADP}} = 0.005$ [23]; $K_m^{\text{KG}_{\text{in}}} = 0.03$; $K_m^{\text{CoA}} = 0.002$; $K_m^{\text{NAD}} = 0.93$; $K_i^{\text{SuccCoA}} = 0.011$; $K_i^{\text{NADH}} = 0.0018$; $K_i^{\text{ATP}} = 0.01$; $K_i^{\text{ADP}} = 0.56$ [35]; $K_i^{\text{Sal}} = 0.001$ [5]
STK	$K_m^{\text{Succ}} = 0.4 - 0.8$; $K_m^{\text{CoA}} = 0.005 - 0.02$; $K_m^{\text{SuccCoA}} = 0.01 - 0.06$; $K_m^{\text{GDP}} = 0.002 - 0.008$; $K_m^{\text{GTP}} = 0.05 - 0.01$; $K_m^{\text{P}} = 0.2 - 0.7$ [48]; $k_f = 10,780$ [49]; $K_{\text{eq}} = 3.7$ [50]	$K_m^{\text{Succ}} = 0.81$; $K_m^{\text{CoA}} = 0.017$; $K_m^{\text{SuccCoA}} = 0.024$; $K_m^{\text{GDP}} = 0.007$; $K_m^{\text{GTP}} = 0.000068$; $K_m^{\text{P}} = 1.5$; $k_1 = 1,700,000$; $k_{-1} = 1,149$; $k_2 = 10,000$; $k_{-2} = 1,990,000$; $K_d^{\text{SuccCoA}} = 0.029$; $K_{d,E-GDP-CoA} = 0.00038$; $K_d^{\text{GDP}} = 0.14$; $K_{d,E-GTP-SuccCoA} = 0.49$ [48]; *STK = 1 [23]
SDH	SDH = 0.05; $k_f = 10,000$; $k_r = 102$ [34]; $K_m^{\text{Succ}} = 0.13$; $K_m^{\text{Q}} = 0.0003$; $K_m^{\text{OH}_2} = 0.0015$; $K_m^{\text{Fum}} = 0.025$ [53]; $K_m^{\text{Succ}} = 0.01$; $K_m^{\text{Fum}} = 0.29$ [52]	$K_m^{\text{Succ}} = 0.084$; $K_m^{\text{Succ}} = 0.29$ [52]; $k_f = 1e6$ [23]; $K_i^{\text{Sal}} = 7e-5$ [5]
FUM	FUM = $2.27e-4$; $K_m^{\text{Fum}} = 0.047$; $K_m^{\text{Malin}} = 0.017$; $k_f = 90,721$; $k_r = 71,342$ [54]	*FUM = 0.5; * $K_m^{\text{Fum}} = 0.01$ [23]; $K_m^{\text{Fum}} = 0.036$; $k_f = 90,722$ [55]
MDH	$k_f = 5.4e5$; $k_r = 8.6e3$; MDH = $9.03e-4$; $K_m^{\text{OAA}} = 0.0795$; $K_m^{\text{Malin}} = 0.386$; $K_m^{\text{NAD}} = 0.0599$; $K_m^{\text{NADH}} = 0.26$; $K_m^{\text{OAA}} = 0.0055$; $K_m^{\text{Malin}} = 0.36$; $K_i^{\text{NAD}} = 1.1$; $K_i^{\text{NADH}} = 0.0136$; $K_{\text{eq}} = 8,000$; [56]	*MDH = 1 [23]
KMC	$k_f = 325$; $k_r = 309$; $K_m^{\text{Malout}} = 1.36$; $K_m^{\text{Malin}} = 0.71$; $K_m^{\text{KG}_{\text{in}}} = 0.17$; $K_m^{\text{KG}_{\text{out}}} = 0.31$; $K_{\text{eq}} = 1$ [57]	*KMC = 2 [23]; $k_1 = 858$; $K_i^{\text{KG}_{\text{out}}} = 4.2e-3$ [57]
Oxaloacetate binding to SDH (ISDH)	$k_i = 1,200$ 1/min mM; $k_{-i} = 0.02$ 1/min [34]	
SCL	$V_f = 0.75$ mM/min [26]; $K_m^{\text{Sal}} = 2$ [4]; $K_m^{\text{CoA}} = 0.63$ [58]	$K_i^{\text{CoA}} = 0.63$
SGT	$V_f = 900$ mM/min; $K_m^{\text{SalCoA}} = 0.008$; $K_m^{\text{Gly}} = 20$ [26]	$K_i^{\text{Gly}} = 20$

*Parameters values estimated from verification of the whole model.

does not change with time. Below we describe each individual salicylate impact on the Krebs cycle in more detail:

(A) Salicylate-induced CoA sequestration results from salicylate activation consisting of two processes [26]: the reaction salicyl-CoA formation reaction accompanied by CoA

consumption and catalyzed by salicyl-CoA ligase (SCL in Figure 1) and reaction of glycine acylation accompanied with CoA release and catalyzed by salicylCoA-glycine acyltransferase (SGT in Figure 1). Rate equations of these enzymes catalyzing salicylate activation have been derived (see Appendix). To simulate the influence of salicylate-induced CoA sequestration on the functioning of the Krebs cycle we took these two reactions into account.

- (B) Uncoupling of oxidative phosphorylation results from salicylate transport via the inner mitochondrial membrane. Salicylate is a weak acid which passes through the membrane in a neutral form only. A neutral form of salicylate is formed via binding of an external proton to anionic form of salicylate [27]. This means that transport of salicylate into the mitochondrial matrix is accompanied by simultaneous transport of protons in the same direction. It was shown by Haas *et al.* [7] how salicylate addition influences the transmembrane potential and Δ pH. Two sets of parameter values have been chosen to model the uncoupling effect of salicylate. The first set of parameter values corresponds to the energy state with coupled respiration and oxidative phosphorylation without salicylate addition: $\Delta\psi = 0.139$ V, $H_{in} = 5.2$ nM and $H_{out} = 39.8$ nM [7]. The second set of parameter values describes the functioning of mitochondria in an uncoupled state with added salicylate: $\Delta\psi = 0.135$ V, $H_{in} = 13$ nM; $H_{out} = 39.8$ nM [7]. Using either the first or the second set of values for the parameters in the system of differential equations (1), and calculating the steady-state values of the intermediate concentrations and fluxes, we have estimated the influence of uncoupling on the functioning of the Krebs cycle.
- (C) α -ketoglutarate dehydrogenase and succinate dehydrogenase can be inhibited by salicylates [5]. Since the mechanisms of interaction between salicylates and these enzymes are unknown, we have assumed that both α -ketoglutarate dehydrogenase and succinate dehydrogenase are inhibited by salicylates in an uncompetitive manner [20]. This means that the maximal rates of these enzymes depend on salicylate concentration in accordance with the following equation:

$$V_{\max}^{\text{enzyme}}(\text{Sal}) = V_{\max}^{\text{enzyme}} / \left(1 + \text{Sal} / K_{i,\text{enzyme}}^{\text{Sal}}\right). \quad (2)$$

To estimate the values of the inhibition constants of the two enzymes with respect to salicylates, experimental data [5] have been used where the following experiments have been performed: a suspension of mitochondria respiring on α -ketoglutarate has been incubated in media with and without salicylate. Then, time series of oxygen concentration consumed by these mitochondria have been measured. To quantitatively describe these experiments, a kinetic model of α -ketoglutarate oxidation in the Krebs cycle has been constructed (Figure 3).

