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A new regulatory principle for in vivo biochemistry: Pleiotropic low affinity regulation by the adenine nucleotides – Illustrated for the glycolytic enzymes of *Saccharomyces cerevisiae*

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ABSTRACT

Enzymology tends to focus on highly specific effects of substrates, allosteric modifiers, and products occurring at low concentrations, because these are most informative about the enzyme's catalytic mechanism. We hypothesized that at relatively high in vivo concentrations, important molecular monitors of the state of living cells, such as ATP, affect multiple enzymes of the former and that these interactions have gone unnoticed in enzymology.

We test this hypothesis in terms of the effect that ATP, ADP, and AMP might have on the major free-energy delivering pathway of the yeast *Saccharomyces cerevisiae*. Assaying cell-free extracts, we collected a comprehensive set of quantitative kinetic data concerning the enzymes of the glycolytic and the ethanol fermentation pathways. We determined systematically the extent to which the enzyme activities depend on the concentrations of the adenine nucleotides. We found that the effects of the adenine nucleotides on enzymes catalysing reactions in which they are not directly involved as substrate or product, are substantial. This includes effects on the Michaelis–Menten constants, adding new perspective on these, 100 years after their introduction.

1. Introduction

Systems Biology is the science that aims to discover how biological function emerges from the interactions of components of living systems [1–3]. In order to complement existing top-down and bottom-up systems biology strategies, a 'domino' approach that starts from both the edges and the nodes of the network has been developed [4]. To demonstrate the principles of the approach, we started with ATP (and ADP and AMP) as the most-connected molecule [5] and focused on the main pathways involved in ATP synthesis (catabolism), in ATP consumption for growth (anabolism), and in ATP consumption not coupled to growth (e.g. maintenance). We expected the network around ATP to be limited to those enzyme reactions that consume or produce ATP.

Although abundant experimental data are available on many cellular (sub) systems, including *Saccharomyces cerevisiae* and its glycolysis, combining these can often be near to impossible as each data set stems from a different experimental setup. Hence, integration of experimental, computational, and theoretical approaches within the field of systems biology necessitates a standardization in experimental conditions and procedures [6,7]. For yeast systems biology this has led the yeast systems biology network to produce a

Abbreviations: 2PGA, 2-phosphoglyceric acid; 3PGA, 3-phosphoglyceric acid; ADH, alcohol dehydrogenase (EC 1.1.1.1); ALD, fructose 1,6-bisphosphate aldolase (EC 4.1.2.13); ENO, enolase (EC 4.2.1.11); EtOH, ethanol; F16BP, fructose 1,6-bisphosphate; F26BP, fructose 2,6-bisphosphate; F6P, fructose 6-phosphate; G3PDH, glycerol 3-phosphate dehydrogenase (EC 1.1.1.8); G6P, glucose 6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase (EC 1.1.1.49); GAP, glyceraldehyde 3-phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); Glc, glucose; HXK, hexokinase (EC 2.7.1.1); LDH, lactate dehydrogenase (EC 1.1.1.27); PDC, pyruvate decarboxylase (EC 4.1.1.1); PEP, phosphoenolpyruvate; PFK, phosphofructokinase (EC 2.7.1.11); PGI, phosphoglucose isomerase (EC 5.3.1.9); PGK, 3-phosphoglycerate kinase (EC 2.7.2.3); PGM, phosphoglycerate mutase (EC 5.4.2.1); PYK, pyruvate kinase (EC 2.7.1.40); Pyr, pyruvate; TPI, triosephosphate isomerase (EC 5.3.1.1)

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standard set of chemostat experiments [8] and an international consortium to produce a consensus genome-wide metabolic map [5,9].

However, standardization in itself is not enough. The standards should provide data that represent the actual in vivo situation. For enzyme kinetics assays, a medium has been developed that mimics the cytosolic environment of *S. cerevisiae* [10]. The activities of yeast glycolytic (and ethanol fermentation) enzymes as measured in the assays optimized for the individual enzymes differed from their activities in this in vivo medium. More surprisingly perhaps, some enzyme activities under these in vivo-like conditions were significantly higher than the ones measured in the individually optimized assays. The emphasis of the paper of Van Eunen and co-workers was on effects on the k_{cat} of generic medium conditions such as ionic strength, pH, and inorganic phosphate ('buffer') concentration.

Here we shall take the standardization effort one step further and assess the effects of the concentrations of substrates and cofactors on enzyme activities: the focus of this paper rests on the effect of ATP, ADP, and AMP on the activity of the glycolytic enzymes. The inhibitory or activatory effect of these nucleotides is often only taken into account when they are substrates or products of a particular enzyme. A more general overall regulatory role of these nucleotides is not yet part of the standard biochemical paradigm.

