Glucose and the ATP paradox in yeast

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A sustained decrease in the intracellular ATP concentration has been observed when extra glucose was added to yeast cells growing aerobically under glucose limitation. Because glucose degradation is the main source of ATP-derived free energy, this is a counter-intuitive phenomenon, which cannot be attributed to transient ATP consumption in the initial steps of glycolysis. We present a core model for aerobic growth in which glucose supplies carbon, as well as free energy, for biosynthesis. With Metabolic Control Analysis and numerical simulations, we demonstrate that the decrease in the ATP concentration can be reproduced if the biosynthetic route is more strongly activated by carbon substrates than is the catabolic (ATP-producing) route.

Key words: free energy, glucose pulse, Metabolic Control Analysis, model.

INTRODUCTION

In yeast, glucose acts as a source of free energy. Glucose is oxidized to ethanol (fermentative catabolism) or CO_2 (oxidative catabolism), and this is coupled with ATP synthesis via substrate-level phosphorylation or oxidative phosphorylation respectively. The branch between fermentative and oxidative growth occurs at the level of pyruvate. This metabolite is formed by glycolysis, and can either be converted into ethanol via pyruvate decarboxylase and alcohol dehydrogenase, or oxidized via the pyruvate dehydrogenase complex and the tricarboxylic acid cycle. Some of the intermediary metabolites are substrates for biosynthesis [1]. Pyruvate is a precursor for the synthesis of branched-chain amino acids and CoA. The first steps of glycolysis are coupled with ATP consumption, whereas the later steps yield more ATP than that invested in these first steps. This ‘turbo design’ enables glucose import at low extracellular concentrations, but requires sophisticated regulation [2].

In yeast, ATP is the source of most of the free energy to be invested in anabolism. On the other hand, Lagunas [3] showed that yeast growing on glucose, galactose or ethanol consumed more than 50 % of the ATP produced in catabolism to drive processes other than the production of biomass. It was proposed that micro-organisms need not be optimized solely for maximum energy efficiency, and it was shown that optimization for maximum growth rate might actually imply dissipation of excess ATP free-energy equivalents [4]. Another aspect concerning the concentration of ATP is much less well understood: when the supply of ammonia was reduced in a chemostat at a constant dilution rate, the glycolytic rate increased, with a concomitant decrease in the concentration of ATP [5].

However, a significant role of ATP remains that of acting as the free-energy mediator between catabolism and anabolism (i.e. the endergonic processes accompanying growth). The synthesis of ATP from ADP and inorganic phosphate is powered by the breakdown of glucose. In this regard, it is ATP (ATP/ADP/phosphate ratio) that sets the anabolic flux. In view of the role of glucose as the ultimate free-energy source, one might expect an increase in the internal ATP concentration when a shortage of extracellular glucose is relieved and the flux through catabolic and anabolic pathways is increased. This is, however, not always observed. When aerobically growing cells of *Saccharomyces cerevisiae* (baker’s yeast) were shifted from conditions of glucose limitation to glucose excess, the ATP level decreased by 40 % ([6,7]; also see below). The decreased ATP/ADP ratio was indicative of a decreased free-energy level. After a partial recovery in 30 s, the decrease remained stable for several minutes. The transient initial decrease has been attributed to the increase in phosphorylation of hexoses, stemming from the ‘turbo design’. The sustained decrease cannot be attributed to this design (the overall transit time for glycolysis in these experiments is approx. 10 s; [7]). Since there is an ample amount of phosphate present, one should expect a new steady state at increased flux through both catabolic and anabolic pathways to be accompanied by an increased ATP/ADP ratio, because it is the latter that should drive increased anabolism or further ATP consumption. No explanation has been offered for the decreased steady-state level of ATP. We shall refer to this phenomenon as the ATP ‘paradox’.

It has been shown that control in a metabolic network can be distributed [8]. The control of the ATP concentration could thus be a subtle function of the relative activation of catabolic and anabolic routes. Here, we investigate how this phenomenon affects the free-energy metabolism of the cell, and whether this is sufficient to solve the ATP paradox. First, we present new experimental results in which glucose addition causes a sustained fall in ATP concentration. Then, we introduce a core model for aerobic growth on glucose. We use Metabolic Control Analysis to determine how the ATP concentration changes in the core model after addition of extra glucose, and we extend our approach with numerical simulations. In the final section, we discuss the implications of our findings.

MATERIALS AND METHODS

Fermentation and experimentation

S. cerevisiae CENPK 113 7D was grown aerobically in a glucose-limited chemostat. The dilution rate was 0.1 h^{-1}. The growth medium was on the basis of that described in [9], but with double the amounts of trace elements, vitamins and salts, and a glucose concentration of 30 g/l. The airflow through the chemostat was 3 litres of air/litre of culture per min. A steady state was attained

