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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