This model has been obtained by the following modification of the system of differential equations (1):

- 1) elimination of variables (and corresponding differential equations) corresponding to glutamate, aspartate and oxaloacetate concentrations;
- 2) elimination of all reactions (and corresponding reaction rates) involved in glutamate transport and degradation (AGC, AspAT and MDH reactions in Figure 1);

Figure 3 The scheme of the Krebs cycle oxidizing α -ketoglutarate as substrate showing the influence of salicylates.

- 3) addition of new variables corresponding to the consumed oxygen concentration (O_2^{consumed}) and salicyl-CoA (SalCoA). Time dependences for these variables are determined by the following differential equations:

$$\begin{aligned} \frac{dO_2^{\text{consumed}}}{dt} &= (V_{\text{SDH}} + V_{\text{KGDH}})/2 \\ \frac{d\text{SalCoA}}{dt} &= V_{\text{SCL}} - V_{\text{SGT}}. \end{aligned} \quad (3)$$

The new model contains all mechanisms of salicylate impact on the Krebs cycle (see clauses A, B and C above and Equations (2) and (3)) and includes oxaloacetate as a parameter. As OAA is not consumed by other reactions (when α -ketoglutarate is the only oxidized substrate) the MDH reaction would be in equilibrium. The existence of this reaction would influence OAA concentration. So we have determined the OAA parameter value through the equilibrium constant for the MDH reaction: $OAA = \text{Mal} \times \text{NAD} / (K_{\text{eq}}^{\text{MDH}} \times \text{NADH})$. Time dependences of the oxygen concentration consumed in the experiment, with and without salicylate, have been calculated under the following initial conditions: $\text{Mal}_{\text{in}} = 1.41 \text{ mM}$; $\text{KG}_{\text{in}} = 0.08 \text{ } \mu\text{M}$; $\text{SucCoA} = 0.02 \text{ mM}$; $\text{CoA} = 0.98 \text{ mM}$; $\text{SalCoA} = 0$; $\text{SDH} = 2 \text{ } \mu\text{M}$; $\text{SDH-OAA} = 48 \text{ } \mu\text{M}$; $\text{Suc} = 0.12 \text{ } \mu\text{M}$; $\text{Fum} = 2.4 \text{ mM}$; $O_2^{\text{consumed}} = 0$.

Initial values of the intermediate concentrations have been calculated as steady-state values in the model of α -ketoglutarate oxidation without salicylate addition ($\text{Sal} = 0$) (see Figure 3). To estimate the values of the unknown inhibition constants for salicylate for KGDH and SDH we have fitted the new model to experimentally measured dependences on oxygen consumption. Figure 4 demonstrates that experimental data from [5] (symbols) and theoretical curves corresponding to our model of the Krebs cycle oxidizing α -ketoglutarate closely coincide. Values of the inhibition constants are listed in Table II. The K_i value for succinate dehydrogenase is two orders of magnitude lower than that for α -ketoglutarate dehydrogenase, so succinate dehydrogenase should be inhibited more strongly by salicylate.

Figure 4 Salicylates inhibition of mitochondrial respiration rate via oxidation of α -ketoglutarate described by the model and experimental points from [5] in the following conditions: 1, $\text{KG}_{\text{out}} = 10 \text{ mM}$, $\text{pH}_{\text{out}} = 7.4$ (white squares); 2, $\text{KG}_{\text{out}} = 10 \text{ mM}$, $\text{pH}_{\text{out}} = 7.4$, $\text{Sal} = 6.7 \text{ mM}$ (black squares).

Impacts of different mechanisms of salicylate inhibition on the total adverse effect on the Krebs cycle

To estimate the contribution of each individual mechanism of Krebs cycle inhibition, we have taken into account each inhibitory mechanism one by one. We have calculated the steady-state dependence of glutamate influx on external glutamate concentration. The lower the steady-state influx of glutamate is, the more significant has the individual contribution to the cumulative inhibition of the Krebs cycle been found to be. External metabolite concentrations have been taken from cytosol data available in the literature (values are listed in Table I). Figure 5 demonstrates how the steady-state value of the glutamate influx depends on its extra-mitochondrial concentration without taking into account any mechanism of salicylate influence (curve 1), with incorporation of individual mechanisms of salicylate-induced inhibition (curves 2–5), and when all possible inhibitory mechanisms are accounted for (curve 6). Analyzing the results (Figure 5) we conclude that the single CoA-sequestration mechanism of salicylate (i.e., reactions catalyzed by salicyl-CoA ligase and salicyl-CoA-glycine acyltransferase) only changes the glutamate influx slightly (see curve 2). Moreover, uncoupling of oxidative phosphorylation by salicylates (Figure 5, curve 3) had little effect on the flux. Whereas, in contrast, all other mechanisms substantially decrease

Figure 5 Influence of different mechanisms of salicylate inhibition on Krebs cycle oxidation of glutamate and malate. Dependences of glutamate consumption rates on its extra-mitochondrial concentration were obtained from the models where different mechanisms of salicylate (5 mM) inhibition were taken into account: 1, without salicylate; 2, CoA consumption by salicylate transformation processes; 3, uncoupling effect of salicylates; 4, salicylate inhibition of α -ketoglutarate dehydrogenase; 5, salicylate inhibition of succinate dehydrogenase; 6, all salicylate inhibition mechanisms accounted for.

this flux when they have been incorporated into the model individually. Indeed, taking into account either inhibition of α -ketoglutarate dehydrogenase (Figure 5, curve 4) or succinate dehydrogenase (Figure 5, curve 5) decreased the glutamate influx by approximately a factor of 20. Accounting for all possible inhibitory mechanisms resulted in glutamate influx near zero (Figure 5, curve 6). Thus, comparison of individual impacts of different inhibitory mechanisms allows us to conclude that the most substantial contribution of salicylates to Krebs cycle inhibition is due to inhibition of succinate dehydrogenase and α -ketoglutarate dehydrogenase.

Prediction of possible ways to recover Krebs cycle functioning

In this section we address the following question: Is it possible to identify changes in external substrates that could increase the flux through the Krebs cycle and compensate for the salicylate inhibition? We have modified the model of glutamate and malate oxidation to include all salicylate influences (Figure 1). Figure 6 demonstrates that the simultaneous increase in the external malate concentration (from 0.495 to 10 mM), the decrease in external α -ketoglutarate concentration (from 0.54 to 0 mM), and the decrease in internal glycine concentration (from 1 mM to 0.1 μ M) results in a significant recovery of steady-state glutamate influx. This result can be explained in following manner. As shown in the previous section, all mechanisms of salicylate influence (except salicylate uncoupling) that contribute greatly to the Krebs cycle inhibition, affect the lower part of the Krebs cycle (reactions of KGDH, STK, SDH, FUM in Figure 1) and do not affect its upper part (reactions AspAT, MDH, and KMC). This means that redirection of flux from the lower segment of the Krebs cycle to the α -ketoglutarate–malate carrier shunt may result in a substantial increase of flux via the salicylate-inhibited Krebs cycle. The kinetic model has allowed us to identify such changes in external metabolite concentrations that resulted in desirable redirection of fluxes and, as a consequence, in substantial recovery of flux in the salicylate-inhibited Krebs cycle (see curves 1 and 2 in Figure 6). Indeed, decrease in external α -ketoglutarate (from 0.54 to 0 mM) and increase in external malate (from 0.495 to 10 mM) made the α -ketoglutarate–malate carrier shunt stronger in comparison with the α -ketoglutarate dehydrogenase for their shared substrate α -ketoglutarate. On the other hand, decrease in glycine concentration resulted in CoA trapping in the complex with salicylate, SalCoA. Substantial decrease of free CoA concentration, resulting from its sequestration with salicylate, lead to the slowing down of α -ketoglutarate dehydrogenase operation and, as a consequence, to a weakening of the competition of the enzyme with the α -ketoglutarate–malate carrier for α -ketoglutarate. Thus, synchronous changes in external