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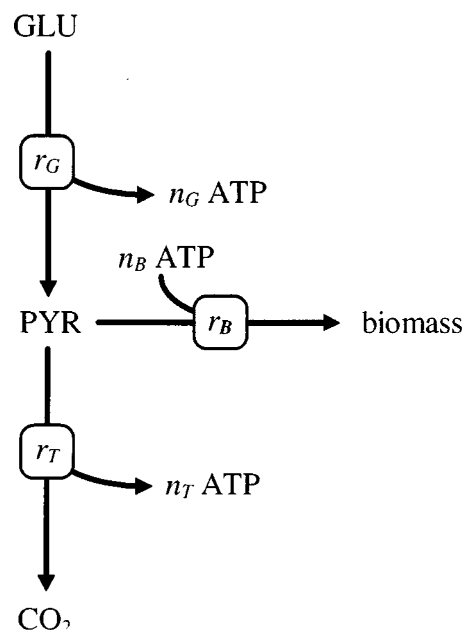
with a cell density of approx. 15 g dry weight/l. After the culture had reached a steady state, the glucose concentration in the chemostat was increased to approx. 2 g/l by addition of glucose. Samples were taken during the steady state and for 15 min after the addition of glucose, at 1 min intervals. Adenine nucleotides were extracted as described by Gonzalez et al. [10].

Chromatography

Samples were analysed for ATP, ADP and AMP by ion-pair reversed-phase HPLC with gradient elution. The HPLC system used consisted of a Waters Alliance 2690 Separations module (Waters, Milford, MA, U.S.A.), a 150 mm × 4.6 mm Supelcosil LC-18-T C18 (particle diameter $d_p = 3\ \mu m$) reversed-phase column with a 2 cm Supelguard LC-18-T guard column (Supelco, Bellefonte, PA, U.S.A.) and a Waters 440 UV-absorbance detector. The initial mobile phase (solvent A) was an aqueous buffer solution of 0.1 M KH_2PO_4 containing tetrabutylammonium bromide at a concentration of 4 mM (pH 6.0). The final mobile phase (solvent B) was a mixture of solvent A and methanol (70:30, v/v), with a pH value of 5.5. The total gradient time (including 5 min of conditioning with solvent A) was 18 min. The mobile-phase composition during the period of 18 min for the gradient was as follows: from zero time to 2.5 min, 100 % solvent A; from 2.5 to 7 min, linear gradient from 100 % solvent A to 100 % solvent B; from 7 to 12 min, 100 % solvent B; from 12 to 13 min, linear gradient from 100 % solvent B to 100 % solvent A; and from 13 to 18 min, 100 % solvent A. The flow rate was equal to 1 ml/min, and UV detection was employed at 254 nm.



Scheme 1 A core model for aerobic growth on glucose

Three essential routes are each represented by a single rate. Glycolysis (r_G) couples the conversion of glucose (GLU) into pyruvate (PYR) with the synthesis of n_G ATP. The tricarboxylic acid cycle (r_T) couples conversion of pyruvate into CO_2 to the synthesis of a further n_T ATP. Biosynthesis (r_B) couples conversion of pyruvate into biomass with the hydrolysis of n_B ATP. The concentrations of the external compounds (GLU, CO_2 and biomass) are regarded as constant in the model.

Sample pretreatment

Before injection, the dried samples (typically 0.1 g) were dissolved in 2 ml of milli-Q water, followed by centrifugation. The (clear) supernatant was set aside for analysis.

RESULTS

Figure 1 shows the results of an experiment where glucose was added to cells of *S. cerevisiae* while growing aerobically in a glucose-limited chemostat. Before the glucose addition, the ATP/ADP ratio was 1.5–2. In the first 10 s after the glucose was added, the ATP/ADP ratio fell by more than a factor of three. This was followed by an incomplete recovery in the subsequent 15 min. The above changes were caused by a fall in the ATP level. While the AMP level increased, the ADP level hardly changed, and the total concentration of adenine nucleotides decreased in the first minute after the glucose addition (results not shown). The mass-action ratio of the adenylate kinase did not change in the first minute after the glucose addition, but increased slightly (from 0.6 to 0.7) during the remainder of the experiment (results not shown).

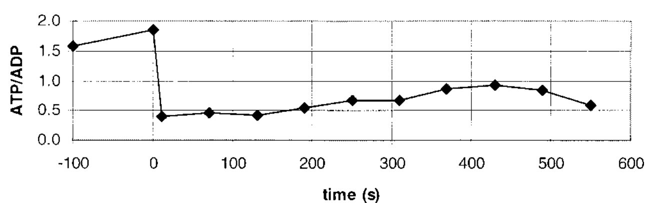


Figure 1 Experimentally observed ATP/ADP ratios before and after glucose was added to cells of *S. cerevisiae* growing aerobically in a glucose-limited chemostat

After glucose addition, at $t = 0$ s, the ATP/ADP ratio decreased by 70%. During the next 15 min, it partially recovered. For a description of the experimental details, see the Materials and methods section.

