The *in situ* Assay of *Candida albicans* Enzymes during Yeast Growth and Germ-tube Formation

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Conditions are described for the preparation of permeabilized cells of *Candida albicans*. This method has been used for the *in situ* assay of enzymes in both yeast cells and germ-tube forming cells. A mixture of toluene/ethanol/Triton X-100 (1:4:0·2, by vol.) at 15% (v/v) and 8% (v/v) was optimal for the *in situ* assay of glucose-6-phosphate dehydrogenase in yeast and germ-tube forming cells, respectively. The concentration of toluene/ethanol/Triton X-100 required for optimal *in situ* activity of other enzymes was influenced by the cellular location of the enzyme, growth phase and morphology. The membrane-bound enzymes (chitin synthase, glucan synthase, ATPase), cytosolic enzymes (glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase, pyruvate kinase, phosphofructokinase, alkaline phosphatase, glucosamine-6-phosphate deaminase and *N*-acetylglucosamine kinase) and wall enzymes (β-glucosidase and acid phosphatase) were measured and compared to the activity obtained in cell extracts. The pattern of enzyme induction and the properties of the allosteric enzymes phosphofructokinase and pyruvate kinase were measured *in situ*. Pyruvate kinase *in situ* was homotropic for phosphoenolpyruvate with a Hill coefficient of 1·9 and a $s_{0.5}$ of 0·6 mM, whereas in cell extracts, it had a Hill coefficient of 1·9 and a $s_{0.5}$ of 1·0 mM. The K_m for ATP was 1·6 mM in cell extracts and 1·8 mM in permeabilized cells. *In situ* phosphofructokinase was homotropic for fructose 6-phosphate ($s_{0.5}$ of 2·3 mM, Hill coefficient of 4·0). The kinetic properties of pyruvate kinase and phosphofructokinase measured *in situ* or *in vitro* were similar for both yeast cells and germ-tube forming cells.

INTRODUCTION

Permeabilized cells provide a convenient system for the assay of intracellular enzymes. The porosity of the cell membrane of the intact cell can be increased by several techniques which allow substrates not normally permeable to cells to enter the intracellular compartment(s). These conditions, designated by Reeves & Sols (1973) as *in situ*, have been used with *Escherichia coli* for measuring the phosphotransferase system (Gachelin, 1969; Kornberg & Reeves, 1972), enzyme induction (Pardee *et al.*, 1959) and catabolite repression (Loomis & Magasanik, 1964). The ease with which some fungal and mammalian cells can be permeabilized has made the *in situ* approach invaluable in the study of enzymes and DNA synthesis during growth (Felix, 1982; Castellot *et al.*, 1978). The permeabilized cell as a system for biochemical study has recently been reviewed in some detail (Felix, 1982).

Permeabilized cells of *Saccharomyces cerevisiae* have been used for the study of glycolytic enzymes (Serrano *et al.*, 1973; Gancedo & Banuelos, 1979), α-glucosidase (Adams, 1972), citrate synthase (Weitzman & Hewson, 1973) and chitin synthase (Flores-Carreon *et al.*, 1980). The conditions devised for studying a particular enzyme *in situ* may not be optimal for other enzymes

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Abbreviations: Fru 6-P, fructose 6-phosphate; FDP, fructose 1,6-diphosphate; Glc-6-P dehydrogenase, glucose-6-phosphate dehydrogenase; PEP, phosphoenolpyruvate; TET, toluene/ethanol/Triton X-100 (1:4:0·2, by vol.).

in the same organism or for studies with different organisms (for review, see Felix, 1982). Often little attention has been given to assessment of the extent of permeabilization of the cells being studied. Permeabilized *Candida albicans* have been used for studies on trehalase (Arnold & McClellan, 1975) and 1,3-β-glucanase (Notario, 1982). In this paper, the optimal conditions for permeabilizing different cell types (yeast cells and germ-tube forming cells) of *Candida albicans* are described. The activities of a number of intracellular enzymes were measured in permeabilized cells.

METHODS

Growth of organism, germ-tube formation and isotope labelling of cells. Yeast cells of *Candida albicans* ATCC 10261 and strain A72 obtained from Dr A. Cassone, Instituto Superiore di Sanita, Rome, Italy, were prepared in shake cultures using a glucose, salts and biotin medium (Shepherd & Sullivan, 1976). Exponential phase yeast cells were harvested after 16 h growth. Yeast cell suspensions were starved for germ-tube formation by aerating (100 cm^3 min^{-1} per 100 ml) for 24 h at room temperature and germ-tube formation was induced in GlcNAc as described by Shepherd *et al.* (1980*a*). Germ-tube forming cells were labelled by adding ^{35}SO$_4$ [0·04 μCi ml^{-1} (1·48 kBq ml^{-1})] to the germination medium. The extent of germ-tube formation was assessed by light microscopy.

Preparation of permeabilized cells. Cells were permeabilized by a modification of the method of Serrano *et al.* (1973). Unless otherwise stated, cells were suspended in 0·1 M-imidazole/HCl buffer, pH 7·0, containing 0·2 M-KCl and 0·1 M-MgCl$_2$ at 1·2–1·6 × 10^9 cells ml^{-1}. Permeabilizing agents were added as indicated, and the suspensions were shaken at maximum speed on a wrist-shaker (Griffin and George Microid shaker) for 5 min at 18 °C. The cells were washed three times by centrifuging (2500 *g* for 10 min) with the buffer to be used in the enzyme assay. The washed cell suspensions were stored in the same buffer at 4 °C. For pyruvate kinase kinetics the imidazole buffer was replaced with 0·1 M-phosphate, pH 7·0. For the measurement of phosphofructokinase, the cells were suspended in 0·1 M-Tris/HCl, pH 7·6, containing 0·1 M-KCl, 0·01 M-MgCl$_2$, 7 mM-β-mercaptoethanol and 10 mM-(NH$_4$)$_2$SO$_4$. Unless otherwise stated, the permeabilizing agent used was toluene/ethanol/Triton X-100 (1:4:0·2, by vol.) (TET). Yeast cells and 1 h germ-tube forming cells were permeabilized with 17% (v/v) and 10% (v/v) of the reagent, respectively.