Figure 6 Effect of changes in external substrate concentrations on salicylate-inhibited Krebs cycle flux: 1, Sal = 5 mM; $\text{KG}_{\text{out}} = 0.54$ mM; $\text{Mal}_{\text{out}} = 0.495$ mM; $\text{Asp}_{\text{out}} = 0$; Gly = 1 mM; 2, Sal = 5 mM; $\text{KG}_{\text{out}} = 0$; $\text{Mal}_{\text{out}} = 10$ mM; $\text{Asp}_{\text{out}} = 0$; Gly = 0.1 μ M.

concentrations of α -ketoglutarate, malate, and internal concentration of glycine predicted by the model resulted in weakening of the inhibitory influence of salicylates on the Krebs cycle.

Salicylic acid therapy remains very popular across the world. More than 100 billion aspirin tablets are consumed worldwide each year. These tablets are used to treat pain and inflammation associated with headaches, toothaches, minor arthritis and muscle or soft tissue injuries, and fever. The drug also prevents blood clots. Moreover, evidence is mounting that regular aspirin usage may reduce the risk of many of today's commonest cancers [28]. But, aspirin is also believed to be a contributory cause of gastrointestinal bleeding and stomach irritation and to be involved in hepatotoxicity. Recent *in vitro* animal studies have shown that the mechanism of diclofenac toxicity relates both to impairment of ATP synthesis by mitochondria, and to production of active metabolites [29]. The mitochondrial permeability transition (MPT) has also been shown to be important in diclofenac-induced liver injury, resulting in generation of reactive oxygen species, mitochondrial swelling and oxidation of NADP and protein thiols.

To compensate for the negative effects of salicylic acids, a new formulation could be introduced. The results presented in this paper suggest a combination of acetylsalicylic acid therapy with compensatory doses of certain Krebs cycle intermediates.

The developed model is not perfect, and could be extended to include metabolic pathways like oxidative phosphorylation and protein interaction networks. This would contribute to better understanding of the mode of action and possible cooperative anti-tumor effects of the aspirin [30].

Conclusion

In this paper we proposed a strategy for investigation of adverse drug effects on cell metabolism. The kinetic modeling approach has been demonstrated by the example of hepatocyte energy metabolism inhibition by salicylates. By investigation of different mechanisms of salicylate influence on the Krebs cycle it was found that its inhibition developed mainly through succinate and α -ketoglutarate dehydrogenase inhibition, whereas CoA consumption in the salicylate-to-salicylurate transformation reactions and uncoupling of oxidative phosphorylation decreased only slightly the Krebs cycle flux with glutamate and malate as oxidized substrates. It was shown that the Krebs cycle flux inhibited by salicylates could be activated through flux redirection by an increase in the external glutamate and malate concentrations and a decrease in external α -ketoglutarate and internal glycine concentration.

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Appendix

Description of individual enzymes of the Krebs cycle

α -ketoglutarate dehydrogenase (KGDH)

KGDH catalyzes the irreversible reaction of oxidative decarboxylation of α -ketoglutarate with the formation of succinyl-coenzyme A and the reduction of NAD. The enzyme is described according to a Ping Pong Ter-Ter mechanism [35]. It is assumed that CO_2

Figure 7 The scheme of the α -ketoglutarate dehydrogenase catalytic cycle.

concentration does not influence the reaction rate. The influence of activators (ADP and Ca^{2+}), inhibitors (ATP) and products (NADH, SucCoA) is taken into account. We assume that the enzyme exists in both active and inactive forms. ADP or Ca^{2+} transforms the enzyme to its active form, whereas ATP transforms the enzyme to its inactive form (see catalytic cycle depicted in Figure 7 for details). The rate equation was derived in the following form:

$$V_{\text{KGDH}} = \frac{\text{KGDH} \cdot \left(1 + \frac{\text{ADP}}{K_i^{\text{ADP}}}\right) \cdot k_f \frac{\text{KG}_{\text{in}}}{K_m^{\text{KG}_{\text{in}}}} \frac{\text{CoA}}{K_m^{\text{CoA}}} \frac{\text{NAD}}{K_m^{\text{NAD}}}}{\frac{\text{CoA}}{K_m^{\text{CoA}}} \frac{\text{NAD}}{K_m^{\text{NAD}}} \left(\frac{\text{KG}_{\text{in}}}{K_m^{\text{KG}_{\text{in}}}} + \frac{1 + \frac{\text{ATP}}{K_i^{\text{ATP}}}}{1 + \frac{\text{Ca}}{K_i^{\text{Ca}}}}\right) + \frac{\text{KG}_{\text{in}}}{K_m^{\text{KG}_{\text{in}}}} \left(\frac{\text{CoA}}{K_m^{\text{CoA}}} + \frac{\text{NAD}}{K_m^{\text{NAD}}}\right) \left(1 + \frac{\text{NADH}}{K_i^{\text{NADH}}} + \frac{\text{SucCoA}}{K_i^{\text{SucCoA}}}\right)}. \quad (4)$$

Here, KGDH is the concentration of α -ketoglutarate dehydrogenase; K_m^{CoA} , K_m^{NAD} , $K_m^{\text{KG}_{\text{in}}}$ are Michaelis constants for substrates; K_i^{ADP} , K_i^{ATP} , K_i^{Ca} , K_i^{NADH} , K_i^{SucCoA} are inhibition constants for effectors; k_f is catalytic constant. Parameters known from the literature are KGDH [38], k_f [39], K_m^{CoA} [40], K_m^{NAD} [41], K_i^{ADP} , K_i^{ATP} , K_i^{Ca} , $K_m^{\text{KG}_{\text{in}}}$ [35]. To estimate inhibition constants for NADH and SucCoA and make values of known parameters more precise, experimental data [35] have been used where the reaction has been started by addition of α -ketoglutarate dehydrogenase to the solution of substrates α -ketoglutarate, CoA and NAD and time dependences of NADH accumulation have been monitored with and without addition of effectors ADP and ATP. To quantitatively describe these experiments, the following system of differential equations has been developed (Figure 8):

$$\begin{aligned} \frac{d\text{KG}_{\text{in}}}{dt} &= -V_{\text{KGDH}}, \quad \frac{d\text{CoA}}{dt} = -V_{\text{KGDH}}, \quad \frac{d\text{NAD}}{dt} = -V_{\text{KGDH}}, \\ \frac{d\text{NADH}}{dt} &= V_{\text{KGDH}}, \quad \frac{d\text{SucCoA}}{dt} = V_{\text{KGDH}} \end{aligned} \quad (5)$$

Here, V_{KGDH} is the rate equation for α -ketoglutarate dehydrogenase given by Equation (4); substrates and products of the reaction catalyzed by α -ketoglutarate dehydrogenase are

Figure 8 The scheme of the α -ketoglutarate dehydrogenase reaction described by the system (5) to describe time dependences of NADH production catalyzed by the enzyme.

the variables of the model (5). Initial conditions for the system of differential equations (5) have been set in accordance with initial concentrations of substrates used in the experiments [35]: $KG_{in} = 0.1$ mM; $NAD = 1$ mM; $CoA = 0.25$ mM; $NADH = 0$; $SucCoA = 0$.