In the experiment shown in Figure 1, glucose addition was followed by a sustained decrease in the ATP/ADP ratio. This is in agreement with Theobald et al. [7]. The fall in the ATP/ADP ratio was more pronounced in our experiments. The partial transient recovery, on a time scale of 30 s, observed by Theobald et al. [7] was not observed in our experiments. This may be due to the longer time interval between the samples in Figure 1. The fact that the ATP/ADP ratio remained low during the experiment, which lasted five times longer than that of Theobald et al., supports our view that metabolism reached a new steady state. Larsson et al. [5] observed that, on a longer time-scale, i.e. after which enzyme concentrations have adjusted, the ATP concentration remained low, but the ATP/ADP ratio recovered. The partial recovery observed in our results might be the initial onset of the recovery observed by Larsson et al. [5]. However, it might also be due to a decrease in the extracellular glucose concentration. Our results are novel because they demonstrate that addition of a non-toxic amount of glucose may lead to a permanent (at least in terms of metabolism) decrease in the ATP/ADP ratio.

Assuming that the intracellular phosphate or pH or magnesium concentration had not changed significantly, the decrease in the ATP/ADP ratio shown in Figure 1 implies that the free energy of ATP hydrolysis had decreased because of glucose addition. As discussed in the Introduction, this is a counter-intuitive result, because more free energy was available to the cell in the form of a non-toxic concentration of extracellular glucose.

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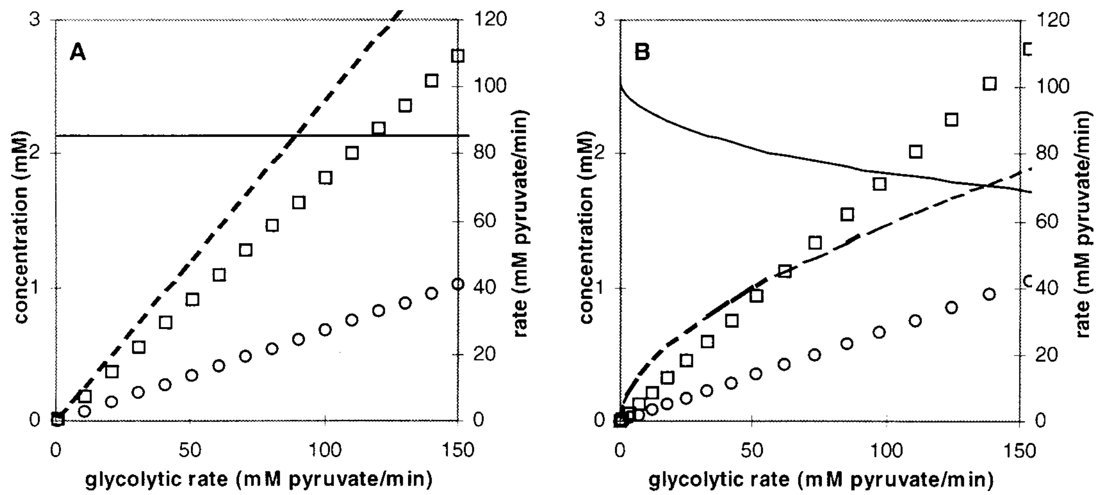

Figure 2 Simulated steady states for the core model of Scheme 1

The concentrations of ATP (continuous line) and pyruvate (broken line) and the fluxes of the tricarboxylic acid cycle (○) and biosynthesis (□) are plotted against the glycolytic flux. (**A**) Mass-action kinetics (eqn 5). (**B**) Increased Hill coefficient for the biosynthesis route (eqn 6). Parameters are described further in the text.

A core model

Since we aim to find a simple metabolic feature that solves the ATP paradox, we have employed a core model. This model includes the following three processes: (i) glycolysis converts glucose into pyruvate and produces ATP; (ii) the tricarboxylic acid cycle dissimilates pyruvate to CO_2 and produces further ATP; (iii) biosynthesis generates biomass from pyruvate, while hydrolysing ATP. As shown in Scheme 1, each process is represented by a single rate. For simplicity, biosynthesis from metabolites other than pyruvate is not included here.

The model presented here is too simple to represent all interactions between glycolysis, the tricarboxylic acid cycle and biosynthesis. For example, it cannot reproduce glycolytic oscillations [11,12] or the 'turbo effect' [2]. However, a model with three reactions and two intermediates is the simplest model that has the potential of reproducing the observed ATP decrease. The approach of replacing a pathway by a single reaction is justified for steady-state calculations if the intermediate metabolites do not affect other reactions. Therefore our conclusions may be extrapolated to a more detailed model. The implications of adding more pathways, such as ethanol production and ATPases, are discussed in the numerical section.

Note that ATP+ADP is conserved by all reactions in the model. To describe the experimentally observed decrease in the total amount of adenine nucleotides, a more complex model would be required, which is outside the scope of this paper and is not necessary to reach our aim of finding a simple metabolic feature that can reproduce the observed decrease in the ATP/ADP ratio. Because of the conservation of ATP+ADP, ADP is not included explicitly as a variable. Rather, a change of ATP levels is representative for ADP, and thus also for the free energy of ATP synthesis. The concentrations of pyruvate (PYR in this and subsequent equations and inequalities) and ATP should each obey a balance equation:

$$\frac{\mathrm{d}ATP}{\mathrm{d}t} = n_G r_G + n_T r_T - n_B r_B$$

$$\frac{\mathrm{d}PYR}{\mathrm{d}t} = r_G - r_T - r_B \quad (1)$$

where concentrations are expressed in italics, and rates are given in units of mol of pyruvate per time unit. The ATP stoichiometries n_G, n_T and n_B are assumed to be constant. The rates r_G, r_T and r_B depend on the concentrations of pyruvate, ATP and glucose (GLU in subsequent equations and inequalities). In the case of a particular steady state, these dependencies may be expressed with elasticity coefficients (ϵ_X^i), which correspond to the percentage by which a rate (r_i) changes, when the concentration of a metabolite (X) increases by 1% [13]. Away from equilibrium, and in the absence of co-operativity, the elasticity of a reaction for any of its substrates tends to range from 1 (at concentrations far below the Michaelis constant, K_m) to zero (at substrate saturation). The elasticity for its products is negative (except in the case of product stimulation). For the core model in Scheme 1, this implies:

$$\epsilon_{GLU}^G \geqslant 0\ (1) \quad \begin{matrix} \epsilon_{ATP}^G \leqslant 0\ (-4.5) & \epsilon_{ATP}^T \leqslant 0\ (-4.5) & \epsilon_{ATP}^B \geqslant 0\ (1) \\ \epsilon_{PYR}^G \leqslant 0\ (0) & \epsilon_{PYR}^T \geqslant 0\ (1) & \epsilon_{PYR}^B \geqslant 0\ (2) \end{matrix} \quad (2)$$

The numerical values between parentheses are extracted from the simulation in Figure 2(B) (at $GLU = GLU_0$), and serve as an illustration. Biomass and CO_2 are not included, because these do not significantly inhibit the pathways that produce them. The model presented here does not include enzyme synthesis or regulation. It can be used to determine the new steady state that is assumed by the metabolism after glucose addition.

Analytical results

Let us now determine how, in the above core model, the steady state changes with the glucose concentration. A basic property of the system in Scheme 1 is that the ratio between the steady-state fluxes (J_G, J_T and J_B) cannot change. Mathematically, this follows from the steady-state solution of eqn (1) (see Appendix, eqn A2). The explanation in physical terms is as follows. Addition of glucose should stimulate the glycolytic flux. The pyruvate concentration should then increase, which, in turn, should stimulate the tricarboxylic acid and biosynthetic fluxes. Initially, the three fluxes should not necessarily increase by the same percentage. If, for example, the elasticity of the biosynthesis

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pathway (for pyruvate) was larger than that of the tricarboxylic acid cycle, the former flux would increase by a larger percentage than the latter. This would, however, result in a net ATP consumption. The decreased ATP concentration would then stimulate the tricarboxylic acid cycle and inhibit biosynthesis, i.e. the change in the flux ratios would be reduced. The ATP decrease would continue until the ratio between the fluxes was the same as before the glucose addition.

In the above qualitative account to account for how the ratio between the steady-state fluxes is constant, we proposed that the ATP concentration should decrease, upon addition of glucose, if biosynthesis was more strongly stimulated by pyruvate than the tricarboxylic acid cycle. If realistic, this sequence of events should solve the ATP paradox that we address in this paper. The experimentally observed ATP decrease is counter-intuitive when glucose is regarded only as a source of free energy, but occurs naturally in the model presented here, where glucose supplies anabolic, as well as catabolic, routes for carbon. The concentrations of ATP and pyruvate adapt to maintain the ratio between the fluxes. Since the above argument is qualitative and hard to generalize, we shall substantiate it by a mathematical analysis and by numerical simulations.

To determine how the steady-state concentrations change upon addition of glucose, we consider an infinitesimal change of the glucose concentration. We apply Metabolic Control Analysis to calculate the co-response coefficients (${}_{GLU}O^{X}_{J_G}$), which express the percentage by which a steady-state concentration (X) changes when, as the result of a change in glucose concentration, the glycolytic flux (J_G) changes by 1% [13]. A straightforward relationship exists between the co-response coefficients and the elasticity coefficients introduced above (see eqn A5):

$$ {}_{GLU}O^{ATP}_{J_G} = \frac{\%\text{ change of } ATP}{\%\text{ change of } J_G} = \frac{\epsilon^{T}_{PYR} - \epsilon^{B}_{PYR}}{\epsilon^{T}_{PYR}\epsilon^{B}_{ATP} - \epsilon^{B}_{PYR}\epsilon^{T}_{ATP}} $$

$$ \left[= \frac{(1)-(2)}{(1)(1)-(2)(-4.5)} = -0.10 \right] $$

$$ {}_{GLU}O^{PYR}_{J_G} = \frac{\%\text{ change of } PYR}{\%\text{ change of } J_G} = \frac{\epsilon^{B}_{ATP} - \epsilon^{T}_{ATP}}{\epsilon^{T}_{PYR}\epsilon^{B}_{ATP} - \epsilon^{B}_{PYR}\epsilon^{T}_{ATP}} $$

$$ \left[= \frac{(1)-(-4.5)}{(1)(1)-(2)(-4.5)} = 0.55 \right] \quad (3) $$

The numerical value, which was obtained with the values from eqn (2), is positive for pyruvate and negative for ATP. The latter implies that, with the indicated set of elasticity coefficients, ATP should decrease with increasing glycolytic flux, upon addition of glucose. This confirms the above qualitative conclusion that our model provides a solution to the ATP paradox.