Preparation of cell extracts. Cells were suspended in the appropriate enzyme assay buffer at the concentration used for permeabilized preparations. Glass beads (0·5 mm) were added to a ratio of 1:2 (v/v) and the suspension shaken at 4000 r.p.m. (2 × 30 s) in a Braun homogenizer with cooling by compressed CO$_2$. The glass beads were separated from the cell extract by centrifuging (2000 *g* for 5 min). The cell extracts for pyruvate kinase and phosphofructokinase studies were chromatographed on a column (9 × 2·5 cm) of Sephadex G-25 using 0·05 M-phosphate, pH 7·0 and 0·1 M-Tris/HCl, pH 7·6, respectively.

Enzyme assays. Acid and alkaline phosphatases (EC 3.1.3.2 and EC 3.1.3.1, respectively) were measured at 30 °C using fixed time assays of 30 min and 10 min, respectively. Each assay (0·5 ml) contained *p*-nitrophenol phosphate (6 mM) in 0·1 M-acetate buffer, pH 4·0 (acid phosphatase) or 0·8 M-Tris/HCl buffer, pH 9·0, containing 0·1 M-MgCl$_2$ (alkaline phosphatase). The reaction was stopped by the addition of 0·5 ml 10% (w/w) TCA and the tubes were chilled on ice. After the addition of 1·5 ml 1·0 M-NaOH, the tubes were centrifuged to remove denatured protein and the nitrophenol produced was measured at 420 nm (ε = 16 × 10^3 mol^{-1} cm^{-1}).

β-Glucosidase (EC 3.2.1.21) was measured in 0·1 M-potassium phosphate, pH 7·0 (30 min at 30 °C in a final volume of 0·5 ml) using *p*-nitrophenol-β-D-glucose at 6 mM. The production of *p*-nitrophenol was measured as described above. Glucosamine-6-phosphate deaminase (EC 5.3.1.10) was assayed as described by Gopal *et al.* (1982*a*), chitin synthase (EC 2.4.1.16) as described by Chiew *et al.* (1980), and 1,3-β-glucan synthase (EC 2.4.1.34) as described by Gopal *et al.* (1982*b*).

ATPase (EC 3.6.1.3) activity was measured by a discontinuous method in an assay volume of 0·5 ml. Each assay contained 10 μmol MgCl$_2$, 100 μmol KCl and 5·0 μmol ATP. Assays were performed in either 0·2 M-acetate buffer, pH 5·6 or 0·2 M-Tris/HCl buffer, pH 9·1. The assay mixtures were incubated for 15 min at 37 °C, and the reaction was stopped by the addition of 0·1 ml 5% (w/w) TCA. After neutralizing and centrifuging the assay mixture, the ADP formed was measured spectrophotometrically by the coupled pyruvate kinase–lactate dehydrogenase assay described by Hess & Wieker (1974).

The following continuous enzyme assays were performed at 30 °C in a spectrophotometer fitted with a jacketed cuvette compartment. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was assayed essentially as described by Broad & Shepherd (1970). Each assay (1·0 ml final volume) contained 0·50 μmol Glc-6-P, 8·0 μmol MgCl$_2$ in 0·08 M-Tris/HCl, pH 7·2. NADP$^+$ isocitrate dehydrogenase (EC 1.1.1.42) was assayed by the method of Flavell & Fincham (1968). Each assay (final volume of 1·15 ml) contained 0·2 μmol NADP$^+$ and 1·8 μmol isocitrate in 0·16 M-potassium phosphate, pH 7·0 and 2·0 mM-MgCl$_2$. Pyruvate kinase (EC 2.7.1.40) was measured by the coupled lactate dehydrogenase method (Hess & Wieker, 1974). Each assay (1·0 ml) was in 0·1 M-phosphate buffer

(pH 6·0) containing 0·1 M-KCl, 0·01 M-MgCl$_2$, 2·5 µmol ADP, 2·5 µmol PEP, 0·2 mM-NADH and lactate dehydrogenase. The lactate dehydrogenase added was sufficient to give an extinction change of 2 extinction units min^{-1} upon the addition of excess pyruvate. 6-Phosphofructokinase activity (EC 2.7.1.11) was measured with a coupled assay system described by Racker (1947). The disappearance of NADH was followed at pH 7·6 in a 1·0 ml assay system which contained 80 µmol Tris/HCl, 80 µmol KCl, 8 µmol MgCl$_2$, 2·0 µmol Fru 6-P, 1·8 µmol ITP, 0·16 µmol NADH, 16·0 µmol (NH$_4$)$_2$SO$_4$ and 5·6 µmol β-mercaptoethanol. Sufficient rabbit muscle aldolase ($\sim$1·0 unit), triose phosphate dehydrogenase ($\sim$0·1 unit) and baker's yeast α-glycerophosphate dehydrogenase ($\sim$1·0 unit) was added to each assay to give an extinction change of 1·6 units min^{-1} upon the addition of excess FDP.