The system of differential equation (4) has four conservation laws:

$$\begin{aligned} KG_{in} + SucCoA &= C_{tot}^{KGDH} && \text{(conservation of four-carbon skeleton)} \\ CoA + SucCoA &= CoA_{tot}^{KGDH} && \text{(conservation of CoA)} \\ KG_{in} + NADH &= e_{tot}^{KGDH} && \text{(conservation of electrons)} \\ NADH + NAD &= NAD_{tot}^{KGDH} && \text{(conservation of pyridine nucleotides)} \end{aligned}$$

Values of NAD_{tot}^{KGDH} , e_{tot}^{KGDH} , CoA_{tot}^{KGDH} , C_{tot}^{KGDH} can be calculated from initial conditions. To estimate parameter values of Equation (4) we have fitted the solution of Equations (5) to experimentally measured time dependences of NADH accumulation. Figure 9 demonstrates a good fit of experimental data from [35] (symbols) to theoretical curves generated by the system of differential equations (5). Values of kinetic parameters are listed in Table II.

Aspartate–glutamate carrier (AGC)

The inner mitochondrial membrane is not permeable to glutamate. Only a membrane carrier, in exchange for an aspartate, could transfer protonated glutamate to the matrix. The membrane electrochemical potential is consumed in the reaction. The rate equation for AGC was derived assuming that it functions according to a random Ter-Ter mechanism [42]. By the Cleland classification [43] this means that substrate binding and product release occur in an arbitrary order (see Figure 10). We have assumed that the affinity of the enzyme for the substrate/product does not depend on the enzyme's state. In this case, dissociation constants for substrates and products are equal to the Michaelis constants. The mechanism includes two slow stages (with rate constants k_1 , k_{-1} , k_2 , k_{-2}) corresponding to reorientation of transporter with respect to the inner mitochondrial membrane. Using these assumptions

Figure 9 Time dependence of NADH production by α -ketoglutarate dehydrogenase reaction presented by experimental data points [35] and described by the system (5) with the following initial values (enzyme concentration was equal to 0.4 nM): 1, $KG_{in} = 0.1$ mM; $NAD = 1$ mM; $CoA = 0.25$ mM; $NADH = 0$; $SucCoA = 0$ (white squares); 2, 1.5 mM ADP was added at the seventh minute (black squares); 3, 1.5 mM ATP was added on the seventh minute (squares with oblique hatching).

and applying rapid equilibrium and quasi-steady state approaches we have derived the following rate equations for AGC:

$$V_{AGC} = AGC \cdot \frac{k_1 k_2 \frac{Glu_{out}}{K_m^{Glu_{out}}} \frac{Asp_{in}}{K_m^{Asp_{in}}} \frac{H_{out}}{K_m^{H_{out}}} - k_{-1} k_{-2} \frac{Glu_{in}}{K_m^{Glu_{in}}} \frac{Asp_{out}}{K_m^{Asp_{out}}} \frac{H_{in}}{K_m^{H_{in}}}}{\left(k_1 \frac{Glu_{out}}{K_m^{Glu_{out}}} \frac{Asp_{in}}{K_m^{Asp_{in}}} \frac{H_{out}}{K_m^{H_{out}}} + k_{-2} \right) \left(1 + \frac{H_{in}}{K_m^{H_{in}}} \right) \cdot \left(1 + \frac{Glu_{in}}{K_m^{Glu_{in}}} \right) \left(1 + \frac{Asp_{out}}{K_m^{Asp_{out}}} \right) + \left(k_1 \frac{Glu_{in}}{K_m^{Glu_{in}}} \frac{Asp_{out}}{K_m^{Asp_{out}}} \frac{H_{in}}{K_m^{H_{in}}} + k_2 \right) \left(1 + \frac{H_{out}}{K_m^{H_{out}}} \right) \cdot \left(1 + \frac{Glu_{out}}{K_m^{Glu_{out}}} \right) \left(1 + \frac{Asp_{in}}{K_m^{Asp_{in}}} \right)}. \quad (6)$$

Here, AGC is the concentration of the aspartate–glutamate carrier, $K_m^{Glu_{out}}, K_m^{Asp_{in}}, K_m^{H_{out}}, K_m^{Glu_{in}}, K_m^{Asp_{out}}, K_m^{H_{in}}$ are the Michaelis constants for intra- and extra-mitochondrial glutamate, aspartate and protons. Since transport of aspartate and glutamate is coupled with proton transport through the membrane, the reaction rate of AGC depends on transmembrane potential. We have assumed that all stages of the catalytic cycle associated with charge transfer across the membrane depend on potential. These are stages of carrier reorientation corresponding to glutamate and aspartate transfer across the membrane (reaction 1 of catalytic cycle characterized by rate constants k_1 and k_{-1} , see Figure 10) and stages of proton binding and release characterized by corresponding Michaelis constants. These parameters depend on electric potential [44, 45]:

$$\begin{aligned} K_m^{H_{in}} &= K_{m,0}^{H_{in}} \times e^{\delta_1 \frac{\Delta\psi}{RT/F}}; K_m^{H_{out}} = K_{m,0}^{H_{out}} \times e^{-\delta_3 \frac{\Delta\psi}{RT/F}}; k_1 \\ &= k_1^0 \times e^{(1-\alpha) \delta_2 \frac{\Delta\psi}{RT/F}}; k_{-1} = k_{-1}^0 \times e^{-\alpha \delta_2 \frac{\Delta\psi}{RT/F}}. \end{aligned}$$

Where $\Delta\psi > 0$ is a transmembrane potential; $\dot{O}$ is the absolute temperature; R is the universal gas constant; F is the Faraday's constant; δ_i is a part of the potential, consumed by the i th stage; α is a part of the potential that influences the reverse reaction. We have assumed that $\delta_1 = 0.1$, $\delta_2 = 0.8$, $\delta_3 = 0.1$, and $\alpha = 0.5$. Values of the Michaelis constants for substrates and products have been taken from the literature [42]. The remaining parameters were unknown

Figure 10 The scheme of the aspartate–glutamate carrier catalytic cycle.

Figure 11 Aspartate–glutamate carrier initial rate dependence on the aspartate concentration presented by experimental data points (obtained from submitochondrial particles with transmembrane potential of 180 mV) [23] and by rate equation (6) in the following conditions (enzyme concentration in submitochondrial particles was defined to be 0.4 μM): 1, $\text{Glu}_{\text{in}} = 0$; $\text{Glu}_{\text{out}} = 96.25$ mM; $\text{pH}_{\text{out}} = 7.2$; $\text{pH}_{\text{in}} = 7.5$ (black squares); 2, $\text{Glu}_{\text{in}} = 0$; $\text{Glu}_{\text{out}} = 96.25$ mM; $\text{pH}_{\text{out}} = 7.2$; $\text{pH}_{\text{in}} = 6$; (white squares); 3, $\text{Glu}_{\text{in}} = 1$ mM; $\text{Glu}_{\text{out}} = 95.75$ mM; $\text{pH}_{\text{out}} = 7.2$; $\text{pH}_{\text{in}} = 7.4$; (squares with oblique hatching); 4, $\text{Glu}_{\text{in}} = 0$; $\text{Glu}_{\text{out}} = 61.25$ mM; $\text{pH}_{\text{out}} = 7.2$; $\text{pH}_{\text{in}} = 6$; (squares with horizontal hatching).

and have been estimated from fitting the model to experimental data [23] (parameters values are listed in Table II). Dependences of initial rates of aspartate influx into submitochondrial particles have been measured at different pH values and glutamate concentrations. Figure 11 demonstrates that experimental data from [23] (symbols) and theoretical curves generated by the rate equation (6) closely coincide. Two parameters could not be estimated from *in vitro* data: the concentration of AGC in mitochondria and the Michaelis constant for external protons.