Our results can be extended further, since we can use eqn (3) to find conditions under which the experimental ATP decrease can occur. In each expression of eqn (3), the numerator and the denominator are a difference between two contributions. With the signs of the elasticity coefficients as given in eqn (2), one can see that the denominators are positive, and also that the co-response coefficient for pyruvate is positive. This implies that the pyruvate concentration can only increase with increasing glycolytic flux, independent of the kinetic parameters, provided that the elasticities do not change sign. The numerator in the co-response coefficient for ATP can change sign, depending on the relative magnitudes of the elasticity coefficients:

$$ {}_{GLU}O^{ATP}_{J_G} < 0 \quad \text{when } \epsilon^{B}_{PYR} > \epsilon^{T}_{PYR} \quad (4) $$

and vice versa. Thus our mathematical results confirm the above quantitative discussion. For straightforward kinetics, i.e. with signs of the elasticities as in eqn (2), the concentration of ATP decreases with increasing glycolytic flux if, and only if, the biosynthesis pathway is more sensitive ('elastic') to pyruvate than the tricarboxylic acid cycle. The result in eqn (4) also implies that the ATP paradox can only be solved by explicitly considering the fact that glucose provides the carbon for biosynthesis, or at least that an ATP-consuming pathway is sensitive to carbon metabolites. If this were not considered, the biosynthesis would be independent of pyruvate, so that the condition in eqn (4) could not be fulfilled.

The manner in which the concentrations of ATP and pyruvate depend on the glycolytic flux is expressed in eqn (3). Strictly speaking, this result can be only used to determine whether ATP and PYR increase upon addition of glucose, if the condition is fulfilled that the glycolytic flux increases with the (extracellular) glucose concentration. This condition is fulfilled for all experiments and calculations discussed in the present paper. Moreover, any steady state for which the glycolytic flux decreases with increasing glucose is unstable (see the Appendix, text below eqn A7). In practice, therefore, the above condition is always fulfilled.

Numerical results

So far we considered infinitesimal changes in the steady state. Let us now use numerical simulations to study finite changes, and to generalize the core model presented in Scheme 1. For simplicity, we shall assume here that all three routes are described by irreversible mass-action kinetics. In such a case, the rate constants can be obtained from the steady-state fluxes and concentrations without any fitting. This approach can also be applied to detailed kinetic models (D. Visser, R. T. J. M. van der Heijden, K. Mauch, M. Reuss and J. J. Heijnen, unpublished work). The balance of ATP and pyruvate is again given by eqn (1), with the following rate equations:

$$ r_G = J_{G0}\left(\frac{GLU}{GLU_0}\right)\left(\frac{ADP}{ADP_0}\right) $$

$$ r_T = J_{T0}\left(\frac{PYR}{PYR_0}\right)\left(\frac{ADP}{ADP_0}\right) $$

$$ r_B = J_{B0}\left(\frac{PYR}{PYR_0}\right)\left(\frac{ATP}{ATP_0}\right) \quad (5) $$

Here, GLU is a parameter that will be varied in the simulations, and $ADP = ADP_0 + ATP_0 - ATP$. The ADP dependence of r_G represents the stimulation by its overall substrate (ADP) and the inhibition by its overall product (ATP). The actual rate equation is more complex, since ATP is consumed in the upper part of glycolysis. Further parameters are taken from [7], pertaining to the steady state before extra glucose is added to the medium. The extracellular glucose concentration, GLU_0, is 0.07 mM. The intracellular concentrations are 2.1 mM, 0.47 mM and 0.79 mM for ATP_0, ADP_0 and PYR_0 respectively. The glycolytic flux (J_{G0} = 33.4 mM pyruvate/min) is derived from glucose uptake. The flux of (carbon usage for) biosynthesis (J_{B0} = 23.7 mM pyruvate/min) is derived from the growth rate and composition of the cells. The tricarboxylic acid flux (J_{T0} = 9.7 mM pyruvate/min) follows from the absence of ethanol production. The ATP stoichiometries (in mol ATP per mol pyruvate) are as follows: for glycolysis, $n_G = 1$; for the tricarboxylic acid cycle, $n_T = 7$, which corresponds to a P/O ratio of 1 [9]; and for biosynthesis, $n_B = 4$. This last value is adapted so as to balance consumption and production of ATP.

Steady-state solutions of eqn (5) were calculated with the program SCAMP [14]. For this particular set of rate equations,

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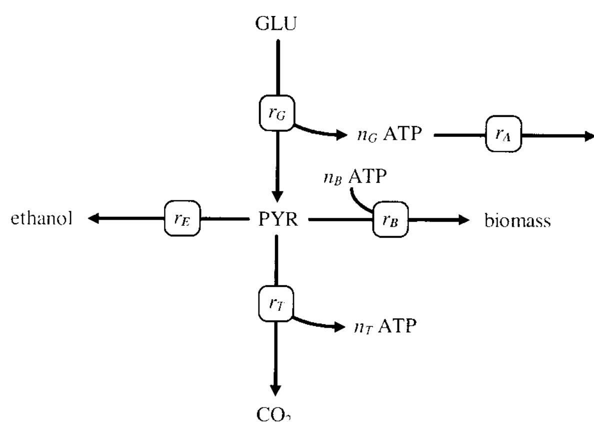

