In situ analysis of methylglyoxal metabolism in *Saccharomyces cerevisiae*

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Abstract Methylglyoxal metabolism was studied during *Saccharomyces cerevisiae* grown with D-glucose as the sole carbon and energy source. Using for the first time a specific assay for methylglyoxal in yeast, metabolic fluxes of its formation and D-lactate production were determined. D-Glucose consumption and ethanol production were determined during growth. Metabolic fluxes were also determined in situ, at the glycolytic triose phosphate levels and glyoxalase pathway. Maximum fluxes of ethanol production and glucose consumption correspond to maxima of methylglyoxal and D-lactate formation fluxes during growth. Methylglyoxal formation is quantitatively related to glycolysis, representing 0.3% of the total glycolytic flux in *S. cerevisiae*.

Key words: In situ metabolic study; Methylglyoxal metabolism; Glycolysis; Glyoxalase pathway; Methylglyoxal; D-Lactate; *Saccharomyces cerevisiae*

1. Introduction

Glycolysis is probably the most thoroughly studied metabolic pathway, where dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde-3-phosphate (GAP) are formed from fructose-1,6-bisphosphate by the action of aldolase (EC 4.1.2.13). Triose phosphate isomerase (EC 5.3.1.1) catalyzes the aldo-ketose isomerization of these triose phosphates and only GAP follows the glycolytic pathway, being converted to 1,3-bisphosphoglycerate in the reaction catalyzed by GAP dehydrogenase (EC 1.2.1.12) [1].

However, triose phosphates are very unstable molecules and the β-elimination reaction of the phosphoryl group from the common 1,2-enediolate of both trioses may occur, leading to methylglyoxal formation [2,3]. This is a non-enzymatic, parametabolic reaction and therefore methylglyoxal occurrence is an unavoidable consequence of glycolytic metabolism [4]. Methylglyoxal can also be formed from the leakage of the 1,2-enediolate intermediate in the active center of triose phosphate isomerase in a paracatalytic reaction [5]. The enzymatic synthesis of methylglyoxal from DHAP, through the action of methylglyoxal synthase (EC 4.2.99.11) was described in bacteria [6]. It was also detected in methylotrophic yeasts [7] and in mutants of *Saccharomyces cerevisiae* [8]. However, methylglyoxal synthase activity was not detected in wild strains of this yeast growing either aerobically or anaerobically, in different carbon sources [9]. Also, no sequences homologous to methylglyoxal synthase in *S. cerevisiae* were revealed from genomic analysis, casting some uncertainty about the existence of this enzyme in yeast [10].

The main methylglyoxal catabolic pathway in eukaryotic cells is the glyoxalase system, comprising the enzymes glyoxalase I (*S*-D-lactoylglutathione:methylglyoxal lyase; EC 4.4.1.5) and glyoxalase II (*S*-2-hydroxyacylglutathione hydrolase; EC 3.1.2.6) [11]. Glyoxalase I isomerizes the hemithioacetal formed non-enzymatically from methylglyoxal and GSH, forming *S*-D-lactoylglutathione while glyoxalase II catalyzes the hydrolysis of this thioester to D-lactate, regenerating GSH [11].

Once formed, methylglyoxal irreversibly modifies macromolecules, forming advanced glycation end-products (AGE) [12]. Methylglyoxal reacts with guanyl nucleotides in nucleic acids to form imidazopurinone adducts and reacts with lysine and arginine residues in proteins forming crosslinks and imidazole derivatives [13]. These protein modifications are associated to the clinical complications of diabetes mellitus, Alzheimer's disease and ageing [14,15].

Despite more than 80 years of research in this field, little is known regarding the quantitative aspects of methylglyoxal metabolism in eukaryotic cells [11]. The main reason is the lack, until recently, of specific and sensitive assays for methylglyoxal [16,17].

In this work, we determined in situ the metabolic fluxes related to methylglyoxal formation and catabolism, using specific assays for methylglyoxal and D-lactate, as well as the glycolytic flux through GAP dehydrogenase. In situ metabolic fluxes were compared with global metabolic fluxes in vivo, during aerobic cell growth on D-glucose.

2. Materials and methods

2.1. Yeast strain and culture conditions

The *S. cerevisiae* strain IGC 4072 was obtained from the Portuguese Yeast Culture Collection, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa. It is a diploid strain that contains no genetic markers.