Enzyme units. A unit of activity in all assays is the amount of enzyme which transforms 1 µmol of substrate per min under the conditions of the assay. In this communication, all results are expressed on a cell number basis (i.e. units per 10^{10} cells). In growing yeast cells of *C. albicans*, 10^{10} cells is equivalent to 58 mg dry weight. With the starved and 1 h germinating cells, 10^{10} cells are equivalent to 44 and 46 mg, respectively (Sundaram *et al.*, 1981). For allosteric enzymes $s_{0.5}$ and h are the substrate concentration for half maximal activity and the Hill coefficient, respectively.

Analytical methods. Oxygen uptake rates were measured in a Clarke type oxygen electrode (Rank Instruments, Cambridge, U.K.). Carbohydrate was determined by the method of Dubois *et al.* (1956), glucose by the glucose oxidase method of Lloyd & Whelan (1969), and reducing sugar by the method of Lever (1973). Protein was determined by the Lowry method using crystalline bovine serum albumin as standard. Radioactively labelled material was counted in Bray's scintillant (Bray, 1960). Dry weights and cell numbers were determined as described by Sundaram *et al.* (1981).

RESULTS

Preparation of permeabilized cells

A variety of reagents were used to permeabilize yeast cells of *C. albicans* (Table 1). The effect of each agent was assessed by comparing the Glc-6-P dehydrogenase activity in a homogenate from an untreated batch of cells with the *in situ* activity. No Glc-6-P dehydrogenase was detected in cells treated with the detergents SDS (2%, v/v) and Triton X-100 (2%, v/v), but more than 90% of the control activity was recovered when the treated cells were homogenized. A similar result was obtained when cell suspensions were freeze-thawed up to eight times in buffer or treated with 50% (v/v) ether, 15% (v/v) dimethylsulphoxide, 10% (v/v) toluene and 10% (v/v) ethanol (data not shown). After nystatin and filipin treatment, less than 2% of the total Glc-6-P dehydrogenase activity could be measured by the *in situ* assay. A combination of toluene and ethanol was found to partially permeabilize *C. albicans* cells. When Triton X-100 was included, higher activities of Glc-6-P dehydrogenase could be measured. Table 1 shows that toluene/ethanol/Triton X-100 as a 1:4:0·2 (by vol.) mixture (TET) was the most effective agent tested for permeabilizing exponential phase yeast cells of *C. albicans*.

Table 1. *Preparation of permeabilized yeast cells of C. albicans*

Buffered yeast cell suspensions (2·5 × 10^9 cells in 2·0 ml) were treated with permeabilizing agents as described in Methods and the washed cells assayed *in situ* for Glc-6-P dehydrogenase activity. A control sample of cells homogenized contained 1·13 units per 2·5 × 10^9 cells.

Permeabilizing agent	Concn (%, v/v)	Glc-6-P dehydrogenase *in situ* (units)	Homogenate activity (%)
Nystatin (300 units ml^{-1})	10·0	0·00	0
Filipin (400 µM)	10·0	0·02	2
Toluene/ethanol 5:4	0·5	0·04	3
Toluene/ethanol 5:4	17·0	0·11	10
	20·0	0·24	20
Toluene/ethanol/Triton X-100 5:4:1	17·0	0·49	44
Toluene/ethanol/Triton X-100 3:4:1	17·0	0·60	54
Toluene/ethanol/Triton X-100 2:4:1	17·0	0·61	56
Toluene/ethanol/Triton X-100 1:4:0·2	17·0	0·72	65
Toluene	10·0	0·00	0
Ethanol	10·0	0·00	0

Table 2. *Extraction of carbohydrate and protein from cells after treatment with TET*

Radioactively labelled cells were treated with different concentrations of TET and the protein and carbohydrate extracted into the permeabilizing buffer were analysed as described in Methods. A sample of the cell suspension was homogenized and analysed as a control. The results are shown from one representative experiment. The data are expressed as the amount extracted from 10^{10} cells. The yeast cell homogenate contained a total of 31 mg protein and 47 mg carbohydrate per 10^{10} cells, the germ-tube forming cell homogenate contained a total of 24 mg protein and 42 mg carbohydrate per 10^{10} cells.

(a) *Yeast cells*

TET concn (%, v/v)	Protein (mg)	Carbohydrate (mg)	Reducing sugar (μg)	Glucose (μg)
0	1·0	0·3	31	28
5	1·4	1·5	56	60
10	1·6	2·2	91	88
15	1·5	2·5	87	90
20	2·0	2·5	84	87

(b) *1 h germ-tube forming cells*

TET concn (%, v/v)	Protein (mg)	Carbohydrate (mg)	Reducing sugar (μg)	Glucose (μg)	$10^{-3} \times$ Radioactivity (c.p.m.)*
0	1·6	0·2	50	0	0·5
4	2·9	1·9	350	94	100
8	3·2	3·2	500	115	120
12	4·0	3·2	860	310	150
16	5·0	3·9	1010	270	160

* Total ^{35}S counts in germ-tube forming cell homogenates was 242×10^3 c.p.m.

The amount of Glc-6-P dehydrogenase activity detected in yeast cells permeabilized with TET was not increased by the inclusion of 10 mM-β-mercaptoethanol, 50 μM-phenylmethylsulphonyl fluoride (PMSF) or both β-mercaptoethanol and PMSF, by shaking cells for longer than 5 min or by a second treatment with the permeabilizing agent. There was no significant difference in the extent of permeabilization over the temperature range 4 to 37 °C.