Four glycolytic enzymes have adenine nucleotides as cosubstrates, but the others do not. For the former enzymes low-affinity effects of the adenine nucleotides in standard enzymology assays must have been obscured by the stronger, more specific, effects. Our hypothesis is therefore most pertinent for interactions of the nucleotides with the latter enzymes. Molecular biochemistry would expect these as specific allosteric interactions, which are traditionally observed at submillimolar concentrations of the effectors. For the yeast glycolytic enzymes, these additional allosteric interactions are unknown. In the case of intracellular free ion concentrations, however, the effects occurred in the multiple millimolar range [10], suggesting that much less affine interactions, usually unnoticed in molecular enzymology, might still be relevant for the enzymes functioning in pathways in vivo. Therefore, to test our hypothesis, we here examined whether in the millimolar concentration range, adenine nucleotides affected the activity of the glycolytic enzymes. We use the standardised in vivo-like assay medium, together with extracts from cells grown under standardized growth conditions [8,10–11] to generate a comprehensive set of kinetic data with regard to the effects of ATP, ADP, AMP on the glycolytic enzymes in *S. cerevisiae*. We validate our hypothesis of pleiotropic effects of the energy-state-monitoring metabolites on the components of the glycolytic and ethanol-fermentation pathway, both experimentally and in silico in the glycolysis model developed by Teusink and co-workers [12].

2. Materials and methods

2.1. Strain and growth conditions

The haploid, prototrophic *S. cerevisiae* strain CEN.PK 113-7D (MATa, MAL2-8^c, SUC2, provided by P. Kötter, Frankfurt, Germany) was used. For each continuous culture, a pre-culture was started from a fresh glycerol stock (stored at −80 °C); and grown in a flask on a rotary shaker at 30 °C in the medium described below. The glycerol stocks had been prepared by adding 30% glycerol (v/v) to a stationary-phase culture started with a colony taken by the provided plate and grown under the same conditions as the pre-culture.

Aerobic glucose-limited chemostat cultivations were carried out in 2 L fermenters (Applikon, Schiedam, the Netherlands), at 30 °C, with a culture volume of 1 L, a stirring rate of 800 rpm, an aeration rate of 0.50 L of air per minute, and a dilution rate of 0.1 h^{-1}. The cultures were fed a defined mineral medium [13], with 42 mM glucose as the growth-limiting nutrient. The volume of the culture was kept constant by an effluent pump, coupled to a preset level sensor. The fermenters were thermostated with water jackets. The extracellular pH was monitored and kept at 5.0 ± 0.1 through automatic drop-wise addition of a 2 M KOH solution. Oxygen saturation was monitored with a dissolved-oxygen electrode as well and found to be sufficient for the cells to grow fully respiratorily. To avoid excessive foaming of the culture, 0.0025% (v/v) anti-foaming agent (Sigma) was added to the medium. Cultures were assumed to be at steady state after at least five volume changes and when the culture dry weight and the specific oxygen consumption rate and carbon dioxide production rate changed less than 2% upon a further full volume change.

2.2. Steady-state measurements

Culture dry weights were determined by filtering, washing, and drying culture samples, essentially as described in [14]. However, here filters were dried overnight in a 60 °C incubator. The oxygen consumption and carbon dioxide production rates were determined by analysing the effluent gas from the fermenters with a gas analyser.

2.3. Preparation of cell-free extracts

Once the culture reached steady state, cells were harvested and prepared for storage by centrifugation (3850×*g* for 5 min at 4 °C), washed twice with 10 mM potassium phosphate buffer (pH 7.5) containing 2 mM EDTA, concentrated 20-fold by centrifugation, and stored as 1.0 ml aliquots at −20 °C. Just prior to use, the 1 ml samples were thawed on ice, washed twice by centrifugation (3850×*g* for 5 min at 4 °C) and resuspended in 1.0 ml of 100 mM potassium phosphate buffer (pH 7.5) containing 2.0 mM $MgCl_2$ and 1.0 mM dithiothreitol (DTT). Cell free extracts were made by disrupting the cells with acid-washed glass beads (425–600 μm) in a FastPrep (Qbiogene) machine, by eight bursts of 10 s each at a speed of 6.0 m s^{-1}. Samples were cooled on ice for 60 s in between bursts. Cell debris was removed from the extracts by centrifugation (3850×*g* for 15 min at 4 °C).

2.4. Measurement of enzymatic rates – general procedure

Reaction rates of enzymes were measured by monitoring the reduction of $NAD(P)^+$ or oxidation of NAD(P)H at 340 nm. The activity of phosphoglycerate mutase (PGM) and enolase (ENO) was measured by monitoring the production of phosphoenolpyruvate (PEP) at 240 nm. As detailed below, to some activity assays, additional enzymes and substrates had to be added to couple the reaction to one that could be monitored.