S. cerevisiae IGC 4072 was maintained in YPGlu −0.1% (w/v) yeast extract (Difco), 0.5% (w/v) peptone (Difco) and 2% (w/v) D-glucose (Mikrobiologie grade, Merck) -agar slopes, at 4°C. After an overnight growth at 30°C in solid medium, the cells were transferred to a minimal liquid medium (K medium, Instituto Gulbenkian de Ciência). Cell cultures were batch grown overnight for 16–18 h, to a concentration of approximately 10^8 cells ml^{-1}. After this first growth, the cell suspension was diluted in fresh minimal medium in order to obtain a cell concentration of about 8×10^6 cells ml^{-1}. Cells were again batch grown at 30°C. Samples were collected at defined times (0, 3, 6, 9 and 12 h) and analyzed for enzyme activities, in situ metabolic fluxes and concentration of metabolites. Cell concentration was calculated with a

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calibration curve relating the absorbance integral in the 400–800 nm range to cell concentration determined by direct counting.

2.2. Cell permeabilization and protein determination for in situ assays

Enzymatic activities were determined in situ using digitonin permeabilized cells, prepared as described [18]. Protein concentration in situ was determined according to an early procedure using Coomassie brilliant blue G (Sigma) [19].

2.3. Determination of enzyme activities and metabolic fluxes in situ

All assays were performed at 30°C in a 2 ml reaction mixture. A Hewlett-Packard HP8451A diode array spectrophotometer with a stirred thermostated multi-cuvette holder (HP 83075A) was used to monitor the reaction course.

Glyoxalase I activity was determined at 240 nm, as described by Racker [20]. Methylglyoxal was prepared by acid hydrolysis of 1,1-dimethoxypropanone (Sigma) [17] and the solutions were calibrated by enzymatic assay with glyoxalase I and II [20]. A $\Delta\varepsilon_{240} = 2.86\ mM^{-1}\ cm^{-1}$ was used [21]. The specific activity was expressed as μmol of *S*-D-lactoylglutathione formed per min per mg protein.

Glyoxalase II activity was determined at 412 nm, as described by Martins et al. [21]. The specific activity was expressed as μmol of 2-nitro-5-thiobenzoic acid formed per min per mg of protein.

D-Lactate dehydrogenase was assayed in situ, using a method previously described [9]. Methylglyoxal synthase activity was determined by comparing the rate of methylglyoxal formation from DHAP (100 μM) in 25 mM KH_2PO_4/K_2HPO_4 buffer, pH 7.4, in the absence and in the presence of permeabilized cells. Methylglyoxal concentration was determined using the HPLC-based assay described elsewhere [17].

To study the activity of the enzymatic system involved in the metabolism of triose phosphates D-fructose-1,6-bisphosphate (tetrahexylammonium salt, grade V, Sigma) was used as substrate and the system was followed at the level of the GAP dehydrogenase catalyzed reaction, measuring NADH formation at 340 nm, using 10 mM D-fructose-1,6-bisphosphate. Specific activity was expressed as μmol of NADH formed per min per mg of protein.

In situ metabolic fluxes for methylglyoxal formation and glycolysis at the triose phosphates level were determined in permeabilized cells harvested at the beginning of growth. D-Fructose-1,6-bisphosphate concentration was varied between 1 to 10 mM. Glycolytic flux and methylglyoxal formation in situ were determined at the same time.

2.4. Determination of metabolites

Samples were deproteinized with 0.5 M perchloric acid, stirred, kept on ice for 10 min and immediately analyzed or frozen in liquid nitrogen (samples could be kept for at least a month prior to assay).

D-Glucose was assayed as described by Bergmeyer et al. [22] and ethanol was determined as described by Beutler [23]. L-Lactate and D-lactate were assayed as described by Noll [24] yet, in the case of D-lactate, the enzyme D-lactate dehydrogenase was used, making the assay specific for this isomer. Methylglyoxal was assayed by derivatization with 1,2-diaminobenzene, using the HPLC-based assay referred above [17].

3. Results and discussion

During the aerobic batch growth of the yeast *S. cerevisiae* [25], D-glucose was consumed while ethanol was produced (Fig. 1a). In these conditions, the time course of ethanol formation followed yeast cell growth reaching a concentration of 110 mM at the end of the exponential phase (12 h).

During yeast growth, methylglyoxal was formed but its concentration was very low, being 0.07 μM at time zero and reaching a maximum concentration of 0.3 μM at the end of the exponential phase (Fig. 1b). The final product of the glyoxalase pathway, D-lactate, was also formed and its concentration increased from undetectable at the beginning of growth to 220 μM at the end of the exponential phase (Fig. 1b). L-Lactate was not detected during growth, indicating that neither racemization nor formation from pyruvate occurred.

