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REVERSIBILITY OF THE AFFINITY-LABELLED BIOTIN TRANSPORT SYSTEM IN YEAST CELLS

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SUMMARY

Transport of biotin by *Saccharomyces cerevisiae* is inhibited by biotynyl *p*-nitrophenyl ester. Conversion of the inhibited cells to spheroplasts or simple treatment with thiols results in a total restoration of vitamin transport. Biotinyl *p*-nitrophenyl ester-induced inhibition is not due to an intracellular accumulation of the vitamin and consequent regulation, but appears to be due to specific labelling of the transport system.

INTRODUCTION

Studies to elucidate the mechanism and control of biotin transport [1, 2] were initiated by Lichstein and his associates first with cells of *Lactobacillus arabinosus* [3–6] and subsequently with the yeast, *Saccharomyces cerevisiae* [7]. The transport was stimulated by glucose and inhibited by biotin analogs, both features consistent with the phenomenon being an active transport process. Further studies [8] indicated that the transport of the vitamin was regulated by the biotin content of the medium. The inhibition of vitamin uptake in the case of cells grown on media with high biotin concentration was shown to be due to the repression of the synthesis of transport component(s) which appear to be firmly attached to the cell membrane [9]. Treatment of cells with biotinyl *p*-nitrophenyl ester (*p*BNP) resulted in a specific and irreversible loss of biotin transport presumably due to the affinity labelling of the transport system [10].

Current studies concern the reversal of *p*BNP-induced inhibition of transport by treatment with thiols. The biotin transport system of *S. cerevisiae* can be selectively turned off or on upon appropriate chemical treatment of the cells.

Abbreviations: *p*BNP, biotinyl *p*-nitrophenyl ester; *o*BNP, biotinyl *o*-nitrophenyl ester; DCC, dicyclohexylcarbodiimide.

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MATERIALS AND METHODS

Preparation of biotinyl nitrophenyl esters: *p*BNP was synthesized by a slight modification of the earlier procedure [10].

To biotin (244 mg, 1.0 mmol) suspended in 3 ml of methylene chloride was added *p*-nitrophenol (175 mg, 1.3 mmol) and dicyclohexylcarbodiimide (DCC) (206 mg, 1 mmol). After stirring the above mixture for 24 h at 25 °C the reaction mixture was taken to dryness under reduced pressure, and the yellow gummy residue was washed several times with absolute ether. The residue was taken up in isopropanol. Following filtration, the solution was reduced to minimum volume and allowed to crystallize overnight. The crystals of *p*BNP were collected by filtration and washed with anhydrous ether. Yield: 120 mg, 33 %; m.p. 156–158 °C. Anal. Calcd. for $C_{16}H_{19}N_3O_5S$: C, 52.60; H, 5.24; N, 11.50; S, 8.76. Found: C, 52.74; H, 5.35; N, 11.30; S, 8.61.

Analysis of the material by thin-layer chromatography in chloroform/methanol (37 : 3, v/v), revealed a single component, $R_f = 0.45$. Biotinyl *o*-nitrophenyl ester (*o*BNP) was synthesized by similar procedure.

The radioactive derivatives were synthesized by treating 0.266 mg (1.1 μmol) [^{14}C] carbonyl-D-biotin (Radiochemical Centre, Amersham, U.K.) with 100 μl of dichloromethane containing 0.27 mg of the appropriate nitrophenol and 0.23 mg DCC. After 24 h of reaction corresponding unlabelled BNP (25 mg) was added and the product recrystallized from isopropanol as described earlier.

Organism

S. cerevisiae, Fleischman strain 139 (ATCC 9896), was grown at 30 °C in Vogel's medium [11], containing inositol (36 μg/ml), calcium pantothenate (2 μg/ml), pyridoxine hydrochloride and thiamine hydrochloride (4 μg/ml each), biotin (0.25 ng/ml and glucose (1 %) instead of sucrose.

Preparation of spheroplasts

The conversion of yeast cells to their spheroplasts was accomplished using glusulase [12, 13]. In a typical experiment, washed cells were subjected to pretreatment with a medium (4.2 ml/g cells wet weight) containing EDTA (0.04 mM) and 2-mercaptoethanol (0.1 M) at pH 4. After pretreatment for 30 min at 30 °C, the cells were collected by centrifugation, washed once with distilled water and washed twice with 0.6 M KCl.

The treated cells were suspended in 0.6 M KCl (6.4 ml/g cells) containing EDTA (1.25 mM), cysteine hydrochloride (6.25 mM), potassium phosphate buffer (pH 6.1, 50 mM) and 0.4 ml glusulase (Endo Laboratories) with continuous gentle shaking at 30 °C for 60 min. Under these conditions, complete conversion of the cells to spheroplasts (as judged by the fragility of the latter to osmotic shock) was achieved. The spheroplasts were collected by centrifugation, washed twice with 0.6 M KCl, and resuspended in the same medium.

Chemical treatment of cells and spheroplasts

Inhibition studies were carried out be treating a washed and packed cell sample (resuspended to 2 mg cells dry weight per ml) with 10 % ethanolic *p*BNP to obtain a

final concentration of 10 μM for 30 min at 30 °C, after which the cells were washed three times and resuspended to the original volume. In the case of spheroplasts, the procedure was identical except for the inclusion of 0.6 M KCl in all the steps. Cells or spheroplasts treated under identical conditions, except for the omission of the biotinyl derivative, served as controls. Reactivation of inhibited cells was achieved by incubation with mercaptoethanol (0.15 M) at pH 4.0.