Removal of material from cells during permeabilization

Depending on the concentration of reagent employed, 1·0–6% of the carbohydrate was extracted from yeast cells (Table 2). All the reducing sugar was accounted for as glucose, but this represented only 6% of the total carbohydrate in the cells. Carbohydrate and protein extraction was higher (18% and 9% maximum, respectively) with germ-tube forming cells. Up to 30% of the total carbohydrate extracted was reducing sugar, but glucose accounted for less than 30% of this fraction. $^{35}SO_4$ was readily incorporated into germ-tube forming cells (Table 2). When these cells were permeabilized, maximum recovery of counts in the TET buffer (65%) corresponded to maximum *in situ* Glc-6-P dehydrogenase activity (Fig. 1). SDS–PAGE of TET extracts of *C. albicans* cells revealed several bands of which five (75, 66, 45, 34 and 18 kDal) comigrated with cytoplasmic proteins (data not shown). Intracellular Glc-6-P dehydrogenase, alkaline phosphatase, isocitrate dehydrogenase and pyruvate kinase were not released during the permeabilizing treatment. Permeabilized cells failed to respire and were not viable (less than 0·1% viable counts of a control) when plated on yeast extract peptone medium (data not shown).

Studies on enzymes in situ

The data given represent typical examples of experiments which have been repeated at least three times with duplicate assays (standard error < 10%). Variability between the different experiments was less than 20% with the exception of pyruvate kinase where the variation was up to 50%.
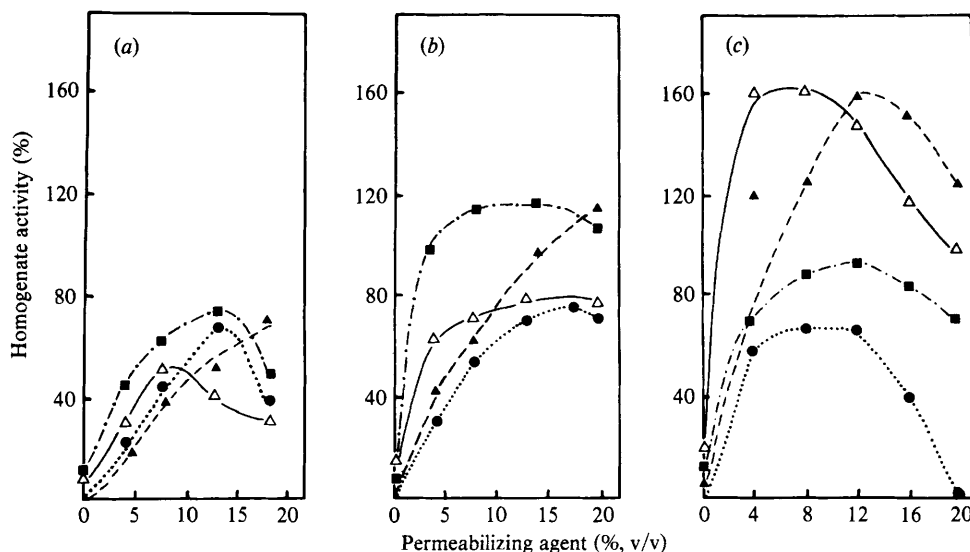
Fig. 1. The effect of varying the concentration of TET on the *in situ* assay of enzymes. Yeast cells (*a*), starved cells (*b*) and 1 h germ-tube forming cells (*c*) were permeabilized with different concentrations of TET as described in Methods. Glc-6-P dehydrogenase (●), alkaline phosphatase (▲), acid phosphatase (△) and β-glucosidase (■) were assayed *in situ* and activities expressed as percentages of the activity in homogenates from the appropriate cell suspension.

Optimum conditions for in situ enzyme assays

The *in situ* activities varied with the concentrations of permeabilizing agent and the type of cell used (Fig. 1). Yeast cells (Fig. 1*a*) showed optimal Glc-6-P dehydrogenase and β-glucosidase activities when cells were permeabilized with 10–15% (v/v) TET. Alkaline phosphatase activity increased over the range of TET shown, but was optimal at 20–25% (v/v). Maximum acid phosphatase was obtained with 7% (v/v) TET. Starved cells and germ-tube forming cells (Fig. 1*b*, *c*) were more susceptible to the permeabilizing agent. With starved cells maximum β-glucosidase and acid phosphatase activities were obtained with 8–15% (v/v) agent. Glc-6-P dehydrogenase activity was optimal around 15% (v/v). Although alkaline phosphatase activity increased over the range of TET shown, maximum activity was assayed at 20–25% (v/v). Glc-6-P dehydrogenase and acid phosphatase activities in germ-tube forming cells are fully exposed at 6% (v/v) TET. Alkaline phosphatase and β-glucosidase are fully expressed by 12% (v/v) TET. High concentrations of TET (greater than 12%, v/v) resulted in a decrease in all *in situ* activities with germ-tube forming cells. The concentrations of TET required for optimal *in situ* assays and the higher activities found for acid and alkaline phosphatases reflect differences in the cell membrane and cell wall of yeast and germ-tube forming cells.