Aspartate aminotransferase (AspAT)

AspAT catalyzes transamination of glutamate and oxaloacetate with formation of α -ketoglutarate and aspartate. AspAT kinetics was described using a Ping Pong Bi-Bi mechanism [46] (see scheme of catalytic cycle in Figure 12).

It was assumed that the mechanism includes two slow stages: the first corresponds to the transformation of glutamate to α -ketoglutarate (reaction 1 in Figure 12) and the second corresponds to formation of aspartate from oxaloacetate (reaction 2 in Figure 12). Using

Figure 12 Scheme of catalytic cycle of aspartate aminotransferase (taken from [46]).

Figure 13 Aspartate aminotransferase initial rate dependence on the concentration of α -ketoglutarate presented by experimental data points [47] and described by the curves according to the rate equation (3) with the following concentrations of aspartate (enzyme concentration was defined to be 1 mM): 1, 50 mM (black squares); 2, 5 mM (white squares); 3, 2 mM (squares with oblique hatching); 4, 1 mM (dotted squares); 5, 0.5 mM (squares with horizontal hatching).

these assumptions and applying rapid equilibrium and quasi-steady state approaches, we have derived the following rate equation for AspAT:

$$V_{\text{AspAT}} = \text{AspAT} \cdot \frac{k_f^2 \frac{\text{OAA}}{K_m^{\text{OAA}}} \frac{\text{Glu}_{\text{in}}}{K_m^{\text{Glu}_{\text{in}}}} - k_r^2 \frac{\text{Asp}_{\text{in}}}{K_m^{\text{Asp}_{\text{in}}}} \frac{\text{KG}_{\text{in}}}{K_m^{\text{KG}_{\text{in}}}}}{\left(k_f \frac{\text{Glu}_{\text{in}}}{K_m} + k_r \frac{\text{Asp}_{\text{in}}}{K_m} \right) \left(1 + \frac{k_r}{k_{-1}} \frac{\text{KG}_{\text{in}}}{K_m} + \frac{k_1 - k_r}{k_1} \frac{\text{OAA}}{K_m} \right) + \left(k_r \frac{\text{KG}_{\text{in}}}{K_m} + k_f \frac{\text{OAA}}{K_m} \right) \left(1 + \frac{k_f}{k_1} \frac{\text{Glu}_{\text{in}}}{K_m} + \frac{k_{-1} - k_r}{k_{-1}} \frac{\text{Asp}_{\text{in}}}{K_m} \right)}. \quad (7)$$

We have obtained the following constraints from this equation:

$$k_1 > k_f; k_{-1} > k_r.$$

Here, AspAT is the concentration of aspartate aminotransferase; K_m^{OAA} , $K_m^{\text{Asp}_{\text{in}}}$, $K_m^{\text{Glu}_{\text{in}}}$, $K_m^{\text{KG}_{\text{in}}}$ are the Michaelis constants for substrates and products; k_f , k_r are turnover numbers in forward and reverse directions, k_1 , k_{-1} are rate constants for individual reaction steps (see catalytic cycle in Figure 12). Parameters known from literature are AspAT [38], $K_m^{\text{Asp}_{\text{in}}}$, $K_m^{\text{KG}_{\text{in}}}$, k_r [47], K_m^{OAA} [32], K_{eq} [31]. The parameter k_{-1} value has been estimated from experimental data [47] where dependence of the initial rate of the reverse reaction on α -ketoglutarate concentration has been measured at different concentrations of aspartate. All parameter values are listed in Table II. Figure 13 demonstrates good fitting of experimental data from [47] (symbols) to theoretical curves generated by Equation (7).

Other parameters ($K_m^{\text{Glu}_{\text{in}}}$, k_f and k_1) could not be estimated from the available *in vitro* data.

Succinate thiokinase (STK)

STK catalyzes the reaction of succinyl-CoA decomposition coupled with GDP phosphorylation. The catalytic mechanism of the STK reaction has been published in [48]. However, we have found that the rate equation derived in accordance with the mechanism could not describe reciprocal plots of initial velocities versus reciprocal concentrations of GDP and CoA measured with four fixed concentrations of phosphate (Figures 1 and 9 from [48]). To avoid these discrepancies we have suggested that the STK kinetics should be described in

accordance with a Random Bi Uni Uni Bi mechanism (see Figure 14). According to this mechanism, substrates GDP and SucCoA bind to the enzyme in a random order. The decomposition of SucCoA occurs at the catalytic site followed by succinate release. The next step of the catalytic cycle is phosphate binding followed by GDP phosphorylation and release of CoA and GTP in random order. The catalytic cycle depicted in Figure 14 has two dead-end complexes, E-GDP-CoA and E-SucCoA-GTP. The rate equation derived in accordance with the new mechanism allows us to describe almost all reciprocal plots of initial velocities versus substrates from [48]:

$$V_{\text{STK}} = \frac{\text{STK} \left(k_1 k_2 \frac{\text{SucCoA}}{K_{\text{E-GDP-SucCoA}}} \frac{\text{GDP}}{K_{\text{GDP}}^{\text{GDP}}} \frac{\text{P}}{K_{\text{P}}^{\text{P}}} - k_{-1} k_{-2} \frac{\text{Suc}}{K_{\text{E-Suc}}^{\text{Suc}}} \frac{\text{GTP}}{K_{\text{E-GTP-CoA}}^{\text{GTP}}} \frac{\text{CoA}}{K_{\text{E-CoA}}^{\text{CoA}}} \right)}{\left(k_1 \frac{\text{SucCoA}}{K_{\text{E-GDP-SucCoA}}} \frac{\text{GDP}}{K_{\text{GDP}}^{\text{GDP}}} + k_{-2} \frac{\text{GTP}}{K_{\text{E-GTP-CoA}}^{\text{GTP}}} \frac{\text{CoA}}{K_{\text{E-CoA}}^{\text{CoA}}} \right) \left(1 + \frac{\text{Suc}}{K_{\text{E-Suc}}^{\text{Suc}}} + \frac{\text{P}}{K_{\text{P}}^{\text{P}}} \right)} + \left(1 + \frac{\text{GDP}}{K_{\text{GDP}}^{\text{GDP}}} + \frac{\text{SucCoA}}{K_{\text{E-GDP-SucCoA}}} + \frac{\text{SucCoA}}{K_{\text{E-GDP-SucCoA}}} \frac{\text{GDP}}{K_{\text{GDP}}^{\text{GDP}}} + \frac{\text{GDP}}{K_{\text{E-GDP}}^{\text{GDP}}} \frac{\text{CoA}}{K_{\text{E-GDP-CoA}}^{\text{CoA}}} + \frac{\text{CoA}}{K_{\text{E-CoA}}^{\text{CoA}}} \right. \quad (8)$$

$$\left. + \frac{\text{GTP}}{K_{\text{E-GTP}}^{\text{GTP}}} + \frac{\text{GTP}}{K_{\text{E-GTP-CoA}}^{\text{GTP}}} \frac{\text{CoA}}{K_{\text{E-CoA}}^{\text{CoA}}} + \frac{\text{SucCoA}}{K_{\text{E-GTP}}^{\text{GTP}}} \frac{\text{GTP}}{K_{\text{E-GTP-SucCoA}}^{\text{GTP}}} \right) \left(k_2 \frac{\text{P}}{K_{\text{P}}^{\text{P}}} + k_{-1} \frac{\text{Suc}}{K_{\text{E-Suc}}^{\text{Suc}}} \right).$$