In order to mimic the in vivo situation as much as possible, the standardised in vivo-like assay medium as described in [10] was used. This medium (pH 6.8) contained 300 mM potassium, 245 mM glutamate, 50 mM phosphate, 20 mM sodium, an estimated 2 mM "free" magnesium (not bound to adenosine nucleotides, $NADP^+$ or TPP), 2.5–10 mM sulphate (depending on total magnesium addition) and 0.5 mM calcium. All substrate, co-factors, and coupling enzyme concentrations, including NADH etc., were checked to be sufficient and, if needed, altered compared to previously described assay concentrations, as described below. To determine the effect of ATP, ADP, and AMP on the enzyme reaction rates, these compounds were added to the assay mixture in a series of concentrations up to 10 mM (0.1, 0.5, 1–3, 5, and 10 mM, respectively) together with the same concentration of magnesium sulphate. Other additions to the assay varied with the individual assays (see below). In this series of assays, the enzyme substrates were varied individually at concentrations around their original

K_M value. Since some of the coupling enzymes used here turned out to be inhibited by the adenosine nucleotides, we double-checked these enzyme concentrations to be sufficient in the assays in which additional adenosine nucleotides were added. For each assay, cell-free extracts were diluted such that three or two dilutions fell in the linear range of the assay.

The assays were carried out in a Spectramax plate reader (Molecular Devices) at 30 °C, using 96-wells plates with a flat bottom (Greiner 655101/655191 for the assays at 340 nm and Greiner UV-Star for the ones at 240 nm). The standard assay volume was 300 μl, including 5.0 μl sample and 30 μl start reagent. Under the conditions used here the 'extinction coefficient' of NADH for a volume of 300 μl was determined to be 4.54 mM^{-1} by measuring the absorption at a series of NADH concentrations in a final volume of 300 μl. Please note that this value is for a fixed volume and hence is a combination of the actual extinction coefficient and the path length. Determining the coefficient for a fixed volume eliminates the large effect the meniscus might have on the path length in such small volumes. For the assays based on the monitoring of PEP production at 240 nm, the assay volume was reduced to 75 μl and the maximum concentration of ATP/ADP/AMP to 5 mM, since the absorption of these nucleotides at 240 nm rendered a too high background signal otherwise, impairing the linearity of the assay. The 'extinction coefficient' of PEP for a volume of 75 μl was 0.554 mM^{-1} (again, this value is a combination of the actual extinction coefficient and the path length). During the assay, first a base line was measured with only the sample and reagent present. Then the machine was paused and the start reagent added quickly. Absorbance was measured at 10 s intervals. All determinations were done in triplicate using cell extracts from three different cultures.

Table 1
Concentrations of substrates, co-factors and coupling enzymes used in the assays of the glycolytic enzymes and the enzymes of the ethanol fermentation pathway in cell-free extracts of *S. cerevisiae* described here ([new]) compared to the "traditional" ones set up for maximal activity (see text) ([old]). The substrate concentrations given here are the ones suggested for use in assays determining maximum enzymatic rates. To determine the effects of the adenine nucleotides, substrate concentrations were varied. Changes are marked in bold.

Enzyme	Component	[New]	[Old]
HXK	Glc	10 mM	10 mM
	ATP	**3 mM**	1 mM
	$NADP^+$	1 mM	1 mM
	G6PDH	1.8 U ml^{-1}	1.8 U ml^{-1}
PGI	G6P	**25 mM**	5 mM
	ATP	1 mM	1 mM
	NADH	0.15 mM	0.15 mM
	PFK	1 U ml^{-1}	1 U ml^{-1}
	ALD	0.45 U ml^{-1}	0.45 U ml^{-1}
	TPI	1.8 U ml^{-1}	1.8 U ml^{-1}
	G3PDH	0.6 U ml^{-1}	0.6 U ml^{-1}
PFK	F6P	**2 mM**	0.25 mM
	ATP	**1 mM**	0.5 mM
	AMP	**2 mM**	–
	F26BP	**–**	0.1 mM
	NADH	0.15 mM	0.15 mM
	ALD	0.45 U ml^{-1}	0.45 U ml^{-1}
	TPI	1.8 U ml^{-1}	1.8 U ml^{-1}
	G3PDH	0.6 U ml^{-1}	0.6 U ml^{-1}
ALD	F16BP	**20 mM**	2 mM
	NADH	0.15 mM	0.15 mM
	TPI	1.8 U ml^{-1}	1.8 U ml^{-1}
	G3PDH	0.6 U ml^{-1}	0.6 U ml^{-1}
TPI	GAP	**24 mM**	6 mM
	NADH	0.15 mM	0.15 mM
	G3PDH	8.5 U ml^{-1}	8.5 U ml^{-1}
GAPDH	GAP	**4 mM**	6 mM
	NAD^+	**5 mM**	1 mM
	ADP	**0.5 mM**	10 mM
	PGK	22.5 U ml^{-1}	22.5 U ml^{-1}
PGK (reverse)	3PGA	**25 mM**	25 mM
	ATP	**2 mM**	2 mM
	NADH	0.15 mM	0.15 mM
	GAPDH	8 U ml^{-1}	8 U ml^{-1}
PGM	3PGA	**10 mM**	5 mM
	23BPGA	**1 mM**	1.25 mM
	ENO	**2 U ml^{-1}**	1 U ml^{-1}
ENO	2PGA	**3 mM**	1 mM
PYK	PEP	2 mM	2 mM
	ADP	**5 mM**	10 mM
	NADH	0.15 mM	0.15 mM
	F16BP	1 mM	1 mM
	LDH	13.8 U ml^{-1}	13.8 U ml^{-1}
PDC	NADH	0.15 mM	0.15 mM
	TPP	20 mM	20 mM
	Pyr	50 mM	50 mM
ADH	NAD^+	**2 mM**	1 mM
	EtOH	**1 M**	0.1 M