In Table 3 and Fig. 1, the activity of several enzymes are compared. With exponential phase yeast cells, only 55–75% of the *in vitro* activities of Glc-6-P dehydrogenase, isocitrate dehydrogenase, acid phosphatase and pyruvate kinase could be detected in the permeabilized cells. Alkaline phosphatase and ATPase activity in permeabilized yeast cells was 70–100% of the *in vitro* activity. With starved yeast cells, the total *in vitro* isocitrate dehydrogenase, ATPase and alkaline phosphatase could be detected *in situ*; however, *in situ* assays accounted for only 70–90% of Glc-6-P dehydrogenase, acid phosphatase and pyruvate kinase. In germ-tube forming cells, the activity of Glc-6-P dehydrogenase, isocitrate dehydrogenase and ATPase were found to be similar in permeabilized cells and homogenates, However, acid and alkaline phosphatase activities *in situ* were 1·6 times higher than the *in vitro* activity.

In situ measurement of membrane-bound enzymes

ATPase activity in permeabilized cells and homogenates was assayed at pH 5·6 and 9·1. ADP formed in the assay was linear with time for 20 min and proportional to the cell number up to

Table 3. *Comparison of in vitro and in situ enzyme activities*

Cells were treated with TET at concentrations which gave the maximum *in situ* activity for Glc-6-P dehydrogenase, acid and alkaline phosphatase (Fig. 1). For isocitrate dehydrogenase, pyruvate kinase and ATPase, the TET concentration was 17% (v/v). Enzyme assays were performed as described in Methods and activities are expressed as units per 10^{10} cells. The *in vitro* activity is from a homogenate.

Enzyme	Homogenate activity	*In situ* activity	Ratio of *in situ* : *in vitro* activity
Glc-6-P dehydrogenase			
yeast cells	4·50	2·90	0·65
starved cells	6·04	4·61	0·76
1 h germ-tube forming cells	4·01	4·11	1·02
Isocitrate dehydrogenase			
yeast cells	0·32	0·18	0·56
starved cells	0·33	0·34	1·03
1 h germ-tube forming cells	0·31	0·30	0·98
Acid phosphatase			
yeast cells	0·23	0·19	0·65
starved cells	0·38	0·28	0·74
1 h germ-tube forming cells	0·27	0·50	1·60
Alkaline phosphatase			
yeast cells	3·64	4·08	1·12
starved cells	2·40	2·46	1·03
1 h germ-tube forming cells	1·50	2·40	1·60
Pyruvate kinase			
yeast cells	15–30	13–25	0·5–0·8
starved cells	15–31	14–34	0·7–0·9
1 h germ-tube forming cells	14–30	10–40	0·7–1·03
ATPase, pH 5·6			
yeast cells	1·30	1·01	0·77
starved cells	1·23	1·13	0·92
1 h germ-tube forming cells	1·23	1·20	0·98

1.4×10^9 cells. The ATPase activity in four different cell samples at pH 5·6 and pH 9·1 was 1·3 (± 0.3) and 4·2 (± 0.6) units per 10^{10} yeast cells, respectively. Dicyclohexyl carbodiimide (760 µg ml^{-1}) and oligomycin (150 µg ml^{-1}) had no effect on the pH 5·6 ATPase, but inhibited the pH 9·1 activity (87% and 90%, respectively). Chitin synthase and 1,3-β-glucan synthase could be measured after treatment with either 4% (v/v) TET or 4% (v/v) toluene/ethanol (1:1). Higher concentrations of TET gave reduced activities. The 1,3-β-glucan synthase activity was 17·5 units (g dry wt)$^{-1}$ with exponential phase yeast cells. Chitin synthase activity in yeast cells of strain A72 was 12·7 (± 1.0) units (g dry wt)$^{-1}$. The activity decreased in starved cells to 8·4 (± 0.4) units (g dry wt)$^{-1}$, but was 33 (± 1.0) units (g dry wt)$^{-1}$ in germ-tube forming cells. These changes in chitin synthase *in situ* are similar to the changes in the expressed activity of membrane preparations (Chiew *et al.*, 1980).

In situ measurement of glucosamine-6-phosphate deaminase and GlcNAc kinase induction

Glucosamine-6-phosphate deaminase and GlcNAc kinase in *C. albicans* are inducible (Gopal *et al.*, 1982*a*; Shepherd *et al.*, 1980*b*). A low basal level of deaminase activity was present in non-induced cells (Table 4) but after incubation with 2·5 mM-GlcNAc for 3 h, a 22-fold higher activity was found in permeabilized cells. Since *C. albicans* cells contain approximately 20% protein, the deaminase activity of 1·34 units per 10^{10} cells translates to 0·087 units (mg protein)$^{-1}$. Values of 0·06–0·09 units (mg protein)$^{-1}$ are obtained in cell extracts (Gopal *et al.*, 1982*a*). The activity of GlcNAc kinase in non-induced cells (0·26 units per 10^{10} cells) increased to 3·1 units per 10^{10} cells after 3 h incubation in 2·5 mM-GlcNAc.

Properties of pyruvate kinase in situ

Pyruvate kinase in permeabilized cells showed similar characteristics to the enzyme in homogenates. When assayed with 2·5 mM-ADP and PEP, activity at pH 6·0 was twice that at

Table 4. *In situ assay of glucosamine-6-phosphate deaminase induction in C. albicans*

Starved yeast cells of *C. albicans* were incubated with 2·5 mM-GlcNAc at 30 °C. Portions (5·8 × 10^9 cells) were removed at 0, 45, 90, 135 and 180 min and permeabilized with TET at a final concentration of 9% (v/v) in 0·1 M-imidazole/HCl buffer, pH 7·0, containing 0·1 M-KCl and 10 mM-MgCl₂. Glucosamine-6-phosphate deaminase was assayed as described in Methods.