Here, STK is a concentration of succinate thiokinase, and k_1, k_{-1}, k_2, k_{-2} are rate constants; K_{E}^{S} is the dissociation constant for compound S from enzyme form E. Parameters known from the literature are: $K_{\text{m}}^{\text{CoA}}, K_{\text{m}}^{\text{Suc}}, K_{\text{m}}^{\text{SucCoA}}, K_{\text{m}}^{\text{GDP}}, K_{\text{m}}^{\text{GTP}}, K_{\text{m}}^{\text{P}}$ – the Michaelis constants for CoA, Suc, SucCoA, GDP, GTP and P [48]; catalytic constant k_f [49]; and the equilibrium constant K_{eq} [50]. Using the approach suggested in [51] we have expressed a number of parameters from Equation (8) in terms of kinetic parameters known from the literature. Rate constants k_2 and k_{-1} have been expressed from k_f and K_{eq} values: $k_2 = \frac{k_f k_1}{k_1 - k_f}$; $k_{-1} = \frac{k_{-2} \sqrt{W}}{1 - \sqrt{W}}$ where

$$W = \frac{k_f^3 K_{\text{m}}^{\text{Suc}} K_{\text{m}}^{\text{GTP}} K_{\text{m}}^{\text{CoA}}}{K_{\text{eq}} k_1 k_{-2} K_{\text{m}}^{\text{SucCoA}} K_{\text{m}}^{\text{GDP}} K_{\text{m}}^{\text{P}}}.$$

These expressions have given us the following constraints for the parameters: $k_1 > k_f$, $W > 1$.

Figure 14 The scheme of the succinate thiokinase catalytic cycle.

Six dissociation constants have been expressed through Michaelis constants:

$$\begin{aligned} K_{E-GDP-SucCoA}^{SucCoA} &= K_m^{SucCoA} \frac{k_1 + k_2}{k_2}; K_E^{GDP} = K_m^{GDP} \frac{k_1 + k_2}{k_2}; K_{E-GDP-CoA-P}^P \\ &= K_m^P \frac{k_1 + k_2}{k_2}; K_E^{Suc} = K_m^{Suc} \frac{k_{-1} + k_{-2}}{k_{-2}}; K_{E-GTP-CoA}^{GTP} \\ &= K_m^{GTP} \frac{k_{-1} + k_{-2}}{k_{-2}}; K_E^{CoA} = K_m^{CoA} \frac{k_{-1} + k_{-2}}{k_{-2}}. \end{aligned}$$

Taking into account these relationships we have decreased the number of unknown parameters of Equation (8) from 14 to 6. The remaining undetermined parameters were: k_1 , k_{-2} , $K_{E-SucCoA}^{SucCoA}$, $K_{E-GDP-CoA}^{CoA}$, K_{E-GTP}^{GTP} , $K_{E-GTP-SucCoA}^{SucCoA}$. We have estimated their values from experimental data [48] where dependences of the initial rate of succinate thiokinase on substrates and products have been measured. Moreover, the fitting of the rate equation (8) to experimental data has allowed us to identify Michaelis constants for substrates and products more precisely (see Table II). Figure 15 demonstrates that experimental data from [48] (symbols) and theoretical curves generated by Equation (8) closely coincide.

Succinate dehydrogenase (SDH)

SDH catalyzes the reaction of succinate oxidation to fumarate coupled with reduction of ubiquinone to ubiquinol. The enzyme is described according to Random Bi-Bi mechanism. Applying a rapid equilibrium approach, the following rate equation has been derived:

$$V_{SDH} = SDH \frac{k_f \frac{Suc}{K_{E-Suc}^{Suc}} \frac{Q}{K_m^Q} - k_r \frac{Fum}{K_{E-Fum}^{Fum}} \frac{QH_2}{K_m^{QH_2}}}{1 + \frac{Suc}{K_{E-Suc}^{Suc}} + \frac{Q}{K_m^Q} + \frac{K_m^{Suc}}{K_{E-Suc}^{Suc}} + \frac{Fum}{K_{E-Fum}^{Fum}} + \frac{QH_2}{K_m^{QH_2}} + \frac{K_m^{Fum}}{K_{E-Fum}^{Fum}} + \frac{Fum}{K_{E-Fum}^{Fum}} \frac{QH_2}{K_m^{QH_2}}} \quad (9)$$

Here, SDH is the concentration of succinate dehydrogenase; k_f , k_r are turnover numbers in the forward and reverse directions; K_E^S is the dissociation constant for compound S from enzyme form E; K_m^Q , $K_m^{QH_2}$, K_m^{Suc} , K_m^{Fum} are Michaelis constants for ubiquinone, ubiquinol, succinate and fumarate. The values of all parameters of Equation (9) are available from the

Figure 15 Succinate thiokinase initial rate dependence on the concentration of substrates and products presented by experimental data points [48] and described by the curves according to the rate equation (8) under the following conditions (enzyme concentration was defined to be 0.05 μ M): (a) GDP = 0.05 mM; SucCoA, mM: 1, 0.05 (white squares); 2, 0.03 (black squares); 3, 0.02 (squares with oblique hatching); (b) Suc = 1 mM; CoA = 0.1 mM (white squares).

Figure 16 Succinate dehydrogenase initial rate dependence on the concentration of substrate succinate presented by experimental data points [52] and described by the curves according to the rate equation (9) with 20 μM malonate ($K_i = 1.2 \mu\text{M}$ [34]) and the following concentrations of the second substrate phenasinemethosulfate (enzyme concentration in submitochondrial particles was defined to be 0.1 mM): 1, 250 μM (white squares); 2, 160 μM (black squares); 3, 50 μM (squares with oblique hatching); 4, 20 μM (dotted squares); 5, 10 μM (squares with vertical hatching).

literature (see Table II). However, having fitted the rate equation (9) to experimental data published in [52] where dependences of SDH initial rate on succinate concentration have been measured at different concentrations of phenasine methosulfate (acceptor of electron analogous to ubiquinone) and malonate (inhibitor competitive to succinate) we found that values of K_m^{Suc} and $K_{E-\text{Suc}}^{\text{Suc}}$ had to be changed substantially (see Table II) to provide the best coincidence between experimental data and the theoretical curves shown in Figure 16.