All enzyme activities were calculated as micromoles of substrate converted per minute per milligram of extracted protein (U (mg protein)$^{-1}$). Total extracted protein was determined with a bicinchoninic acid kit (BCA Protein Assay Kit, Pierce, Thermo Fisher Scientific) with bovine serum albumin (BSA, 2 g L^{-1} stock solution, Pierce) as standard. Since the DTT present in the extracts influences the assay, it was added to the standards as well (1 mM).

2.5. Measurement of enzymatic rates – individual enzymes

The substrates, co-factors, and enzymes for coupled reactions are given in Table 1. The final column gives the concentrations used in previous assays [10,15].

The activator of phosphofructokinase (PFK), fructose 2,6-bisphosphate (F26BP), was not commercially available anymore, thus AMP was used (and checked) as an activator instead [16]. ADP is an inhibitor of glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) (GAPDH), but was needed for the coupling reaction. Hence it was used anyway, but at a far lower concentration than previously in [10,15]. Monitoring the production of PEP when measuring the activity of PGM and ENO obviated the coupled reaction of pyruvate kinase (EC 2.7.1.40) (PYK) which has ADP as a substrate.

2.6. Calculations

Calculation of the kinetic parameters and determination of the type of inhibition from the experimental data was done according to the methods described in [17], using Hanes plots, Dixon plots and plots suggested by Cornish-Bowden in Section 5.3 of the book.

2.7. Mathematical modelling

Model calculations were done in COPASI [18]. The glycolysis model of Teusing and co-workers [12] was used as the basis of the model used here. The inhibition of hexokinase by trehalose 6-phosphate and activation of pyruvate kinase by fructose 1,6-bisphosphate (F16BP) were introduced according to [19]. The fluxes to acetate, glycogen, succinate, and trehalose were set to be simple mass action rates, as was the ATPase rate accounting for ATP utilisation (cf. the original model [12]).

All experimental data was converted to units related to the intracellular volume (mM for concentrations and mM min^{-1} for rates) by assuming a cytosolic volume of 3.75 ml (mg cell protein)$^{-1}$ [20]. The values of the kinetic parameters determined here were introduced in the model. Other parameters were kept the same, with the exception of the (arbitrary) value of the kinetic constant of the succinate flux, which was set at 0.5 mM to preserve a balanced system. The various inhibitory and activatory effects of the presence of ATP, ADP, and AMP reported here were introduced in the rate equations as specified in [17]. More specifically, for

allosteric inhibition of V_{max} (in the case of mixed, non-competitive, and uncompetitive inhibition), the rate equation was divided by the factor 1 + *phi* with *phi* = [ATP]/K_{IUATP} + [ADP]/K_{IUADP} + [AMP]/K_{IUAMP} and K_{IU} the inhibition constant of uncompetitive component of the inhibition by the particular nucleotide (if applicable). In the case of uncompetitive or mixed inhibition, the K_M of the substrate was divided by the factor 1 + *phi*, while in the case of competitive or mixed inhibition the K_M was multiplied with a similar factor 1 + *phi*, but now including the inhibition constant of the competitive component of the inhibition, instead of the uncompetitive one. In keeping with the second law of thermodynamics, the K_M of the corresponding product was divided or multiplied by the same factor as well. The experimentally observed activatory effects of the nucleotides were introduced by multiplying the rate equations by a factor $1 + n[ATP]/(K_{A\text{-}ATP} + [ATP]) + n[ADP]/(K_{A\text{-}ADP} + [ADP]) + n[AMP]/(K_{A\text{-}AMP} + [AMP])$ with $n = 2$ for the nucleotides involved.