Time (min)	Glucosamine-6-phosphate deaminase activity units per 10^{10} cells	Fold increase above basal activity
0	0·06	–
45	0·66	11
90	0·84	14
135	1·10	18
180	1·34	22

Table 5. *Kinetic properties of pyruvate kinase measured in vitro and in situ*

Homogenates and permeabilized cells were prepared as described in Methods. Pyruvate kinase was measured in (a) with 2·5 mM-ADP, varying concentrations of phosphoenolpyruvate (PEP) ±0·2 mM-fructose-1,6-diphosphate (FDP), and in (b) with 2·5 mM-PEP, varying ADP and ±1·96 mM-ATP. Hill plots were used to determine the apparent $s_{0.5}$ value for phosphoenolpyruvate and for the value of h. Lineweaver–Burke plots were used to determine the apparent K_m for ADP.

(a) *Variable PEP concentration*

	$s_{0.5}$ (mM PEP)		h value	
	No FDP	0·2 mM-FDP	No FDP	0·2 mM-FDP
1 h germ-tube forming cells				
in vitro	0·50	ND	2·0	ND
in situ	0·54	0·41	3·4	1·3
Yeast cells				
in vitro	0·60	0·40	1·9	1·2
in situ	1·0	0·50	1·9	1·2

(b) *Variable ADP concentration*

	K_m apparent (mM ADP)	
	No ATP	1·96 mM-ATP
1 h germ-tube forming cells		
in vitro	1·6	ND
in situ	1·4	ND
Yeast cells		
in vitro	1·6	1·8
in situ	1·8	2·0

ND, Not determined.

pH 7·0. The activity was stable to storage at 4 °C and was stimulated by the following: MgCl₂ (eightfold at 6 mM); NH₄Cl (eightfold with 100 mM) and KCl (fourfold with 100 mM). Under standard assay conditions with 0·4 mM-PEP, a twofold activation of pyruvate kinase was obtained with the addition of FDP at 0·5 μM.

Plots of pyruvate kinase activity *in situ* against PEP were sigmoidal, confirming the homotropic interaction for this substrate (data not shown). The properties of pyruvate kinase in permeabilized cells and homogenates are summarized in Table 5. A value of 0·5 mM-PEP was obtained for the apparent $s_{0.5}$ for the enzyme from germ-tube forming cells. The inclusion of FDP at 0·2 mM in the assays lowered the $s_{0.5}$ value to 0·4 mM for the *in situ* enzyme from germ-tube forming cells. Similar results were obtained with the enzyme from yeast cells except that the $s_{0.5}$ value for PEP (1 mM) was higher than the value for the enzyme in extracts (0·6 mM).

Hill plots for the enzyme from yeast cells gave similar h values in the *in vitro* and *in situ* assays

(1·90 and 1·95). Pyruvate kinase *in vitro* and *in situ* showed similar changes in the *h* value with the addition of 0·2 mM-FDP (1·30 and 1·20, respectively). With germ-tube forming cells, however, the Hill coefficient ($h = 3$) for the *in situ* enzyme was higher than the value ($h = 2$) obtained with cell extracts. The high value ($h = 3$) decreased to 1·3 when 0·2 mM-FDP was included in the assays.

Plots of velocity with varying ADP concentrations were hyperbolic (data not shown). When germ-tube forming cells were used, apparent K_m values of 1·6 mM and 1·4 mM were obtained with cell extract and permeabilized cells, respectively. For yeast cells, the apparent K_m values were 1·6 mM and 1·8 mM (Table 5). V_{max} for pyruvate kinase in both assays was reduced by 50% with 4 mM-ATP. The apparent K_m value for ADP did not change significantly by the inclusion of 1·96 mM-ATP. Dixon plots (not shown) of the inhibition of pyruvate kinase with ATP were non-linear. Pyruvate kinase activity was inhibited 50% by the presence of 4 mM-ATP in all assays.

Properties of phosphofructokinase in situ

Phosphofructokinase in permeabilized cells and homogenates showed similar profiles for activity versus pH. When assayed with 2·5 mM-ITP in either 0·1 M-Tris/HCl or 0·1 M-imidazole/HCl, activity at pH 6·5 was 0·13 of the activity at pH 7·6. The enzyme in both preparations was stimulated by: KCl (twofold with 50 mM), NH_4Cl (fourfold with 20 mM), $(NH_4)_2SO_4$ (threefold with 20 mM) and inhibited 50% by 20 mM-sodium phosphate (pH 7·0). Phosphofructokinase in permeabilized cells and cell extracts of 1 h germ-tube forming cells had the same stability (half-life of ~30 h at 4 °C) as the *in situ* yeast enzyme. However, the enzyme in yeast cell homogenates was unstable (half-life of 1·5 h at 4 °C).

The ATP inhibition *in situ* of phosphofructokinase from 1 h germ-tube forming cells was dependent upon the Fru 6-P concentration and similar to the inhibition observed *in vitro*. Under the standard assay conditions, 50% inhibition of activity was observed with 2 mM-ATP and 9 mM-ATP when the Fru 6-P concentration in the assay was 0·4 mM and 0·8 mM, respectively. When assayed *in situ* under the former conditions, the inhibition was relieved by either AMP or UMP (at 2 mM), but not CMP (Fig. 2a). Similar results were obtained with the enzyme from cell extracts (data not shown).
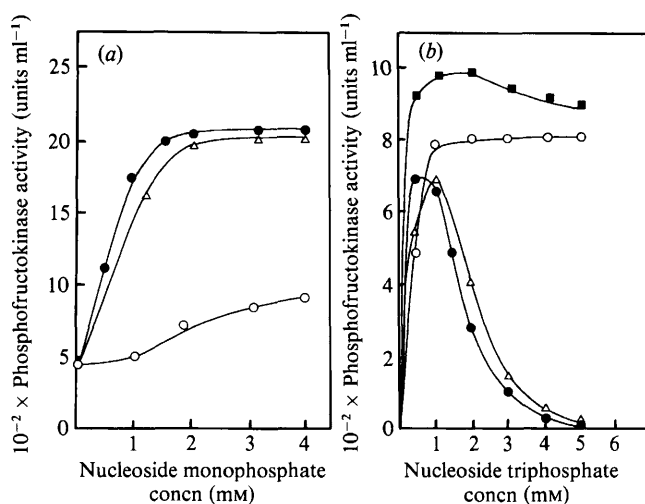