Scheme 2 Extension of the core model for aerobic growth on glucose with a reaction (r_E) that produces ethanol from pyruvate, and a reaction (r_A) that represents pyruvate-independent ATP consumption, e.g. by an ATPase

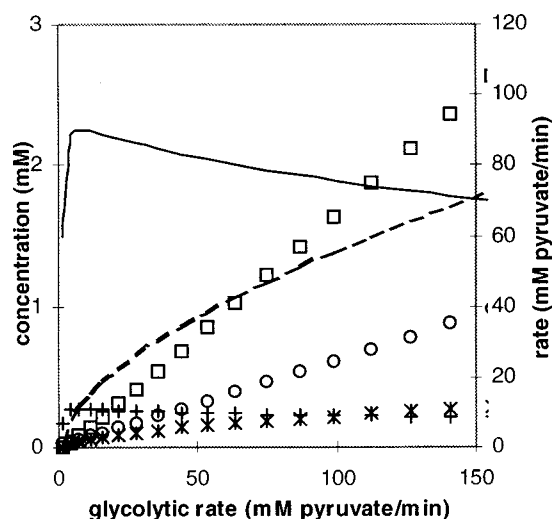

Figure 3 Simulated steady states for the core model adapted from Scheme 1, with added routes for ethanol production and ATP consumption

The concentrations of ATP (continuous line) and pyruvate (broken line) and the fluxes through the tricarboxylic acid cycle (○), biosynthesis (□), ethanol production (×) and the ATPase (+) fluxes are plotted against the glycolytic flux. The model is described by eqns (5–7), incorporating the modifications of eqn (7). Parameters are as described in the text.

an analytical solution is also possible. In Figure 2(A) the steady-state concentrations and fluxes are plotted against the glycolytic flux. Experimentally, ATP decreased by over 20% as the glycolytic flux increased by a factor of five ([7], and E. Esgalhado, O. J. G. Somsen, J. L. Snoep and H. V. Westerhoff, unpublished work). In our simulation (Figure 2A), the pyruvate concentration increased linearly, but the ATP level was not affected by the addition of glucose. This is in accordance with the analytical results in the previous section: since we used mass-action kinetics, biosynthesis and the tricarboxylic acid cycle are equally stimulated by an increase in pyruvate concentration. Consequently, a change in ATP is not required to maintain the constant flux ratios discussed in the previous section.

According to our analytical results, the experimentally observed ATP decrease can occur only when the biosynthesis route is more sensitive to pyruvate than is the tricarboxylic acid cycle. This may be achieved by increasing the Hill coefficient (h) of the former route, i.e. by adapting eqn (5) as follows:

$$r_B = J_{B0}\left(\frac{PYR}{PYR_0}\right)^h\left(\frac{ATP}{ATP_0}\right) \tag{6}$$

with $h = 2$. The results are shown in Figure 2(B). In this case, the ATP concentration decreased with increasing glycolytic flux. The pyruvate concentration increased, but the increase was no longer linear with the glycolytic flux. As J_G increased from J_{G0} to $5J_{G0}$, ATP decreased by 23%, which is sufficient to explain the experimental observations. We conclude that the experimentally observed ATP changes can be explained by including, in a simple model, the metabolic feature that glucose is a substrate for anabolic as well as catabolic routes, provided that the former route is more strongly stimulated by an increase in the pyruvate concentration than the latter. The tricarboxylic acid and biosynthesis fluxes are the same in both panels of Figure 2, and increase linearly with the glycolytic flux. This confirms the analytical result of the previous section, that the ratio between the fluxes is not affected by the glucose addition.

The constant ratio between the steady-state fluxes forms a basic property of our core model. However, when additional routes are active, such as the production of ethanol from pyruvate, or a pyruvate-independent ATP consumption (e.g. an ATPase), the flux ratio is no longer constant. To test whether our results remain valid in this more general case, we include the above two routes in our core model. This is shown in Scheme 2. The balance equations are modified as follows:

$$\frac{dATP}{dt} = n_G r_G + n_T r_T - n_B r_B - r_A$$

$$\frac{dPYR}{dt} = r_G - r_T - r_B - r_E$$

$$r_E = J_{E0}\left(\frac{PYR}{PYR_0}\right)$$

$$r_A = J_{A0}\left(\frac{ATP}{ATP_0}\right) \tag{7}$$

The other rate equations are as given in eqns (5) and (6). We use a value of J_{E0} of 5 mM pyruvate/min (for ethanol production) and a value of J_{A0} of 10 mM ATP/min (for ATP consumption). The results for the adapted core model are shown in Figure 3. The ethanol production increased but, in contrast with experimental results obtained previously ([7], and E. Esgalhado, O. J. G. Somsen, J. L. Snoep and H. V. Westerhoff, unpublished work), it remained low compared with the other fluxes. The results for ATP changes were similar to those shown in Figure 2(B), but the decrease was somewhat less pronounced. This can be explained as follows. Since the ATPase was taken as being insensitive to pyruvate, it reduces the overall pyruvate sensitivity of ATP consumption. This relaxes the condition of eqn (4), and reduces the ATP decrease. The effect was most strongly observed in the region of low glycolytic flux, i.e. when the ATPase dominated the ATP consumption. In this region, the ATP increased with increasing glucose. We conclude that, to predict ATP changes, one should consider the carbon substrate-dependence (here, pyruvate) of all ATP-producing and -consuming pathways.