3. Results

3.1. Determination of the maximum rates (V_{max}) of the glycolytic enzymes

First of all, we determined what the maximum rates (V_{max}) of the glycolytic enzymes in the cell-free extracts were when the previously established in-vivo-like assay medium was used [10]. By definition, such a V_{max} value only represents the maximum reaction rate of an enzyme, measured in the presence of substrates in saturating concentrations and the complete absence of products. So-called optimal conditions for each enzyme used traditionally [15], do not always give the highest maximum rate [10]. Hence, to ensure that the concentrations of the individual substrates (including coenzymes such as NADH), co-factors, and coupling enzymes were saturating, we first varied these concentrations, both below and above the ones prescribed by the assays set up for maximal activity [15], until we found saturating concentrations. To exclude the effect of product inhibition, we only looked at initial reaction rates. Since we used cell-free extracts for our assays and not purified, reconstituted single enzymes, the rates given here are for the pool of isoenzymes present in the extracts.

Surprisingly, we found that several substrate concentrations as used in the 'traditional' assays set up for maximal activity (Table 1) were not saturating; substrate concentration had to be increased for the measured rates to become independent of these concentrations and to thus establish the maximum enzyme activity under the in vivo-like conditions used here (Table 2). These increased concentrations amounted to up to ten times the original ones. In addition, we observed that GAPDH is inhibited by ADP, a substrate for the coupled reaction driven by 3-phosphoglycerate kinase (EC 2.7.2.3) (PGK) and needed to have the reaction remove the product of GAPDH. Lowering the ADP concentration resulted in a significant increase in the rate of GAPDH. The concentration of the substrate glyceraldehyde 3-phosphate (GAP) could be lowered and still be saturating. A practical problem we encountered was the lack of available (pure) fructose 2,6-bisphosphate, an activator of PFK typically used in the PFK activity assay. We confirmed that the absence of any activator resulted in an extremely low, almost undetectable enzyme activity, even at very high substrate concentrations (data not shown). Therefore, we used AMP as an activator instead [16], which gave a physiologically relevant rate that would support the glycolytic flux under the growth conditions used here.

Table 2
V_{MAX} values (mean and their S.E.M.) for the glycolytic enzymes and the enzymes of the ethanol fermentation pathway in cell-free extracts of *S. cerevisiae* as measured in the in-vivo-like assay medium with the new set of saturating substrate, co-factor, and coupling enzyme concentrations. Three series of independent measurements of a relevant series of substrate, co-factor, or coupling enzyme concentrations were carried out in extracts of three separate cultures each.

Enzyme	V_{MAX} (mmol min^{-1} (g protein)$^{-1}$)
HXK	1.45 ± 0.086
PGI	1.95 ± 0.18
PFK	1.17 ± 0.10
ALD	3.90 ± 0.049
TPI	64.0 ± 6.4
GAPDH	5.74 ± 4.8
PGK (reverse rate)	12.1 ± 0.74
PGM	31.1 ± 5.1
ENO	5.90 ± 0.67
PYK	6.12 ± 0.45
PDC	1.37 ± 0.12
ADH (reverse rate)	21.1 ± 2.7

3.2. Determination of Michaelis constants (K_M) of enzyme vis-à-vis their substrates

Next, we determined the affinity of the substrates for their enzymes. For most of the enzymes, we assumed Michaelis–Menten-type kinetics as given in [12,17]. For PFK a two-substrate Monod, Wyman, Changeux model for allosteric enzymes was used (see for a description [12]).

To determine the Michaelis constants (K_M) of the individual substrates for the enzymes, the enzyme rates at various substrate concentrations were measured and the K_M calculated [17]. The resulting values are given in Table 3. None of the results stand out and – as discussed above – comparison with other datasets will be hard, since the experimental conditions were different.

3.3. Determination of inhibition or activation of enzyme activities by ATP, ADP, and AMP

In order to determine systematically whether, as in the hypothesis we meant to test, ATP, ADP, and AMP affected enzymatic rates also of reactions in which they were not involved as substrates or products, the adenine nucleotides were added to the assay mixtures at concentrations ranging from 0 to 10 mM. We determined the type of inhibition or activation (if present), by varying the concentrations of the substrates other than the nucleotides individually in a range relevant for that particular substrate (as described in [17]) and the implications of our findings are shown in Table 4. Since ATP, ADP, or AMP are typically available as sodium phosphate salts, we

Table 3
Substrate affinities of the glycolytic enzymes and of the enzymes of the ethanol fermentation pathway in cell-free extracts of *S. cerevisiae* as measured in the in-vivo-like assay medium with the new set of saturating substrate, co-factor, and coupling enzyme concentrations. The affinities are given as mean K_M values with their S.E.M., $n = 9$.