It was found [53] that the mitochondrial succinate dehydrogenase is strongly inhibited by oxaloacetate. To take this fact into account we have assumed that catalytically active succinate dehydrogenase, SDH, could bind oxaloacetate to form a catalytically inactive complex, SDH-OAA. The process of SDH inactivation is described in accordance with mass action law:

$$V_{\text{ISDH}} = k_i \cdot \text{SDH} \cdot \text{OAA} - k_{-i} \cdot (\text{SDH} - \text{OAA}). \quad (10)$$

The values of the rate constants in Equation (10) have been taken from [34] (see Table II). Taking into account both rate equations (9) and (10) we have reproduced experimental time dependences of SDH-catalyzed succinate consumption [53] on different concentrations of oxaloacetate where the reaction has been started by addition of succinate dehydrogenase to the solution of substrates succinate and ubiquinone and time dependences of succinate consumption have been monitored at different concentrations of added oxaloacetate. To describe qualitatively these experiments, the following system of differential equations has been developed (Figure 17):

$$\begin{aligned} \frac{d\text{Suc}}{dt} &= -V_{\text{SDH}}; \quad \frac{dQ}{dt} = -V_{\text{SDH}}; \quad \frac{d\text{Fum}}{dt} = V_{\text{SDH}}; \quad \frac{d\text{QH}_2}{dt} = V_{\text{SDH}}; \\ \frac{d\text{SDH}}{dt} &= -V_{\text{ISDH}}; \quad \frac{d\text{OAA}}{dt} = -V_{\text{ISDH}}; \quad \frac{d(\text{SDH} - \text{OAA})}{dt} = V_{\text{ISDH}}. \end{aligned} \quad (11)$$

Here, V_{SDH} , V_{ISDH} are rate equations for succinate dehydrogenase, and for the process of its inactivation given by Equations (9) and (10); substrates, products and two forms of SDH

Figure 17 The SDH reaction scheme described by the system (11), constructed to reproduce time dependences of the succinate concentration in the succinate dehydrogenase reaction with added oxaloacetate.

(free and bound with OAA) are variables of the minimodel (11). Initial conditions for the system of differential equations (11) have been set in accordance with initial concentrations of substrates used experimentally [53]: Suc = 1.33 mM; Q = 10 μ M; Fum = 0 mM; QH₂ = 0 mM; SDH = 1.5 nM; SDH-OAA = 0.

The system of differential equations (11) has five conservation laws:

$$\text{Suc} + \text{Fum} = C_{\text{tot}}^{\text{SDH}} \quad (\text{conservation of four-carbon skeleton})$$

$$\text{Suc} + \text{QH}_2 = e_{\text{tot}}^{\text{SDH}} \quad (\text{conservation of electrons})$$

$$\text{Q} + \text{QH}_2 = Q_{\text{tot}}^{\text{SDH}} \quad (\text{conservation of ubiquinone})$$

$$\text{SDH} + \text{SDH-OAA} = \text{SDH}_{\text{tot}}^{\text{SDH}} \quad (\text{conservation of succinate dehydrogenase})$$

$$\text{OAA} + \text{SDH-OAA} = \text{OAA}_{\text{tot}}^{\text{SDH}} \quad (\text{conservation of oxaloacetate})$$

Values of $C_{\text{tot}}^{\text{SDH}}$, $e_{\text{tot}}^{\text{SDH}}$, $Q_{\text{tot}}^{\text{SDH}}$, $\text{SDH}_{\text{tot}}^{\text{SDH}}$, $\text{OAA}_{\text{tot}}^{\text{SDH}}$ can be calculated from initial conditions. Figure 18 demonstrates the reproduction of experimental time dependences from [53].

Fumarase (FUM)

The reaction in which fumarate is transformed to malate, catalyzed by fumarase, is described according to the Uni-Uni mechanism [54]:

$$V_{\text{FUM}} = \text{FUM} \frac{k_f \frac{\text{Fum}}{K_m^{\text{Fum}}} - k_r \frac{\text{Mal}_{\text{in}}}{K_m^{\text{Mal}_{\text{in}}}}}{1 + \frac{\text{Fum}}{K_m^{\text{Fum}}} + \frac{\text{Mal}_{\text{in}}}{K_m^{\text{Mal}_{\text{in}}}}} \quad (12)$$

Here, FUM is a concentration of fumarase; k_f , k_r are catalytic constants in the forward and reverse reactions; K_m^{Fum} , K_m^{Mal} are Michaelis constants for fumarate and malate.

Figure 18 Succinate concentration time dependence in succinate dehydrogenase reaction under the following conditions: 1, Suc = 1.33 mM, Q₂ = 10 μ M (ubiquinone homolog), OAA = 0; 2, Suc = 1.33 mM, Q₂ = 10 μ M, OAA = 0.6 μ M; 3, Suc = 1.33 mM, Q₂ = 10 μ M, OAA = 6 μ M.

Parameter values of Equation (12) are available from the literature [54] (see Table II). However, having fitted the rate equation (12) to experimental data published in [55], we have found that values of $K_m^{\text{Fum}}k_f$ had to be changed (see Table II) to provide good fit (Figure 19).

Malate dehydrogenase (MDH)

The mechanism of the enzyme was described according to the ordered Bi-Bi mechanism with enzyme–NAD complex isomerization [56]:

$$V_{\text{MDH}} = \frac{\text{MDH} \cdot k_f k_r (\text{Mal}_{\text{in}} \cdot \text{NAD} / K_{\text{eq}} - \text{NADH} \cdot \text{OAA})}{K_i^{\text{NADH}} K_m^{\text{OAA}} k_r + K_m^{\text{OAA}} k_r \cdot \text{NADH} + K_m^{\text{NADH}} k_r \cdot \text{OAA} + k_r \cdot \text{NADH} \cdot \text{OAA} + K_m^{\text{NAD}} k_f \cdot \text{Mal}_{\text{in}} / K_{\text{eq}} + K_m^{\text{Mal}} k_f \cdot \text{NAD} / K_{\text{eq}} + k_f \cdot \text{Mal}_{\text{in}} \cdot \text{NAD} / K_{\text{eq}} + K_m^{\text{NAD}} k_f \cdot \text{NADH} \cdot \text{Mal}_{\text{in}} / K_i^{\text{NADH}} K_{\text{eq}} + K_m^{\text{NADH}} k_r \cdot \text{OAA} \cdot \text{NAD} / K_i^{\text{NAD}} + k_r \cdot \text{NADH} \cdot \text{OAA} \cdot \text{Mal}_{\text{in}} / K_i^{\text{Mal}} + k_f \cdot \text{OAA} \cdot \text{Mal}_{\text{in}} \cdot \text{NAD} / K_i^{\text{OAA}} K_{\text{eq}}} \quad (13)$$

Here, MDH is the concentration of malate dehydrogenase; k_f , k_r are catalytic constants in forward and reverse directions; K_{eq} is equilibrium constant; K_m^{OAA} , K_m^{NADH} , K_m^{NAD} , $K_m^{\text{Mal}_{\text{in}}}$ are Michaelis constants for substrates and products; K_i^{OAA} , K_i^{NADH} , K_i^{NAD} , $K_i^{\text{Mal}_{\text{in}}}$ are inhibition constants for substrates and products. All parameter values from Equation (13) are available from the literature [56] (see Table II).

α -ketoglutarate–malate carrier (KMC)

KMC catalyzes the exchange of external malate for intra-mitochondrial α -ketoglutarate. The rate equation for KMC was derived by assuming that it functions according to the random Bi-Bi mechanism [57] (see Figure 20). The mechanism includes two slow stages (with rate constants k_1 , k_{-1} , k_2 , k_{-2}) corresponding to the reorientation of the transporter with respect to

Figure 19 Fumarase initial rate dependence on fumarate concentration described by the rate equation (10) and experimental data points from [55] (enzyme concentration was defined to be 0.15 μM).