DISCUSSION

In the present paper we have discussed the ATP paradox. This is the counter-intuitive experimental observation that the ATP/

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ADP ratio may decrease upon addition of glucose to the external medium, and that this decrease may last too long to be explained by transient ATP consumption in the initial steps of glycolysis.

First, we confirmed and extended the experimental observations of Theobald et al. [7]. We then set up a core model for aerobic growth in which glucose supplies both the carbon and the free energy for biosynthesis. The model describes how the steady state of the metabolism changes after addition of extra glucose, i.e. before enzyme synthesis and regulation can take effect. With Metabolic Control Analysis and numerical simulations, we determined that the experimentally observed ATP decrease can be reproduced if the biosynthesis pathway is more strongly activated by increases in the pyruvate concentration than is the tricarboxylic acid cycle.

Of course, the living cell is more complicated than the core model presented in this paper, and other solutions may be offered for the ATP paradox. Our results imply that the observed ATP decrease can be explained by simple metabolic effects, if the anabolic route is more sensitive to a carbon substrate than the catabolic route. This 'carbon substrate' does not have to be pyruvate. Other metabolites, e.g. from glycolysis or the tricarboxylic acid cycle, might cause qualitatively the same phenomena. Redox balancing might also be relevant, in which case the role of pyruvate is taken by NADH. Essential in our result is that, upon glucose addition, the ATP-consuming (anabolic) pathways are stimulated in other ways than via the ATP level, and more strongly than the ATP-producing (catabolic) pathways. The implication that anabolism is sensitive to its substrates may be remarkable because, most often, one considers only the regulation of these routes by demand for their products [15,16]. Our results might be significant for the design of detailed kinetic models ([17], and D. Visser, R. T. J. M. van der Heijden, K. Mauch, M. Reuss and J. J. Heijnen, unpublished work), since they indicate that the experimental behaviour of the ATP level can only be reproduced if the sensitivities of ATP-consuming routes are properly included.

The origin of the proposed substrate sensitivity of the anabolic route has not yet been established. Recent experiments have shown that the additional glucose is exported largely as ethanol, while the glycolytic flux increased by a factor of five ([7], and E. Esgalhado, O. J. G. Somsen, J. L. Snoep and H. V. Westerhoff, unpublished work). However, this effect does not offer a solution to the ATP paradox, since the increased glycolytic flux should create a net ATP production. For the ATP concentration to fall, this would have to be compensated for by an even stronger activation of an ATP-consuming process. This ATP consumption might be due to anabolism, i.e. a change in the growth rate. Such a change will, however, be difficult to observe, because the experiment is short compared with the generation time of the cells. van Urk et al. [18] demonstrated that the rate of biomass production in *S. cerevisiae* increased by less than 20 % in the first 30 min after a glucose pulse. This is not sufficient to compensate for the increased ATP production by fermentation. The ATP consumption might also be due to a stress response, e.g. a decrease in the intracellular pH [19,20].

Alternatively, the ATP decrease could be explained by inhibition of ATP production, e.g. due to inhibition of the tricarboxylic acid cycle and oxidative phosphorylation [21]. This possibility, which is known as the short-term Crabtree effect, is also accounted for by the explanation presented in this paper. Because the elasticity of the tricarboxylic acid cycle for carbon substrates would be negative, the condition in eqn (4) would be satisfied, at least as long as the elasticity of the biosynthesis route is positive. Addition of glucose would lead initially to a net ATP consumption, after which a decrease in ATP would restore the balance of ATP production and consumption. However, in the experiments quoted here respiration was not lowered by the glucose addition ([5,22], and E. Esgalhado, O. J. G. Somsen, J. L. Snoep and H. V. Westerhoff, unpublished work). Although further experiments are necessary, our results might provide an heuristic tool to investigate this problem. It remains to be seen whether the Pasteur effect, in which the ATP/ADP ratio falls upon anaerobiosis [23], can also be partly explained in similar terms.

Although we cannot yet provide a unique explanation for the observed ATP decrease after addition of glucose, our results do indicate that ATP-consuming routes are more strongly stimulated by carbon metabolites than ATP-producing routes. This does not mean that overall ATP consumption increases more than its production, which would be an inaccurate contention: because the system is at steady-state, ATP consumption and production are equal. When the former is more strongly stimulated by carbon metabolites than the latter, the ATP level must decrease to maintain a steady-state.

