32 Assessing the control of fermentative free-energy metabolism in yeast: a modelling exploration

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Introduction

Free-energy metabolism (i.e., the synthesis and utilisation of ATP) is pivotal to all living systems. Its efficient regulation is important for living cells to survive and to adapt to changing circumstances such as reduced availability of nutrients. This means that ATP should be synthesised when needed by the cell, but that ATP synthesis should be inhibited under conditions of free-energy excess (high concentrations of ATP). In the parlance of control analysis, if the pathway fulfils its functional criterion to supply free energy when needed, the demand for ATP should control the glycolytic flux [1].

An experimental analysis of the control of fermentative free-energy metabolism is sorely needed because to this day textbook dogma pertains that the glycolytic flux is controlled by glycolytic enzymes themselves, with the irreversible steps of hexokinase, phosphofructokinase and pyruvate kinase being identified as "pacemakers" and regulatory enzymes owing to their allosteric effects (cf. [2]). However, single or pairwise overexpression of most of the glycolytic enzymes did not affect the glycolytic flux to any significant extent [3]. To explain this result, Hofmeyr argued that "steady-state glycolytic flux is controlled by reactions outside of what has traditionally been regarded as glycolysis; more specifically, it is controlled by those cellular processes that consume the key product of glycolysis, ATP" [1].

Baker's yeast (*Saccharomyces cerevisiae*) is a good model organism for performing a control analysis of fermentative free-energy metabolism, for two reasons: (i) Yeast is able to grow fermentatively in the absence of oxygen, converting

glucose to ethanol and carbon dioxide. Under these conditions, the system of free-energy supply is well-defined as it only comprises the reactions of the glycolytic pathway and the amount of available glucose will determine or limit the amount of ATP the yeast can generate. (ii) Being a microorganism, yeast is amenable to cultivation in liquid media and specifically in chemostat cultures. The chemostat enables the controlled growth of microorganisms at a defined rate under steady-state conditions, which is a prerequisite for doing controlled metabolic studies.

The sensitivities of the rates of the glycolytic supply and the demand for ATP towards the [ATP]/[ADP] ratio determine how glycolytic flux control is distributed between supply and demand, and the extent to which [ATP]/[ADP] ratio will be homeostatically buffered in response to a varying demand for ATP [1]. The control of yeast fermentative free-energy metabolism can thus be analysed and visualised by constructing a rate characteristic around ATP and ADP, i.e., by plotting in double-logarithmic space how both the ATP supply and ATP demand vary with the [ATP]/[ADP] ratio [1]. The intersection of the supply and demand curves signifies the steady state, and the elasticities of the supply and demand rates towards [ATP]/[ADP] can be read off directly from the slopes of the respective rate curves at the steady-state point. This in turn enables direct calculation of the flux- and ATP/ADP concentration-control coefficients of the supply and the demand reactions.

The supply rate characteristic can be determined by manipulating the demand and measuring the concomitant changes in glycolytic flux and [ATP]/[ADP]; likewise, the demand characteristic is given by the change in flux with [ATP]/[ADP] upon manipulation of the supply. This is the original double-modulation method of Kacser and Burns [4], which has since been extended to modular [5] and top-down [6] control analysis and co-response analysis [7]. All these approaches are in essence identical for a simple two-step linear pathway with a single metabolic intermediate.

Whilst the rate characteristics of ATP supply and demand are extremely informative, their experimental determination is no trivial matter. First, this requires independent manipulation of the supply and demand, and the ability to measure ATP, ADP and the glycolytic flux. Secondly, the system should be at steady state for each determination. The chemostat is an invaluable tool for such an analysis, as it enables the cultivation of microorganisms at steady state. However, as growth rate is set in a chemostat (it equals the dilution rate), independent manipulation of supply (glycolysis) and demand (growth) is difficult and requires an indirect approach [8,9]. Here we describe a strategy for performing an experimental ATP supply-demand analysis in fermenting yeast, illustrating our argument with a kinetic model.