Enzyme	K_M (mM)	
HXK	Glc	0.10 ± 0.012
	ATP	0.37 ± 0.025
PGI	G6P	1.8 ± 0.16
PFK	See text	
ALD	F16BP	8.5 ± 0.91
TPI	GAP	4.2 ± 0.68
GAPDH	GAP	0.45 ± 0.060
PGK	3PGA	1.3 ± 0.089
	ATP	0.31 ± 0.065
PGM	3PGA	5.1 ± 0.91
	23BPGA	0.10 ± 0.018
ENO	2PGA	0.21 ± 0.036
PYK	PEP	0.36 ± 0.20
	ADP	1.2 ± 0.092
PDC	Pyr	15.2 ± 2.1
ADH	EtOH	233 ± 35

Table 4
Kinetic constants and their S.E.M. with regards to the effect of ATP, ADP, and AMP on the activities of the glycolytic enzymes and the enzymes of the ethanol fermentation pathway in cell-free extracts of *S. cerevisiae* as measured in the in-vivo-like assay medium with the new set of saturating substrate, co-factor, and coupling enzyme concentrations. The effects are listed as seen when individual substrates are varied. In case of inhibition, the inhibition type is given as well. "None" means no effect of the nucleotide on the enzyme activity was seen in the concentration range used here. K_{IU} and K_{IC} denote the constants for uncompetitive and competitive components of the inhibition, resp. ((*) activity of ALD is 0 in the presence of 10 mM ATP; (**) maximum at 3 mM; (***) activity of pyruvate decarboxylase (EC 4.1.1.1) (PDC) was undetectable in the presence of 10 mM AMP.)

Enzyme	Varied substrate	Kinetic constants (mM)			Inhibition type
			K_{IU} or K_A	K_{IC}	
HXK	Glc	ATP	12 ± 0.18	–	Uncompetitive
		ADP	7.2 ± 1.1	–	Uncompetitive
		AMP	27 ± 3.6	–	Uncompetitive
	ATP	ATP	–	–	–
		ADP	7.9 ± 1.1	5.4 ± 1.2	Mixed
		AMP		5.5 ± 0.79	Competitive
PGI	G6P	ATP	3.9 ± 0.47	3.2 ± 0.67	Mixed
		ADP	18 ± 2.1	4.2 ± 0.48	Mixed
		AMP	14 ± 2.2	3.7 ± 0.49	Mixed
PFK	See text	See text			
ALD	F16BP	ATP	1.1 ± 0.14		Non-competitive (*)
		ADP	–		None
		AMP	9.4 ± 1.1		Uncompetitive
TPI	GAP	ATP	12 ± 1.8	8.4 ± 1.7	Mixed
		ADP	19 ± 3.1	15 ± 2.0	Mixed
		AMP	22 ± 3.0	8.5 ± 2.3	Mixed
GAPDH	GAP	ATP	5.9 ± 0.57	–	Uncompetitive
		ADP	11 ± 1.5	–	Uncompetitive
		AMP	12 ± 1.8	–	Uncompetitive
PGK	3PGA	ATP	16 ± 2.6	–	Uncompetitive
		ADP	1.6 ± 0.28	–	Non-competitive
		AMP	19 ± 2.7	–	Uncompetitive
	ATP	ATP	–	–	–
		ADP	–	3.3 ± 0.46	Competitive
		AMP	–	3.2 ± 0.41	Competitive
PGM	3PGA	ATP	–	–	None
		ADP	10 ± 1.3	–	Activation
		AMP	–	–	None
	23BPGA	ATP	–	–	None
		ADP	7.8 ± 1.0	–	Activation
		AMP	–	–	None
ENO	2PGA	ATP	–	3.2 ± 1.2	Competitive
		ADP	2.1 ± 0.45	–	Activation (**)
		AMP	4.3 ± 0.80	–	Activation
PYK	PEP	ATP	–	–	None
		ADP	–	–	None
		AMP	–	–	None
	ADP	ATP	–	–	None
		ADP	–	–	–
		AMP	–	–	None
PDC	Pyr	ATP	13 ± 2.4	–	Activation
		ADP	21 ± 3.1	–	Activation
		AMP	29 ± 3.7	6.4 ± 1.3	Mixed (***)
ADH	EtOH	ATP	5.9 ± 1.0	–	Uncompetitive
		ADP	17 ± 2.1	–	Uncompetitive
		AMP	19 ± 2.5	–	Uncompetitive

Table 5
Steady state values of (A) metabolite concentrations and (B) enzymatic rates of glycolysis and the fermentation pathway for the two versions of the model (base Model 1 with no adenine nucleotide effects other than given in the model by Teusink and co-workers and Model 2 with inhibitions and activations by adenine nucleotides reported here added), as well as experimentally determined values from [25].