The ATP paradox is an example of a complex phenomenon, i.e. that a reaction might have a negative control on the concentration of its product. Such phenomena might also occur in a different context. Our results demonstrate that they can be explained by a model and calculation analogous to the one presented in this paper. Complex phenomena are not due to the properties of any individual enzyme or pathway, but rather to subtle interactions in a metabolic network. Such phenomena occur when two modules are connected by two intermediate variables. As we have demonstrated here, and elsewhere for a different scenario [2], such a system can respond counter-intuitively to parameter changes. Here we found that the level of an intermediate variable (the ATP/ADP ratio) may decrease, even though its production is activated. Such a phenomenon is possible in the presence of two or more intermediate variables. It could be understood by considering that metabolite concentrations must adapt to realize steady-state flux changes, i.e. that changing metabolite concentrations may also be the cause and not just the effect of flux changes.

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APPENDIX

We use Metabolic Control Analysis to determine how the steady-state concentrations of pyruvate (PYR) and ATP depend on the glucose concentration (GLU), for the core model of Scheme 1. We shall calculate response coefficients and co-response coefficients. The response coefficients (R^{X}_{GLU}) express the percentage by which a variable (X, usually a flux of concentration) changes, when GLU changes by 1 % [11]. The co-response coefficients (${}_{GLU}O^{X}_{J_G}$) express the percentage by which a variable changes when J_G changes by 1 %, as the result of a change of GLU [11].

A simple relation exists between response coefficients and co-response coefficients [11]:

$$ {}_{GLU}O^{ATP}_{J_G} = R^{ATP}_{GLU}/R^{J_G}_{GLU} \quad \text{and} \quad {}_{GLU}O^{PYR}_{J_G} = R^{PYR}_{GLU}/R^{J_G}_{GLU} \qquad \text{(A1)} $$

In the steady-state solution of eqn (1), the distribution of the glycolytic flux over the tricarboxylic acid cycle and the biosynthesis pathway is fixed by the ATP stoichiometries of the three pathways.

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$$J_T = pJ_G \quad J_B = (1-p)J_G \quad \text{with } p = \frac{n_B - n_G}{n_T + n_B} \tag{A2}$$

Since the ATP stoichiometries are constant, eqn (A2) implies that the flux co-response coefficients are equal to unity:

$$_{GLU}O^{J_T}_{J_G} = {}_{GLU}O^{J_B}_{J_G} = 1 \tag{A3}$$

By means of the elasticity coefficients, the flux co-response coefficients are related to those of the concentrations. Since the parameter *GLU* does not have a direct effect on the tricarboxylic acid cycle of the biosynthesis pathway, this can be expressed as:

$$\begin{pmatrix} _{GLU}O^{J_T}_{J_G} \\ _{GLU}O^{J_B}_{J_G} \end{pmatrix} = \begin{pmatrix} \epsilon^T_{ATP} & \epsilon^T_{PYR} \\ \epsilon^B_{ATP} & \epsilon^B_{PYR} \end{pmatrix} \begin{pmatrix} _{GLU}O^{ATP}_{J_G} \\ _{GLU}O^{PYR}_{J_G} \end{pmatrix} \tag{A4}$$

By solving eqns (A4) with (A3), we obtain the concentration co-response coefficients:

$$_{GLU}O^{ATP}_{J_G} = \frac{\epsilon^T_{PYR} - \epsilon^B_{PYR}}{\epsilon^T_{PYR}\epsilon^B_{ATP} - \epsilon^B_{PYR}\epsilon^T_{ATP}}$$

$$_{GLU}O^{PYR}_{J_G} = \frac{\epsilon^B_{ATP} - \epsilon^T_{ATP}}{\epsilon^T_{PYR}\epsilon^B_{ATP} - \epsilon^B_{PYR}\epsilon^T_{ATP}} \tag{A5}$$

Finally, we calculate the response coefficients with respect to the glucose concentration. Since the glycolytic pathway is directly affected by the glucose concentration, the relationship between the response coefficient of this flux, and that of the concentrations, is given by:

$$R^{J_G}_{GLU} = \epsilon^G_{ATP} R^{ATP}_{GLU} + \epsilon^G_{PYR} R^{PYR}_{GLU} + \epsilon^G_{GLU} \tag{A6}$$

By combining eqn (A6) with (A1), we obtain:

$$R^{J_G}_{GLU} = \frac{\epsilon^G_{GLU}}{1 - \epsilon^G_{ATP\,GLU}O^{ATP}_{J_G} - \epsilon^G_{PYR\,GLU}O^{PYR}_{J_G}}$$

$$\left(= \frac{(1)}{1 - (-4.5)(-0.10) - (0)(0.55)} = 0.69 \right) \tag{A7}$$

The numerical result in parentheses is obtained with the numerical values from eqns (2) and (3). The result can be expressed in terms of the elasticity coefficients by substituting eqn (A5), and the concentration response coefficients can be obtained by substituting eqn (A7) in eqn (A1). The result in eqn (A7) creates a potential problem. One would anticipate that the response coefficient is positive, i.e. that the glycolytic flux increases with the extracellular glucose concentration. This is, however, not the case; the second term in the denominator can be either positive or negative. If its magnitude is sufficiently large, the response coefficient in eqn (A7) may become negative. Although parameters can be found for which this is the case, this does not happen for any parameter set in this paper. Moreover, a stability analysis indicates that a negative response coefficient occurs only for unstable steady states. Therefore, the aforementioned problem does not arise in practice.

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