Toxicity of 3-*O*-methyl glucose in yeast is due to its phosphorylation by glucokinase

José M. Siverio+, Marta D. Valdés-Hevia and Carlos Gancedo*

Instituto de Investigaciones Biomédicas del CSIC, Facultad de Medicina UAM, Arzobispo Morcillo 4, 28029 Madrid, Spain

Received 18 October 1985

3-*O*-Methyl glucose inhibited the growth of *S.cerevisiae* on non-fermentable carbon sources only in strains possessing glucokinase. Accumulation of a phosphorylated derivative of 3-*O*-methyl glucose occurred only in strains carrying glucokinase. Phosphorylation of the sugar in vitro was observed with partially purified preparations of glucokinase but not with equivalent preparations of isoenzymes PI or PII of hexokinase. The K_m value of glucokinase for 3-*O*-methyl glucose was 5 mM and the V_{max} was ~ 1/100 of that found for glucose. A mutant resistant to 3-*O*-methyl glucose was isolated from a strain possessing only glucokinase. This mutant had only about 10% of the glucokinase found in the parental strain.

3-O-Methyl glucose *Glucokinase* *Phosphorylation* *(Yeast)* *Hexokinase*

1. INTRODUCTION

3-*O*-Methyl glucose (3-MG) is a structural analogue of glucose that is toxic to yeast cells growing on non-fermentable carbon sources [1]. This toxicity has been ascribed to the accumulation of a phosphorylated derivative of the analogue that could interfere with the derepression of certain enzymes necessary for growth on these carbon sources as mutants resistant to the toxic effect of the analogue did not accumulate the phosphorylated derivative [1]. The yeast *Saccharomyces cerevisiae* possesses 3 isoenzymes able to phosphorylate glucose, 2 hexokinases named PI and PII and a glucokinase [2,3]. Since contradictory results exist in the literature about the ability of glucose phosphorylating enzymes to phosphorylate 3-MG [4,5] it appeared of interest to study in particular detail the phosphorylation of 3-MG observed in yeast. To this end we used mutants possessing only one of each glucose phosphorylating enzyme.

We present here evidence showing that only glucokinase is able to phosphorylate 3-MG.

2. MATERIALS AND METHODS

Four strains of *S. cerevisiae* were used: F-11 (J. Conde, Sevilla, Spain) was used as wild type, P1T8C has only hexokinase PI activity, P2T22D possesses only hexokinase PII and D-308 exhibits only glucokinase activity (these last strains were kindly provided by K.D. Entian, Tübingen, FRG). Yeasts were grown on rich medium with 2% glucose or ethanol as carbon sources. Growth was followed turbidimetrically at 660 nm. Extracts for determination of enzymatic activity were obtained as in [6]. Glucokinase, hexokinase PI and PII were partially purified as described [7]. Paper chromatography and extraction of metabolites was performed as in [1]. Phosphorylating activity on glucose or on fructose was followed spec-

This paper is dedicated to S.P. Datta in acknowledgement of his dedication to FEBS

* To whom correspondence should be addressed
+ On leave of absence from Departamento de Bioquímica, Facultad de Biología, La Laguna, Tenerife, Spain

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

trophotometrically as described in [2]. It was checked that the incubation mixture used yielded essentially the same results as that described below for phosphorylation of 3-MG. Phosphorylation of 3-MG in vitro was determined as follows: in a final volume of 1 ml, 0.5 units (measured with glucose as substrate) of partially purified preparations of hexokinase PI, PII or glucokinase were incubated at 30°C in a reaction mixture at pH 7 containing 25 mM ATP, 25 mM PEP, 5 units pyruvate kinase, 100 mM K_2HPO_4, 1 mM NaF, 50 mM 3-MG (0.5 Ci/mol) (free of glucose by treatment with glucose oxidase). At appropriate times aliquots of 25 μl were withdrawn, boiled for 5 min, centrifuged and chromatographed on paper as in [1].

To determine the K_m of glucokinase for 3-MG several samples containing different concentrations of labelled 3-MG were incubated in a mixture similar to that described above. Every hour aliquots of 50 μl were withdrawn from every sample and diluted with the same volume of ice-cold 1 M glucose. 50 μl of this mixture were applied to anion exchanger paper discs (Whatman DE-81). The filters were washed with 250 ml water, dried under an infrared lamp and counted in a scintillation counter. To determine the specific radioactivity of the 3-MG in each sample, 20 μl of the reaction mixture were withdrawn at time 0, applied to DE-81 discs, dried and counted in a scintillation counter. The velocities of phosphorylation were calculated from the slope of the line obtained plotting the radioactivity retained in the filters against time.

3-Methyl [1-^{3}H]glucose (3 Ci/mmol) was from Amersham (England). Unlabelled 3-MG was from Sigma (MO, USA). It contained about 0.2% glucose that was eliminated by treatment with glucose oxidase. Proteins were determined according to [8].