	Model 1	Model 2	Experiment
(A) Rates (mM min^{-1})			
v_{GLT}	53	24	
v_{HXK}	58	29	94 ± 4
$v_{PGI-vALD}$	57	28	82 ± 3
$v_{GAPDH-vPYK}$	103	53	147 ± 4
v_{PDC}	98	51	
v_{ADH}	98	51	
$v_{Glycerol}$	12	4.0	
(B) Metabolites (mM)			
GLC_i	4.6	10	
G6P	0.65	0.41	3.8 ± 0.1
F6P	0.10	0.032	0.74 ± 0.01
F16BP	35	4.5	12 ± 1
DHAP + GAP	7.2	2.2	
BPG	1.1E-04	4.8E-05	
3PGA	0.19	0.061	0.78 ± 0.02 (2PGA + 3PGA)
2PGA	0.022	0.0069	
PEP	0.036	0.022	0.1 ± 0.01
PYR	4.6	1.5	2.8 ± 0.6
ACALD	0.17	0.25	
ATP	2.3	1.2	
ADP	2.6	2.6	
AMP	1.4	2.5	
NAD	1.6	1.8	
NADH	0.024	0.017	

measured the effect of the additional sodium ion concentrations as well and found them to be negligible for all enzymes. The same was the case for magnesium sulphate (data not shown).

The results show that, with the exception of PYK, the activities of all enzymes were indeed affected by the presence of ATP, ADP, or AMP, also when they did not act as a substrate for the enzyme (Table 4). Some effects were small, though. In general, the effect of an adenine nucleotide as a substrate was (competitively) inhibited by the others. In addition, some general, mixed-type inhibition was seen with values for the inhibition constants (K_{IU} and K_{IC}) well above 10 mM. An example is triosephosphate isomerase (EC 5.3.1.1) (TPI), of which all three inhibition constants were above 10 mM. Since the intracellular ATP, ADP, and AMP concentrations tend to be below 10 mM [21–23], such high inhibitory constants preclude large effects on enzyme activity.

However, some results stood out. The measurement of hexokinase (EC 2.7.1.1) (HXK) activities revealed substrate inhibition by ATP. In addition, even though the adenosine nucleotides are not substrates of the enzyme, fructose 1,6-bisphosphate aldolase (EC 4.1.2.13) (ALD) was severely inhibited by ATP. A K_I (non-competitive) of only 1.1 mM was calculated, and at an ATP concentration of 10 mM, no ALD activity could be detected. Phosphoglucose isomerase (EC 5.3.1.9) (PGI) activity was also severely inhibited by ATP. ADP acted as an activator of PGM and ENO and AMP as one of ENO.

The kinetics of PFK are rather complex (see for a discussion [12]). Within the range of substrate concentrations used here (0.5–5 mM ATP and 0.5–5 mM fructose 6-phosphate (F6P)), no inhibitory effect of any of the three nucleotides was seen. At an AMP concentration of 0.5 mM, already full activity was found, while an (activating) effect of ADP was only detected when AMP was absent (60% of full activity at 2 mM).

3.4. In silico determination of the potential physiological relevance

The low-affinity effects of adenine nucleotides on the rates of glycolytic enzymes we observed should be relevant for the relationships between the rates and the product/substrate concentrations of the individual enzymes. However, this does not prove that the effects would also be important for the physiological function of these enzymes in vivo, which is to produce the glycolytic flux resulting in glucose consumption and free-energy transduction, and perhaps to establish robust steady state concentrations of glycolytic metabolites and of the adenine nucleotides themselves. For one, effects on enzymes without flux control should not be important for pathway flux [24]. In addition, effects of adenine nucleotides on multiple enzymes might cancel each other when pathway flux or metabolite concentrations are involved.

In order to examine the effects of the inhibition and activation of the glycolytic and fermentation enzymes by the adenine nucleotides might have on the fluxes of the system, we employed a kinetic model of the glycolytic pathway. We used the well-established model of Teusink and co-workers [12] as a starting point, but made a few updates as described in Section 2. We introduced the K_M and V_{max} values determined here, as well as the inhibitions and activations by ATP, ADP, and AMP (see Section 2for the modifications of the rate equations) and compared the results of the model with the additional inhibitory and activatory effects of the nucleotides present, to the one without these effects.

As shown in Table 5, the effects were substantial; in the version of the model where the inhibitory and activatory effects have been introduced, the fluxes are only about half of the fluxes in the model without these activatory and inhibitory effects. The ATP concentration, together with most intracellular metabolites has dropped considerably (Table 5); the ATP/ADP ratio decreased from approximately 1 to 0.5. Clearly, the effects on pathway flux and metabolite concentrations of the adenine nucleotide inhibitions and activations of the enzymatic rates should be expected to be substantial in the context of what we know about the pathway, which is represented by the model.

4. Discussion

From a scientific point of view, standardisation is important. From a physiology point of view however, standardisation is important but not enough. Measurements need to be done under physiologically relevant conditions. When Van Eunen and co-workers [10] developed an assay medium that mimics in vivo conditions for the cytosolic enzymes of *S. cerevisiae* they compared enzyme activities measured under these in vivo conditions to those measured in established assays under so-called 'optimal' conditions for each individual enzyme and found major differences. These authors only considered the effect of changes in the assay medium; the concentrations of substrates, effectors, and coupling enzymes remained unchanged. Here, we have also examined the substrate concentrations forthe enzymes to reach their maximum rates under this in vivo-like assay condition (Table 1) and found that the V_{max} values were higher than Van Eunen and co-workers [10] found. The implication of applying in vivo-like conditions is even greater than they may have realised.