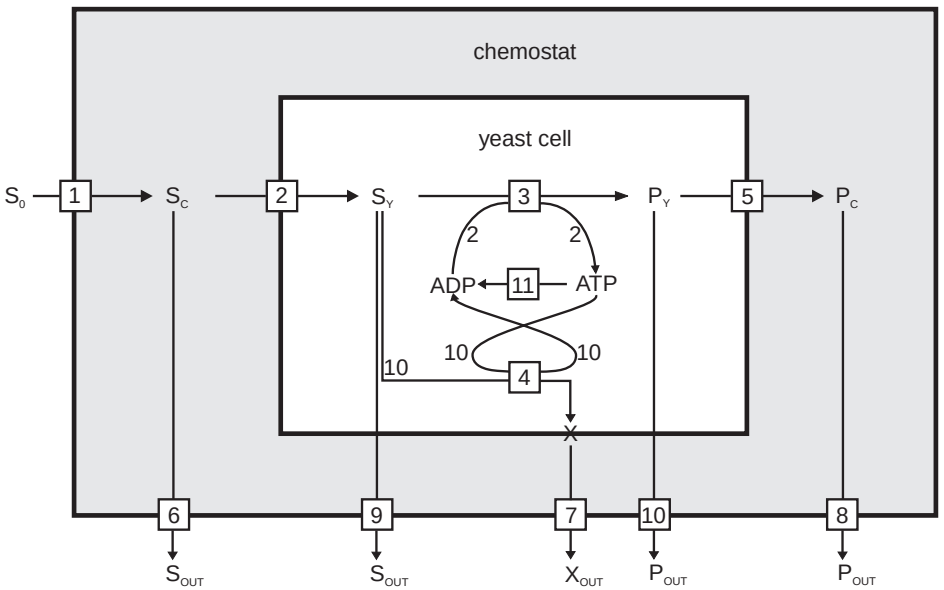

Fig. 32.1 "Open-box" model of yeast growing in a chemostat. The shaded part represents the extracellular growth medium in the chemostat; the inner box represents the intracellular environment. Reactions: *1,* substrate (glucose) feed from medium reservoir; *2,* substrate (S) uptake; *3,* glycolysis; *4,* growth; *5,* product (P) export; *6,* washout of substrate from chemostat; *7,* washout of biomass (X); *8,* washout of product from chemostat; *9 and 10,* washout of intracellular substrate and product, respectively, as a result of biomass washout; *11,* non-growth ATP demand. Subscripts: OUT, external (waste), C, chemostat; Y, yeast. Stoichiometric coefficients are indicated next to the arrows (reactions 3 and 4). The activities of the cellular reactions (2–5 and 11) are proportional to the amount of biomass (x). Inside the yeast cell, reactions 3 and 5 constitute the supply of ATP, while reactions 4 and 11 constitute the demand for ATP. Reaction 2 cannot be uniquely assigned to the supply or demand in this model, as S_Y is used for both glycolysis and growth (see discussion in main text).

Methods

To devise our experimental strategy, we used a kinetic model of yeast growing in a chemostat (Fig. 32.1). The model treats internal cellular reactions explicitly ("open-box") and makes use of our newly developed method for modelling compartmentalised systems with variable volumes (Hofmeyr, J.-H.S., Snoep, J.L. and Rohwer, J.M., in preparation).

The core model in Fig. 32.1 includes the most important cellular reactions relevant to this discussion, *viz.* uptake of substrate, glycolysis, export of product, growth and other ATP demand processes. Full details will be described elsewhere.

Numerical simulation, steady-state calculations and control analysis were performed with the computer program WINSCAMP [10,11].