The accumulation of D-lactate suggests that this compound

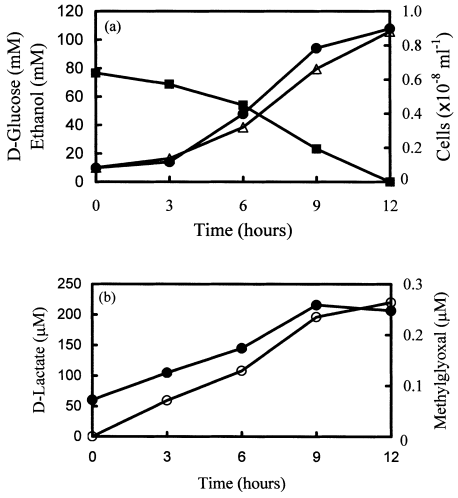

Fig. 1. In vivo time courses of metabolites. a: D-Glucose (■) consumption and ethanol (●) production during growth. Cell concentration (△) was calculated with a calibration curve relating the absorbance integral in the 400–800 nm range with cell number. b: Methylglyoxal (●) and D-lactate (○) formation during the catabolism of D-glucose in *S. cerevisiae*. All metabolites were determined as described in the text, in samples taken at defined times (0, 3, 6, 9 and 12 h). Results obtained for cell concentration, glucose consumption and ethanol production are from a representative experiment from a set of 10 independent experiments. Results shown for methylglyoxal and D-lactate formation were obtained in a representative experiment of a set of five independent experiments.

is not metabolized by *S. cerevisiae* in our growth conditions. In fact, D-lactate dehydrogenase was measured in situ but no activity could be found. Three genes were described that encode D-lactate dehydrogenase proteins [26]. Only one of those proteins is cytoplasmic but its gene is only expressed when functional mitochondria are present in the cells [26].

From these data, it is possible to determine the amount of D-glucose metabolized by the glyoxalase pathway. Using the initial concentration of D-glucose and the concentration of D-lactate formed during yeast growth, it was found that 0.3% of glucose was converted to methylglyoxal and subsequently metabolized to D-lactate by the glyoxalase system.

Metabolic fluxes during growth were determined at each time, by numerically calculating the slope of the progress curves. As observed in Fig. 2a the rates of consumption of D-glucose and the rates of formation of ethanol, methylglyoxal and D-lactate exhibit a maximum at late exponential phase. In situ glycolytic flux at the triose phosphate level increased during growth and reached a steady value of 1.8 mmol NADH min^{-1} at the end of the exponential phase (Fig. 2b).

The above results clearly show a direct relation between methylglyoxal formation during glycolysis and its catabolism by the glyoxalase system. Methylglyoxal and D-lactate formation rates followed the rate of ethanol formation and of D-glucose consumption, being maximal at late exponential phase (Fig. 2a).

The specific activity of the glyoxalase enzymes remained almost constant during growth, in the range of 1.0–1.2 μmol min^{-1} mg^{-1} for glyoxalase I and 0.030–0.045 μmol min^{-1} mg^{-1} for glyoxalase II. The glyoxalase II form we are dealing with is the cytosolic one, encoded by the GLO2 gene, since the mitochondrial enzyme, encoded by the GLO4 gene, is only expressed when yeast cells are grown aerobically, in media

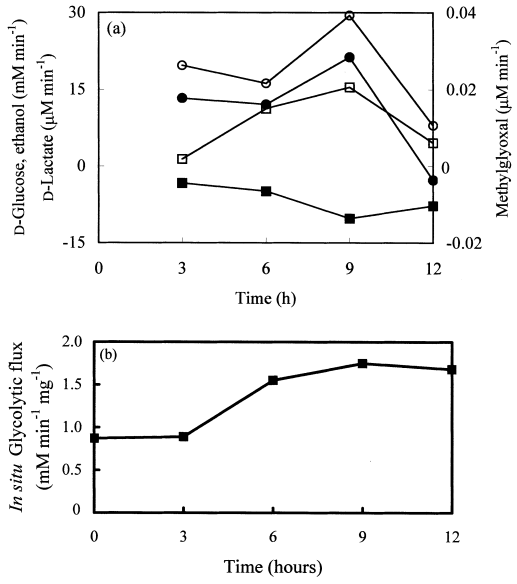

Fig. 2. In vivo and in situ metabolic fluxes. a: Time course for D-glucose (■) consumption rate and ethanol (□), methylglyoxal (●) and D-lactate (○) formation rates. b: Specific in situ glycolytic flux during *S. cerevisiae* growth, determined with 10 mM D-fructose-1,6-bisphosphate. Results are from a representative experiment of a set of five independent experiments.

containing glycerol [27]. The activities of both glyoxalase I and glyoxalase II only increase significantly after the diauxic shift [28].

Our results are in agreement with a previously described study of methylglyoxal and D-lactate formation in erythrocytes, where 0.09–0.4% of D-glucose was converted to D-lactate via the glyoxalase pathway [29]. However, they are in sharp contrast with the results obtained in *Desulfovibrio gigas*, where 40% of D-glucose was degraded by the methylglyoxal bypass [30]. While the cellular extracts of these bacteria exhibited a specific activity of methylglyoxal synthase of about 2–24 mU per mg of protein, we could not detect any activity of this enzyme in *S. cerevisiae*. The late observation, combined with the low percentage of D-glucose metabolized to D-lactate, clearly shows that methylglyoxal formation must occur mainly in a non-enzymatic way.