Fig. 2. (a) Effect of nucleoside monophosphates on phosphofructokinase. Varying concentrations of CMP (○), UMP (△) and AMP (●) were added to the standard assay containing 2·5 mM-ATP and 0·4 mM-fructose 6-phosphate. The *in situ* enzyme preparation contained 2·3 × 10⁸ germ-tube forming cells ml⁻¹. (b) Comparison of nucleoside triphosphates as substrates for phosphofructokinase. *In situ* assays were performed as described in Methods with varying concentrations of ATP (●), CTP (△), UTP (■) and ITP (○). An enzyme preparation containing 2·1 × 10⁸ germ-tube forming cells ml⁻¹ was used.

Table 6. *Apparent K_m and V_{max} values of nucleoside triphosphates for phosphofructokinase*

Substrate	Yeast cells *in situ*	1 h germ-tube-forming cells	
		in situ	*in vitro*
	(a) Apparent K_m (mM nucleoside triphosphate)		
ATP	0·07	0·06	0·07
ITP	0·28	0·30	0·31
UTP	0·35	0·27	0·30
CTP	ND	0·41	0·50
	(b) V_{max} (μmol min⁻¹ per 10^{10} cells)		
ATP	40	30	42
ITP	43	40	43
UTP	30	32	34
CTP	ND	31	27

ND, Not determined.

Table 7. *Kinetic properties of phosphofructokinase in vitro and in situ*

Permeabilized cells and homogenates were prepared and assayed as described in Methods and Fig. 3. Hill plots were used to determine (a) the apparent $s_{0.5}$ value and (b) h value for fructose 6-phosphate in assays using either ITP or ATP ($\pm$ AMP).

Substrate	Yeast cells *in situ*	1 h germ-tube-forming cells	
		in situ	*in vitro*
	(a) Apparent $s_{0.5}$ value (mM Fru b-P)		
ITP	0·8	0·8	0·7
ATP + AMP	1·4	1·7	ND
ATP	2·3	2·2	1·6
	(b) h value		
ITP	2·0	1·6	1·9
ATP + AMP	3·3	2·2	ND
ATP	4·0	3·0	3·5

ND, Not determined.

The *in situ* phosphofructokinase in the germ-tube forming cells exhibited a broad specificity with respect to the phosphate donor (Fig. 2b). ATP, UTP, CTP and ITP were substrates. At a concentration greater than 2 mM, CTP and ATP markedly reduced the *in situ* activity while UTP and ITP were without effect. Similar results were obtained with cell extracts. K_m values for the nucleoside triphosphate substrates are shown in Table 6.

Figure 3 shows a representative plot of velocity with varying Fru 6-P concentration for the enzyme from 1 h germ-tube forming cells. With ATP as the second substrate, the plot was sigmoidal and the co-operativity was decreased by the addition of AMP. Plots with ITP were essentially hyperbolic. Similar results were obtained for the enzyme in homogenates. A comparison of the kinetic properties of phosphofructokinase *in vitro* and *in situ* for different types of cells is summarized in Table 7.
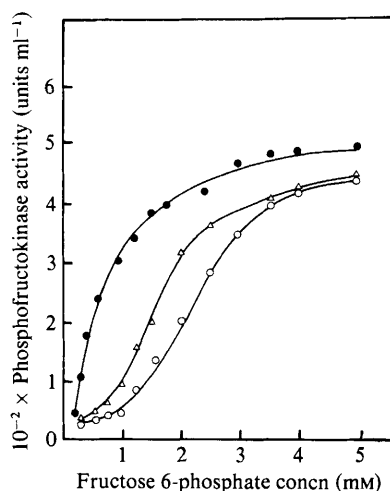
Fig. 3. Effect of fructose 6-phosphate on *in situ* phosphofructokinase activity. Phosphofructokinase activity was measured with varying Fru 6-P concentrations in assays which contained 0·5 mM-ATP (○), or 0·5 mM-ATP and 0·5 mM-AMP (△), or 0·5 mM-ITP (●). The *in situ* enzyme preparation contained 2·6 × 10⁸ germ-tube forming cells ml⁻¹.

DISCUSSION

Numerous reports exist on the permeabilization of *S. cerevisiae* (reviewed by Felix, 1982). By contrast, the optimal conditions for permeabilizing *C. albicans* have not been documented. Notario (1982) and Arnold & McClellan (1975) have measured *in situ* exo-1,3-β-glucanase and trehalase, respectively. However, no assessment of the extent of permeabilization or the utility of the method has been reported.