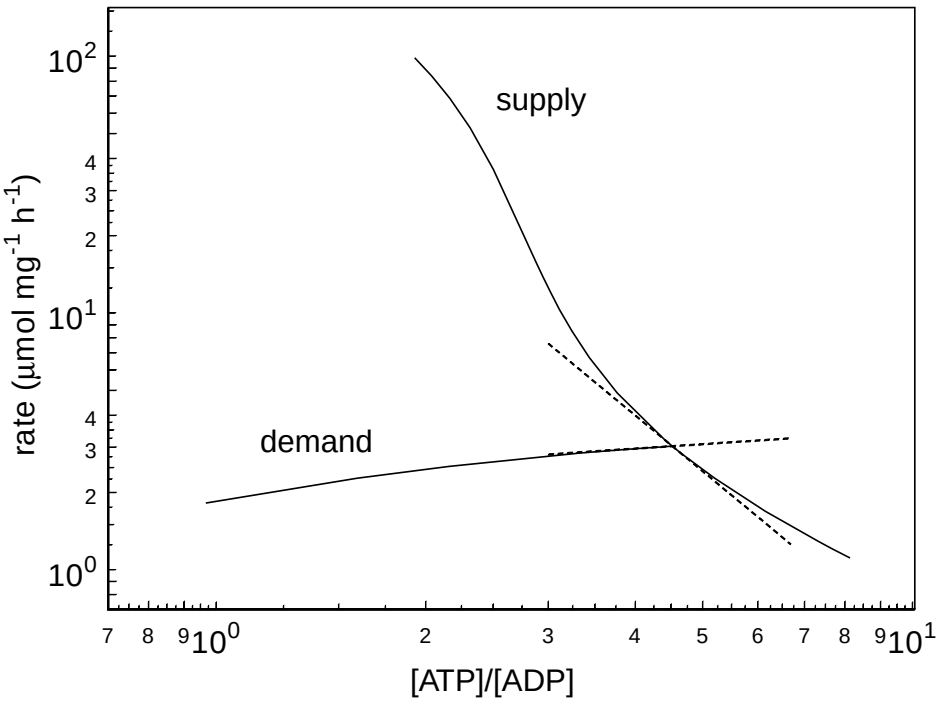

Fig. 32.2 Rate characteristics of ATP supply and demand. Calculations were performed with the kinetic model as described in the text. The intersection of the "supply" and "demand" curves indicates the reference steady state. The dotted lines are tangents to the supply and demand curves at the steady-state point. Because the graph is in double-logarithmic space, the slopes of the tangents equal the elasticities of supply and demand, respectively, with respect to the [ATP]/[ADP] ratio.

Results and discussion

To construct the rate characteristics of free-energy supply and demand with respect to the [ATP]/[ADP] ratio, we performed a number of manipulations on the kinetic model and calculated the resultant steady states. In the first *in silico* experiment, the value of k_{cat11} (i.e., the rate constant of the non-growth ATP demand) was increased over a 100-fold range, simulating an increased demand of ATP. In an experimental setup such a manipulation can be achieved by the addition of benzoic acid (a weak organic acid) to the culture medium, which acts as an uncoupler and results in an increased ATP expenditure to maintain the proton gradient across the plasma membrane [12]. We then correlated the steady-state ATP supply flux (2 times the glycolytic flux J_3) with the calculated [ATP]/[ADP] ratio; the result is shown by the "supply" curve in Fig. 32.2.

The supply curve in Fig. 32.2 will only be a valid ATP supply rate characteristic if changes in the demand are communicated to the supply block only through the [ATP]/[ADP] ratio. However, Fig. 32.1 shows that a second variable (i.e., s_y,

the concentration of S in the yeast cell) links reactions 11 and 3 in the sense that changes in k_{cat11} can be transmitted to reaction 3 via v_4 and resulting changes in s_y. To circumvent this problem, we adjusted the dilution rate after each manipulation of k_{cat11} until s_y returned to its original value while constructing the supply rate characteristic in Fig. 32.2.

In the second *in silico* experiment we manipulated the ATP supply to investigate the dependence of the demand on the [ATP]/[ADP] ratio. To this end, k_{cat3} (the rate constant of the glycolytic reaction) was manipulated over a range. Experimentally, such a manipulation could be achieved by altering the expression level of one or more of the glycolytic enzymes. In addition to the supply and demand being linked not only by ATP and ADP, but also by S_Y, the growth rate (v_4) (as part of the ATP demand) is *fixed* by the dilution rate set in the chemostat. To circumvent this, we adjusted the dilution rate after each manipulation of k_{cat3} until s_y returned to its original value, using a similar approach as in the manipulations of k_{cat11} above. The total ATP demand ($10 \times J_4 + J_{11}$) was then correlated to the [ATP]/[ADP] ratio (the "demand" curve in Fig. 32.2).