3. RESULTS

3.1. *Growth inhibition by 3-MG in yeast mutants possessing only one glucose phosphorylating isoenzyme*

3-MG is toxic to wild-type yeast growing on non-fermentable carbon sources and its toxicity appears to depend on its phosphorylation [1]. To determine if all the glucose phosphorylating isoenzymes are able to phosphorylate 3-MG we tested the growth inhibitory effect of the sugar on mutants possessing only one of the enzymes able to phosphorylate glucose. As can be seen in table 1, 3-MG inhibited growth only in the mutant harboring glucokinase.

3.2. *In vivo and in vitro phosphorylation of 3-MG*

When wild-type yeast is incubated with 3-MG, a phosphorylated derivative, likely 3-MG 6P, accumulates [1]. Incubation of the mutants mentioned above with 3-MG showed that the compound accumulated only in the mutant possessing glucokinase (table 2). Partially purified preparations of glucokinase phosphorylated 3-MG while no phosphorylation was observed with preparations of hexokinase PI or PII (fig.1).

3.3. *Kinetic parameters of glucokinase for 3-MG*

Determination of the maximal rate of glucokinase on 3-MG showed that it was 1/100 of that observed on glucose. K_m was calculated directly from results of phosphorylation of 3-MG (fig.2a) and as K_i of the competitive inhibition of the phosphorylation of glucose (fig.2b). Both methods gave a similar value of 5 mM.

3.4. *Mutants resistant to 3-MG*

In cultures of strain D-308 with ethanol and 3-MG growth could be observed after several days. This growth was due to the appearance of mutants resistant to the analogue. One of these mutants was isolated and purified on plates with ethanol and 0.1 M 3-MG. This mutant termed D-308/R

Table 1

Effect of 3-MG on the growth of yeast strains having only one hexose phosphorylating activity

Strain with	Generation time (h)	
	Without 3-MG	With 0.1 M 3-MG
Hexokinase PI	4	4.5
Hexokinase PII	4	5
Glucokinase	4	>24

Yeasts were grown on rich medium with ethanol as carbon source with the addition of 3-MG as indicated. Growth was followed as described in section 2

Table 2

Accumulation of the phosphorylated derivative of 3-MG in yeast strains having only one hexose phosphorylating activity

Strain with	Radioactivity (cpm)	
	3-MG	3-MG-P
Hexokinase PI	900	<20
Hexokinase PII	500	<20
Glucokinase	1200	1000

Suspensions of the different strains (20 mg/ml) were incubated with 40 mM 3-MG (0.5 Ci/mol) at 30°C in a shaker. After 2 h, 0.5 ml were withdrawn and extracted as described in section 2. An amount of extract corresponding to 80 μl of the original suspension was chromatographed on paper as described in section 2. A standard of 3-MG was run in parallel. Strips of the chromatogram were counted in a scintillation counter. Radioactivity appeared only in 2 spots, one corresponding to 3-MG and the other to its phosphorylated derivative

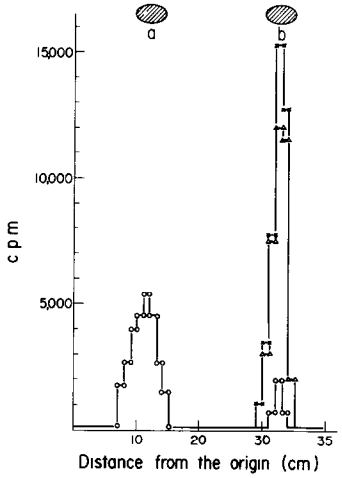

Fig.1. Paper chromatography of the products of phosphorylation of 3-MG by purified preparations of different hexokinase isoenzymes. Partially purified preparations of hexokinase PI (×—×), hexokinase PII (Δ—Δ) or glucokinase (○—○) were incubated with labelled 3-MG for 18 h as described in section 2. After chromatography on paper a part of the chromatogram was cut into 1 cm strips that were counted in a liquid scintillation counter. The other part of the chromatogram was tested with the aniline-diphenylamine reagent [9]. The shaded spots indicate the position of 3-MG (b) and its phosphorylated derivative (a) as detected with the sugar reagent. Time of chromatography 15 h.

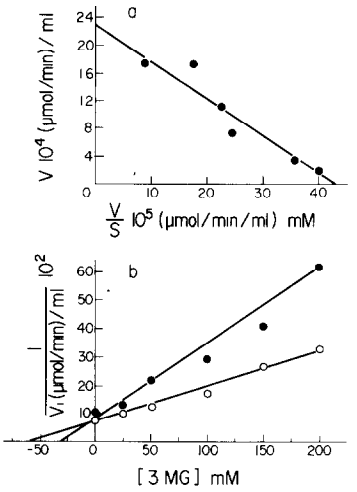

Fig.2. Determination of kinetic parameters of glucokinase for 3-MG. A partially purified preparation of enzyme was used. (a) Data obtained measuring phosphorylation of 3-MG. (b) Data obtained measuring the inhibition of phosphorylation of glucose (●—●, 0.03 mM; ○—○, 0.06 mM).

grew on glucose although more slowly than the parental (generation time 5 vs 3 h). No difference was observed when the growth substrate was ethanol. When glucokinase was assayed in the mutant it was found that its levels were about 10-times lower than in the wild type (15 vs 200 mU/mg protein, yeasts grown on ethanol). The mutant did not accumulate the phosphorylated derivative of 3-MG. Preliminary results suggest that the glucokinase of the mutant has a lower affinity for 3-MG than the parental one. Genetic studies with the mutant have been hampered by the fact that mutants affected in hexose phosphorylating enzymes present difficulties in sporulation [10] and also because on agar plates with 0.1 M 3-MG there is always a residual growth that makes difficult the observation of true resistants.