In addition to determining the maximal rates of the enzymes, we determined the affinity coefficients (Michaelis–Menten constants; K_M) of the various substrates for their enzymes, which is what Van Eunen and co-workers [10] did not do. In light of the on-going discussion on the need of standardisation, it should not come as a surprise that we found it hard to compare this data subset to previous data. For instance, in the case of hexokinase, reportedK_M values for the substrates glucose and ATP in a series of publications range from 0.1 to 0.24 mM and from 0.063 to 2.9 mM, respectively [12,26–29]. This example suggests that not using standardised, in vivo-like assay conditions not only leads to k_{cat} values unrepresentative for the in vivo functioning of the enzymes, but also to unrepresentative K_M values. Where k_{cat} values pertain to the maximum capacity of metabolic functions, the

affinity constants are important not only for these capacities, but also for determining the in vivo concentrations of the metabolites in the pathways. In turn – and more so than the fluxes – these concentrations are important for the cross-regulation of and by other pathways, for cell signalling, and for gene expression.

The findings of this paper suggest a new standard experimental design for the characterization of enzymes. One should not measure an enzyme's properties in fairly indeterminate conditions such as defined by ionic strength, pH, and buffer conditions. Rather, one should also determine the low affinity effects of many functional components of the intracellular milieu. In addition, medium effects should not only be measured for k_{cat}, but also for Michaelis–Menten constants. Our experimental conditions were still a bit in limbo; the advice would be to measure effects both under conditions where substrates are well above K_M values (for effects on k_{cat}) and under conditions where they are below/around K_M values (for effects onK_M).

Most importantly perhaps, we have provided a comprehensive set of quantitative data on the inhibitory and/or activatory effect of the adenosine nucleotides ATP, ADP, and AMP on the individual glycolytic enzymes. Besides giving general insight into the regulatory role of the nucleotides on the glycolytic rate, this set shows that their effect on enzymes in the reactions of which they are not directly involved, can be substantial and should hence not be disregarded. An example is the strong inhibition of fructose 1,6-bisphosphate aldolase by ATP.

That this is important for pathway function, is illustrated further by our in silico modelling results; glycolytic fluxes are strongly affected when introducing these inhibitory and activatory effects.

We initiated this study on the basis of the hypothesis that molecular monitors of important aspects of the cell'sstate may regulate cell function highly pleiotropically, i.e., by working on virtually all enzymes. We proposed that this regulation could well be due to low-affinity interactions with many enzymes for which these monitors are neither a substrate nor a product. In this paper we have validated this hypothesis for molecular monitors of perhaps the most important aspect of the cell's state, its Gibbs free energy potential. Indeed our hypothesis was validated for this case; ATP, ADP, and AMP, affect the activity of most enzymes of the major catabolic pathway of yeast and most often not through substrate or product action.

The concentrations at which these energy monitors exercise their pleiotropic effects are typically much higher than their K_M values as substrate or product for the enzyme reactions they participate in. These low affinities explain why from the point of view of enzyme mechanisms these effects have notbeen considered important. However, the concentrations of these energy metabolites are often in the millimolar range and their variations may therefore *regulate* cell function through these low affinity pleiotropic effects more than through their highly affine substrate/product effects. This offers an entirely new perspective on metabolic regulation.

It should be noted that such pleiotropic effects may not be confined to the adenine nucleotides. Inorganic phosphate, nicotinamide adenine nucleotides and may other molecules may exert similar effects. It is for this reason that we refrained from comparing the new model predictions that incorporate the pleiotropic effects of the adenine nucleotides, to in vivo biochemistry. Table 5 does show relevant experimental in vivo data. It shows that the pleiotropic effects are of the order of magnitude that could bridge the existing discrepancies between in vivo and in vitro, but it also suggests that the effects of the adenine nucleotides alone are not enough to account for the discrepancy.

The adenine nucleotides may well affect many enzymes for which they are neither substrate nor product by competing with a different substrate or product with which they share a limited amount of structural analogy. One may indeed expect ATP at high concentrations to interfere slightly with the binding of any phosphorylated molecule to an enzyme. In this case we expect ATP merely to increase the Michaelis–Menten constant by the factor $1 + [ATP]/K_{ATP}$. Even though the affinity for ATP of the binding site might be low (e.g. 5 mM), since the physiological concentration of ATP is often close to 5 mM it would well increase the K_M for the specific substrateby a factor of two and decrease the rate by almost that same factor. This analysis shows that even a full century later, the work of the Canadian biochemist *avant la lettre* Miss. Maud Menten in the centre of Berlin [30], continues to enable us to understand how biological function can be regulated through the Michaelis–Menten constant too often named only after her colleague Michaelis.

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