Understanding how cells produce methylglyoxal during glycolysis requires an approach to reconstitute metabolism at the triose phosphate levels in glycolysis. The low intracellular concentration of methylglyoxal hinders the use of presently available non-invasive techniques such as NMR spectroscopy. On the other hand, previously used methods for methylglyoxal assay are prone to overestimation due to uncontrolled non-enzymatic conversion of triose phosphates to this 2-oxoaldehyde.

A reliable way to probe cell metabolism is using permeabilized cells [31,32]. Under these in situ conditions, enzymes and other macromolecules remain within the cell boundaries at unchanged concentrations. Low molecular weight molecules, such as substrates and products, freely diffuse across the cell boundary. Any metabolic state, characterized by a given set of enzyme activities can therefore be studied in controlled conditions as regards the concentration of metabolites, pH and reaction medium composition. The cells ability to provide a given glycolytic flux was investigated in situ, by following the dependence of metabolic flux on the concentration of D-fructose-1,6-bisphosphate. The concentration range for D-fructose-1,6-bisphosphate and other reaction conditions were chosen according to in vivo NMR measurements of the concentration of glycolytic intermediates [33] and rapid analysis techniques of intracellular metabolites in *S. cerevisiae* [34]. At the same time, the flux of methylglyoxal formation was followed in situ. Since the glyoxalase enzymes are inactive due to the absence of glutathione, the measurements made truly represent the maximum potential for methylglyoxal formation under glycolytic conditions (Fig. 3a). The relation between glycolytic flux and methylglyoxal formation flux is not linear and appears to follow a second order curve (Fig. 3a). It suggests that methylglyoxal formation kinetics from triose phosphates in situ is more complex than anticipated from a simple first order kinetics. Autocatalysis or paracatalytic reactions may be involved in this process, which requires further investigation.

To address the question of what is the net flux of methylglyoxal when the glyoxalase enzymes are active, methylglyoxal formation in situ was probed in the physiological concentration range of GSH, in order to activate the glyoxalase enzymes. Under these conditions, methylglyoxal concentration decreases considerably to values similar to the ones measured during growth (Fig. 3b).

The importance of the glyoxalase enzymes in keeping very low intracellular methylglyoxal concentrations and the catalytic role of GSH in the process can be fully appreciated by these results. Nevertheless, null mutants for both glyoxalases are viable, revealing extreme sensitivity to extracellular methylglyoxal concentration or when growing in glycerol [9,27]. In the latter case, the triose phosphates pool is probably increased, with a consequent increase of methylglyoxal formation [35].

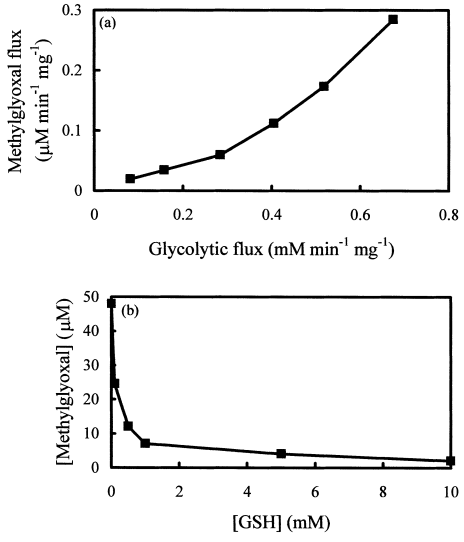

Fig. 3. In situ methylglyoxal flux. a: Quantitative relation between methylglyoxal formation flux and glycolytic flux at GAP dehydrogenase. For each concentration of D-fructose-1,6-bisphosphate (1 to 10 mM) glycolytic flux and methylglyoxal formation were determined simultaneously in situ. Cells were harvested at the beginning of growth. b: Effect of glyoxalase enzymes activation by GSH on methylglyoxal formation. Methylglyoxal concentration after a 2 h incubation of permeabilized cells in the presence of 1 mM of D-fructose-1,6-bisphosphate. Results are from a representative experiment of a set of three independent experiments.

Our results also show the absence of enzymatic synthesis of methylglyoxal and, under these conditions, 0.3% of the glycolytic flux is diverted through the glyoxalase pathway to D-lactate. This is also true for red blood cells [29] and most likely applies to other eukaryotic cell types. The in situ flux analysis shows a defined and quantitative relation between methylglyoxal formation flux and glycolytic flux. It also stresses the importance of the glyoxalase pathway in keeping the intracellular concentration of methylglyoxal in the μM range.

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