The elasticities of supply and demand to the [ATP]/[ADP] ratio could be read off from the slopes of the supply and demand rate characteristics (indicated by dotted lines): the supply elasticity was −2.25 and the demand elasticity 0.18. Using these values, the flux-control coefficients were calculated using the well-known identities:

$$C^J_{\text{supply}}\Big|_{\text{rate characteristic}} = \frac{\varepsilon^{v_{\text{demand}}}_{atp/adp}}{\varepsilon^{v_{\text{demand}}}_{atp/adp} - \varepsilon^{v_{\text{supply}}}_{atp/adp}} = 0.07$$

$$\text{and} \quad C^J_{\text{demand}}\Big|_{\text{rate characteristic}} = \frac{-\varepsilon^{v_{\text{supply}}}_{atp/adp}}{\varepsilon^{v_{\text{demand}}}_{atp/adp} - \varepsilon^{v_{\text{supply}}}_{atp/adp}} = 0.93 \qquad (32.1)$$

The model predicts that most of the flux control resides in the ATP demand (Eq. 32.1), i.e., in reactions outside glycolysis, in agreement with the hypothesis set out in [1]. To validate our approach and to confirm this result, we "took the bug out of the machine". This involved deleting all reactions involving only the chemostat (reactions 1, 6, 7 and 8) and clamping the values of s_c, p_c and the biomass concentration x at the values observed during the reference steady state in the complete model. The steady-state properties (fluxes and intracellular concentrations) of this yeast-only model were identical to those of the complete model (data not shown). The flux-control coefficients on glycolysis could now be computed directly with the model:

$$C^J_{\text{supply}}\Big|_{\text{yeast only}} = C^{J_3}_3 + C^{J_3}_5 = 0.002$$

$$\text{and} \quad C^J_{\text{demand}}\Big|_{\text{yeast only}} = C^{J_3}_4 + C^{J_3}_{11} = 0.996 \qquad (32.2)$$

The flux-control coefficients of reactions 9 and 10 were negligible ($< 10^{-6}$). The value of $C^{J_3}_2$ was 0.002; this was added to neither the supply nor the demand

control coefficients, because S_Y, the product of reaction 2, serves as a substrate for both the supply and the demand.

The differences between the results from the rate-characteristic analysis (Eq. 32.1) and the direct determination in the yeast-only model (Eq. 32.2) are the result of feedback inhibition from P_C in the complete chemostat model. The manipulations in k_{cat11} caused p_c to vary between 13 and 40 mM. When performing the same calculations with p_c fixed at its reference value of 24 mM, the resulting supply rate characteristic was much steeper, and the calculated control coefficients matched those for the yeast-only model in Eq. 32.2 (data not shown). Whether the feedback from P_C presents a serious problem to experimental analysis, depends on the kinetics of the reactions involved. In this model, it is the result of reaction 5 being catalysed by facilitated diffusion and reaction 3 being close to equilibrium; however, in the yeast cell this problem may not be as serious because glycolysis as a whole is far from equilibrium. Clearly the present model is too much of a simplification to investigate this effect in detail.

The model presented in Fig. 32.1 may be unrealistic insofar as that yeast not only uses intracellular glucose, but also 3C- and 4C-metabolites, for biosynthesis and growth. We are currently developing a kinetic model that uses P_Y instead of S_Y for the growth reaction v_4 and will compare those results to the present analysis. Because S_Y would then be localised to the supply block, such a model would obviate the need to re-adjust s_y to its original value (by changing the dilution rate) after a perturbation in the supply or demand.

The approach advocated here for manipulation of the supply may be difficult to implement in experimental practice, as intracellular glucose is hard to measure. Furthermore, as pointed out above, growth also depends on other 3C- and 4C-metabolites, and the question arises how one can be sure to include all of the relevant ones in the analysis, and whether it is indeed possible to keep them all at their original values by changing the dilution rate. In this case, it may be more feasible to cultivate the yeast in rich medium under conditions such that glucose is used only as energy source and not as carbon source. It should then be easy in principle to manipulate the supply by varying the dilution rate. These experiments are currently under way in our laboratory.

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