Measurement of biotin uptake

Cells or spheroplasts treated with the biotinyl derivative as described above were mixed with an equal volume of 0.1 M potassium phosphate solution (pH 4.0) containing glucose (2 %) and radioactive biotin (1.7 μM), 0.6 M KCl being present in experiments with spheroplasts. In view of the fragility of the spheroplasts (or of the cells treated with EDTA and mercaptoethanol) to Millipore filters, uptake was measured using a centrifugation procedure. Aliquots drawn at various intervals were transferred to a suspension of washed lyophilized yeast cells (incapable of biotin transport) which served as carriers. The suspension was then centrifuged (3000 rev./min) and the pellet was washed three times with 0.6 M KCl solution. The pellet was suspended in 1 ml of 0.6 M KCl solution and the entire suspension was transferred to scintillation fluid for counting [14].

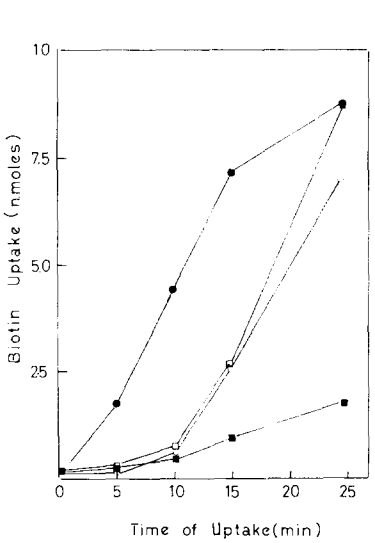

Fig. 1. Biotin uptake by: pBNP-treated cells (■), untreated control cells (●), spheroplasts from *p*BNP-inhibited cells (□) and spheroplasts from control cells (○).

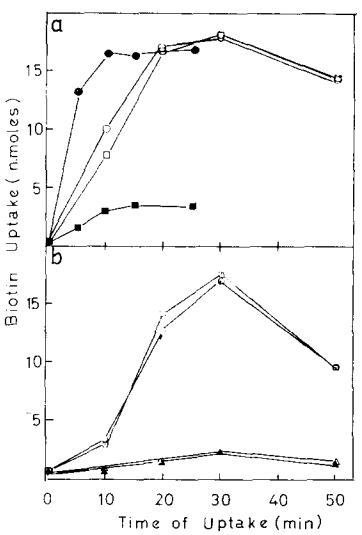

Fig. 2. (a) Biotin uptake by: *p*BNP-treated cells (■), ethanol-treated control cells (●) and by the same cells after treatment with 0.15 M mercaptoethanol (□) and (○), respectively. (b) Biotin uptake by: mercaptoethanol-treated control cells followed by additional treatment with ethanol (○) or *p*BNP (△); and *p*BNP-inhibited cells followed by mercaptoethanol treatment and further incubation with ethanol (⊡) or *p*BNP (▲). The initial lag in uptake noted with spheroplasts and treated cells appears to be due to depletion of energy source by extensive washings following chemical treatments. Equilibration with glucose for 15 min prior to incubation with [^{14}C]biotin abolishes the lag in the uptake.

RESULTS AND DISCUSSION

In an attempt to isolate biotin transport components from the plasma membranes of *p*BNP-treated cells of *S. cerevisiae*, spheroplasts of these affinity-labelled cells were required. Spheroplasts were easily produced by prior treatment of the cells with EDTA and mercaptoethanol followed by the action of glusulase [13].

The production of spheroplasts from the affinity-labelled cells resulted in the restoration of biotin transport (Fig. 1), indicating that during the preparation of the spheroplasts, the cells lost the label. In order to elucidate the stage at which this reversal of *p*BNP-induced inhibition occurred, *p*BNP-treated cells were subjected to treatment with 0.04 M EDTA and 0.15 M mercaptoethanol, under conditions identical with those used in the preparation of spheroplasts (but for the omission of glusulase). Such treatment also resulted in the restoration of the ability to transport biotin. Since treatment with EDTA alone failed to restore uptake, mercaptoethanol appeared to be essential for the regeneration of transport in the case of *p*BNP-inhibited cells. In fact, treatment of *p*BNP-inhibited cells with 0.15 M mercaptoethanol alone, resulted in the complete reactivation of these cells (Fig. 2a). Studies with various concentrations of mercaptoethanol revealed that reactivation was maximal at 0.15 M concentration of the reagent. The reactivated cells were similar to the original control cells, and they could be further inactivated by *p*BNP (Fig. 2b) indicating that the transport component(s) were not drastically altered by mercaptoethanol treatment. This would imply that *p*BNP inhibits biotin transport presumably by covalent attachment to an essential thiol group(s) of the transport component.

In order to elucidate the mechanism of *p*BNP-induced inhibition further, cells were incubated with either a solution of radioactive biotin (40 μM) or *p*BNP (10 μM) (in the absence of glucose) to achieve intracellular concentration of 0.2 nmol and 0.05 nmol of vitamin per mg dry cells, respectively. The transport was not affected by preincubation with biotin while almost complete inhibition was noted in the case of *p*BNP-treated cells even though the intracellular concentration of the vitamin was much less than that in the former. These observations suggest that *p*BNP does not exert its action by intracellular accumulation of the vitamin.

The maintenance of biotin transport activity in 0.15 M mercaptoethanol is in distinct contrast to the recently reported inhibitory effect of this thiol [9]. The deleterious effect of mercaptoethanol reported may well be due to the high pH (7.5) used in the latter study [9]. Indeed, an examination of the pH dependence of the mercaptoethanol-induced restoration of transport has shown that the maximum reactivation occurs between pH values of 4 and 5. Neither reactivation of the inhibited cells nor normal uptake in the case of control cells could be observed with thiols at pH 6.5.

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