An extensive survey of different reagents tested singly and in combination established that TET produced yeast cells with the highest *in situ* Glc-6-P dehydrogenase activity. Different types of *C. albicans* cells responded differently to various combinations of permeabilizing reagents probably because of the differences in the composition of cell walls (Chattaway *et al.*, 1968) and plasma membranes (Marriott, 1975). Exponential phase yeast cells were more resistant than starved yeast cells and germ-tube forming cells. With exponential phase cells the ratio for *in situ* : *in vitro* activity varied from 0·50 to 0·80, while starved and germ-tube forming cells gave values in the range 0·74 to 1·03. The high *in situ* phosphatase activity compared with *in vitro* values suggests that the activity of this enzyme is partially masked in homogenates.

The extensive release of ³⁵S from labelled cells after treatment with TET (66% of the label) shows that the pool of low molecular weight metabolites in permeabilized cells had equilibrated with the extracellular medium. Free glucose found in TET extracts could arise from 1,3-β- and 1,6-β-glucanase, amyloglucosidase or trehalase activities. All carbohydrate present in TET extracts could be derived from the wall but only 3–6% (w/w) of the 20% (w/w) protein extracted could come from this source (P. A. Sullivan, unpublished data). Consistent with observations made with *E. coli* and other yeasts (Felix, 1982) PAGE of TET extracts of *C. albicans* cells confirmed the presence of cytoplasmic proteins.

The *in situ* measurement of chitin synthase confirmed a previous report (Chiew *et al.*, 1980) that a fourfold increase in enzyme activity occurs during germ-tube formation. Since most chitin synthase in *C. albicans* is in an inactive (proenzyme) form, it is likely that activated enzyme is measured. Both glucan and chitin synthases were assayed with ease after cells were treated with 4% (v/v) TET. In contrast to the ATPases, no activity was detected in cells treated with high concentrations of TET. Both the mitochondrial (alkali optimum, oligomycin and dicyclohexyl carbodiimide sensitive) ATPase and plasma membrane (acid optimum) ATPase could be assayed. The activity of both ATPases was similar in homogenates and permeabilized cells.

These results indicate that membrane bound enzymes and their properties can be studied in permeabilized cells.

The glucosamine-6-phosphate deaminase and GlcNAc kinase were previously shown to increase 25- and 20-fold, respectively, when starved cells of *C. albicans* were incubated with 2·5 mM-GlcNAc (Shepherd *et al.*, 1980b; Gopal *et al.*, 1982a). Permeabilization of cells removed from the cells the endogenous hexose phosphates known to interfere with the glucosamine-6-phosphate deaminase assays in cell extracts. The 25- and 15-fold increases in activity of the deaminase and kinase activities after 3 h incubation of cells in 2·5 mM-GlcNAc essentially replicate values obtained with the *in vitro* assays and show that the induction of enzymes can also be reliably monitored with *in situ* assays.

Intracellular enzymes are present in high concentrations (Sols & Marco, 1970) and, thus, protein–protein interactions could change the properties of an enzyme. Therefore, the *in situ* measurement of enzymes involved in the regulation of metabolism, particularly allosteric enzymes is of interest. Pyruvate kinase from both yeast and germ-tube forming cells was activated *in situ* twofold by FDP and showed homotropic interactions for PEP (Hill coefficient and $s_{0.5}$ values of approximately 2·0 and 0·5 mM, respectively). Similar properties have been reported for the pyruvate kinase measured *in situ* in *S. cerevisiae* (Banuelos & Gancedo, 1978). The K_m of pyruvate kinase from *C. albicans* for ADP (1·4–1·8 mM) was higher than that reported for the enzyme from *S. cerevisiae* (0·6 mM). *Candida albicans* phosphofructokinase exhibited the classical allosteric properties described for the enzyme isolated from a number of other sources (Bonnsaeng *et al.*, 1977; Bloxham & Lardy, 1973; Reeves & Sols, 1973; Lindell & Stellwagen, 1968). The degree of co-operativity with respect to Fru 6-P decreased with added AMP. Under similar conditions of assay, the Hill coefficients and $s_{0.5}$ value for Fru 6-P were the same for the *in vitro* and *in situ* preparations.

Phosphofructokinase has previously been studied in cell extracts from yeast, germ-tube and mycelial forms of *C. albicans* (Chattaway *et al.*, 1973). The results from the present study have confirmed that the properties of the enzyme from the different types of cells are similar with the exception that no marked differences were found in V_{max} when ATP rather than ITP was used as the phosphate donor. Further, phosphofructokinase activity remained constant during germ-tube formation.

Saccharomyces cerevisiae phosphofructokinase accounts for less than 2% of the rate at which glucose is utilized (Gancedo & Banuelos, 1979). Yeast cells of *C. albicans* utilize glucose at a rate of 2·8 μmol min⁻¹ per 10^{10} cells and approximately 60% of the assimilated carbon is expired as CO_2 (P. K. Gopal, M. G. Shepherd & P. A. Sullivan, unpublished results). From the amount of phosphofructokinase in *C. albicans* cells it can be calculated that glucose can be metabolized at the rate of 1·2–1·8 μmol min⁻¹ per 10^{10} cells. Since glucose flux through the Embden–Meyerhof pathway is between 60 and 80% (Boonsaeng *et al.*, 1977), phosphofructokinase can account for the rate of glucose utilization found with growing yeast cells of *C. albicans*.

The kinetic properties of pyruvate kinase and phosphofructokinase, are similar when measured either *in situ* or in cell extracts. Thus the *in situ* method of enzyme analysis is a useful alternative approach. This is especially so when an enzyme is labile such as the phosphofructokinase of *C. albicans* and *Candida* 107 (Ratledge & Botham, 1977).

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