Figure 20 The scheme of the α -ketoglutarate–malate carrier catalytic cycle.

the inner mitochondrial membrane. Using these assumptions and applying rapid equilibrium and quasi-steady state approaches we have derived the following rate equation for KMC:

$$V_{\text{KMC}} = \text{KMC} \frac{k_f^2 \frac{k_2}{k_1} \frac{\text{KG}_{\text{in}}}{K_m^{\text{KG}_{\text{in}}}} \frac{\text{Mal}_{\text{out}}}{K_m^{\text{Mal}_{\text{out}}}} - k_r^2 \frac{k_{-2}}{k_{-1}} \frac{\text{KG}_{\text{out}}}{K_m^{\text{KG}_{\text{out}}}} \frac{\text{Mal}_{\text{in}}}{K_m^{\text{Mal}_{\text{in}}}}}{\left(\frac{k_f^2}{k_1} \frac{\text{KG}_{\text{in}}}{K_m^{\text{KG}_{\text{in}}}} \frac{\text{Mal}_{\text{out}}}{K_m^{\text{Mal}_{\text{out}}}} + k_{-2} \right) \left(1 + \frac{k_r}{k_{-1}} \frac{\text{KG}_{\text{out}}}{K_m^{\text{KG}_{\text{out}}}} \left(1 + \frac{\text{KG}_{\text{out}}}{K_i^{\text{KG}_{\text{out}}}} \right) \right) \left(1 + \frac{k_r}{k_{-1}} \frac{\text{Mal}_{\text{in}}}{K_m^{\text{Mal}_{\text{in}}}} \right)} + \left(k_2 + \frac{k_r^2}{k_{-1}} \frac{\text{KG}_{\text{out}}}{K_m^{\text{KG}_{\text{out}}}} \left(1 + \frac{\text{KG}_{\text{out}}}{K_i^{\text{KG}_{\text{out}}}} \right) \frac{\text{Mal}_{\text{in}}}{K_m^{\text{Mal}_{\text{in}}}} \right) \left(1 + \frac{k_f}{k_1} \frac{\text{KG}_{\text{in}}}{K_m^{\text{KG}_{\text{in}}}} \right) \left(1 + \frac{k_f}{k_1} \frac{\text{Mal}_{\text{out}}}{K_m^{\text{Mal}_{\text{out}}}} \right)}. \quad (14)$$

Here, KMC is the concentration of the α -ketoglutarate–malate carrier; k_f , k_r are catalytic constants in forward and reverse directions; $K_m^{\text{KG}_{\text{in}}}$, $K_m^{\text{Mal}_{\text{out}}}$, $K_m^{\text{KG}_{\text{out}}}$, $K_m^{\text{Mal}_{\text{in}}}$ are Michaelis constants for substrates and products; k_1 , k_{-1} , k_2 , k_{-2} are rate constants of individual stages in the catalytic cycle of the carrier. Using the approach described in [51] we have expressed some of the parameters of this catalytic cycle in terms of kinetic parameters known from the literature:

$$k_2 = \frac{k_1 k_f}{k_1 - k_f}, k_{-2} = \frac{k_{-1} k_r}{k_{-1} - k_r}, k_{-1} = \frac{K_{\text{eq}}(k_1 - k_f) + k_f^3 K_m^{\text{KG}_{\text{out}}} K_m^{\text{Mal}_{\text{in}}} / k_r^2 K_m^{\text{KG}_{\text{in}}} K_m^{\text{Mal}_{\text{out}}}}{k_f^3 K_m^{\text{KG}_{\text{out}}} K_m^{\text{Mal}_{\text{in}}} / k_r^3 K_m^{\text{KG}_{\text{in}}} K_m^{\text{Mal}_{\text{out}}}}.$$

So we have obtained the following constraints from these equations:

$$k_1 > k_f; k_{-1} > k_r$$

The parameters known from the literature [57] are: k_f , k_r , $K_m^{\text{KG}_{\text{in}}}$, $K_m^{\text{Mal}_{\text{out}}}$, $K_m^{\text{KG}_{\text{out}}}$, $K_m^{\text{Mal}_{\text{in}}}$. The values of parameters k_1 and $K_i^{\text{KG}_{\text{out}}}$ have been estimated via fitting of the rate equation (14) to experimental dependences of the initial rate of KMC-catalyzed malate efflux on the concentrations of either internal malate or external α -ketoglutarate measured in [57] at different concentrations of the second substrate (Figure 21). The values of the parameters are listed in Table II.

Salicyl-CoA ligase (SCL)

It is known [26] that the mechanism of salicylate (Sal) activation in mitochondria coincides with that of fatty acid activation, and involves ATP consumption and pyrophosphate release

Figure 21 α-ketoglutarate-malate carrier initial rate dependence on substrate concentrations described by rate equation (14) and experimental data points from [57] with the following concentrations of the second substrate (enzyme concentration was defined to be 2 mM): (a) $KG_{out} = 4.84 \mu\text{M}$; (b) Mal_{in} : 1, 2 mM (squares with oblique hatching); 2, 4 mM (black squares); 3, 6 mM (white squares).

and is catalyzed by salicyl-CoA ligase. To describe the functioning of this enzyme we derived the following rate equation:

$$V_{SCL} = \frac{V_f \cdot \text{Sal} \cdot \text{CoA}}{K_m^{\text{Sal}} K_i^{\text{CoA}} + K_m^{\text{Sal}} \text{CoA} + K_m^{\text{CoA}} \text{Sal} + \text{CoA} \cdot \text{Sal}}.$$

Here, V_f is the maximal rate of salicyl-CoA ligase; K_m^{Sal} , K_m^{CoA} are the Michaelis constants for salicylate and CoA; K_i^{CoA} is the inhibition constant for CoA. Since the concentration of ATP in the mitochondrial matrix is much higher than corresponding Michaelis constant of salicyl-CoA ligase [58] we assume that the enzyme is in saturation with respect to ATP. This assumption allows us to ignore the dependence of the enzyme on ATP concentration. The values of parameters known from the literature are: V_f [26], K_m^{Sal} [4]. Because of the lack of experimental data on the dependence of salicyl-CoA ligase on CoA, we have assumed that both the value of Michaelis constant and the value of inhibition constant with respect to CoA for salicyl-CoA ligase were equal to that for acyl-CoA ligase. Parameters values are listed in Table II.

Salicyl-CoA: glycine acyltransferase (SGT)

SGT catalyzes the reaction of salicylurate formation from salicyl-CoA (SalCoA) and glycine. The rate law of the enzyme has been described by the following equation:

$$V_{SGT} = \frac{V_f \cdot \text{SalCoA} \cdot \text{Gly}}{K_m^{\text{SalCoA}} K_i^{\text{Gly}} + K_m^{\text{SalCoA}} \text{Gly} + K_m^{\text{Gly}} \text{SalCoA} + \text{SalCoA} \cdot \text{Gly}}.$$

Here, V_f is the maximal reaction rate; K_m^{SalCoA} , K_m^{Gly} are the Michaelis constants for the substrates; K_i^{Gly} is the inhibition constant for glycine (Gly). The values of the Michaelis constants and maximal reaction rate values have been estimated from the literature [26]. We also assumed that the inhibition constant for glycine was equal to its Michaelis constant (see Table II for values of these parameters).

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