4. DISCUSSION

The results presented show that to exert its toxic effect on yeast 3-MG should be phosphorylated and that this phosphorylation is carried out only by glucokinase. These conclusions are sustained by the results obtained with the mutants lacking glucokinase and by the experiments on phosphorylation in vitro. Mutants lacking glucokinase did

not accumulate the phosphorylated derivative of 3-MG and their growth was unaffected by the sugar. Also hexokinase PI or PII were unable to phosphorylate 3-MG. In contrast mutants with glucokinase accumulated the derivative and had their growth inhibited. This enzyme phosphorylated in vitro 3-MG. Moreover, a mutant with reduced levels of glucokinase was resistant to the analogue and did not accumulate the phosphorylated derivative at appreciable levels. Consistent with the need of phosphorylation of 3-MG to cause physiological effects is our finding that 3-MG is not toxic for *Escherichia coli* (Valdés-Hevía, D.M., unpublished). This sugar is not a substrate for the phosphotransferase system and is therefore not phosphorylated [11].

Several cases of growth stasis caused by accumulation of phosphoric esters have been described in yeast [12,13]. One possibility to account for the growth inhibition could be depletion of ATP. This is however not the case for 3-MG since it has been shown that it causes only a slight decrease of the nucleotide [14]. Another possibility is that the esters act as corepressors inhibiting the synthesis of enzyme necessary for growth.

The kinetic parameters found for the phosphorylation of the sugar analogue may explain previous negative results on the ability of yeast 'hexokinase' to phosphorylate the analogue [4]. Taking into account the different specificities of hexokinases in micro-organisms [15] and the data presented here, results obtained with undefined preparations of hexokinase [16] should be reconsidered.

In vitro phosphorylation of 3-MG by commercial preparations of yeast hexokinase has been reported [1,2]. We have observed that different batches varied widely in their ability to phosphorylate the sugar, many of them being totally inactive. In the light of the experiments reported here the most reasonable explanation will be that different batches may differ in their glucokinase content.

ACKNOWLEDGEMENTS

We thank Dr K.D. Entian (Med. Naturwiss. Forschung Institut, Tübingen, FRG) for the gift of the mutants defective in hexose phosphorylating enzymes and Dr J.J. Aragón (this Institute) for suggestions on the assay of glucokinase by the radioactive method. This work was partially supported by a grant of the Spanish Comisión Asesora de Investigación Científica y Técnica.

REFERENCES

[1] Gancedo, C. and Gancedo, J.M. (1985) Eur. J. Biochem. 148, 593–597.
[2] Lobo, Z. and Maitra, P.K. (1977) Genetics 85, 727–744.
[3] Gancedo, J.M., Clifton, D. and Fraenkel, D.G. (1977) J. Biol. Chem. 252, 4443–4444.
[4] Sols, A. and Crane, R.K. (1954) J. Biol. Chem. 210, 581–595.
[5] Gatley, S.J., Holden, J.E., Halama, J.R., De Grado, T.R., Bernstein, D.R. and Ng, C.K. (1984) Biochem. Biophys. Res. Commun. 119, 1008–1014.
[6] Funayama, S., Gancedo, J.M. and Gancedo, C. (1980) Eur. J. Biochem. 109, 61–66.
[7] Maitra, P.K. (1975) Methods Enzymol. 42, 25–30.
[8] Lowry, O.H., Rosebrough, N.V., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 165–175.
[9] Schwimmer, S. and Bevenue, A. (1956) Science 123, 543–544.
[10] Maitra, P.K. and Lobo, Z. (1983) Genetics 105, 501–515.
[11] Harwood, J.P., Gazdar, C., Prasad, C. and Peterkofsky, A. (1976) J. Biol. Chem. 251, 2462–2468.
[12] Heredia, C.F. and Sols, A. (1964) Biochim. Biophys. Acta 86, 224–228.
[13] Platt, T. (1984) Mol. Cell. Biol. 4, 894–896.
[14] Eraso, P. and Gancedo, J.M. (1985) FEBS Lett. 191, 51–54.
[15] Del Valle, J.A. and Asensio, C. (1978) Biosystems 10, 265–282.
[16] Machado de Domenech, E. and Sols, A. (1980) FEBS Lett. 119, 174–176.