Biochimica et Biophysica Acta, 555 (1979) 169—174

BBA Report

BBA 71395

THE EFFECT OF NYSTATIN ON ACTIVE TRANSPORT IN *RHODOTORULA GLUTINIS (GRACILIS)* IS RESTRICTED TO THE PLASMA MEMBRANE

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(Received March 26th, 1979)

Key words: Active transport; Nystatin; Plasma membrane; Mitochondria; Protoplast; (Rhodotorula)

Summary

The effect of the polyene antibiotic nystatin on the energy-requiring transport of substrates in the obligatory aerobic yeast *Rhodotorula glutinis (gracilis)* is restricted to its interaction with the plasma membrane, whereas the energy metabolism in mitochondria remains unaffected by the antibiotic. A method for preparation of the mitochondrial fraction from this yeast is described.

The obligatory aerobic yeast *Rhodotorula glutinis (gracilis)* has been shown to accumulate monosaccharides and polyoles inside the cell [1,2]. The transport process is strictly coupled to metabolic energy and is completely inhibited by metabolic inhibitors, uncouplers or anaerobiosis [3]. Experiments indicate that an electrochemical gradient of protons is established across the cell membrane [4,5] which is used by the cell to transport monosaccharides uphill by means of H^+ co-transport [6].

The polyene antibiotic nystatin has been shown to interfere with transport of substrates across cell membranes containing sterols [7,8]. In *Chlorella vulgaris* nystatin appears to interact with sterols that play a role in active transport [9].

This report shows that nystatin acts on the plasma membrane of yeast cells, whereas the energy metabolism in mitochondria remains unaffected.

The yeast *Rhodotorula glutinis (gracilis)*, ATCC 26 195 and CBS 6681,

Abbreviation: EGTA, ethyleneglycol bis(aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

was cultivated as described by Kotyk and Höfer [10]. D-Xylose uptake experiments were carried out as described earlier [3]. Protoplasts were obtained by a slightly modified method reported previously [11]: the absorbance (A_{546nm}) was measured from an exponentially growing liquid preculture. A cell suspension ($(10 \times A_{546nm})^{-1}$ml) was inoculated into 100 ml of main culture medium, and grown overnight under continuous shaking at 30°C. Upon harvesting the absorbance was measured again, 1 ml β-glucuronidase/arylsulfatase (Boehringer Mannheim, F.R.G.) were added per $A_{546nm} = 1$ to the cell suspension containing 0.003 M L-cysteine-HCl and 0.9 M sorbitol as osmotic stabilizer and incubated at 30°C. Bovine serum albumin (1 g/l) was added towards the end of incubation. After 90 min the suspension was centrifuged at 2000 × *g* for 10 min, and the pellet washed three times with isolation medium (see below) at 6000 × *g* for 10 min.

Mitochondria from *R. glutinis* were isolated in analogy to the methods developed for *Saccharomyces* yeasts [13—15]: protoplasts were suspended at 0°C in the isolation medium consisting of 0.4 M mannitol, 0.5 M sorbitol, 0.05 M Tris/maleate pH 6.60, 0.001 M EGTA, 2 g/l bovine serum albumin, 0.001 M L-cysteine-HCl and homogenized by a Teflon homogenizer of minimal clearance. Simultaneously, the suspension was diluted from 2 to 3 volumes with distilled water. Three centrifugations, at 10 000 × *g* for 10 min (pellet resuspended in washing medium — see below), at 2000 × *g* for 10 min (pellet discharged), and at 10 000 × *g* for 10 min separated the mitochondria-containing fraction from other cell components. The sediment after the third centrifugation was washed twice in the following medium: 0.26 M mannitol, 0.33 M sorbitol, 0.03 M Tris/maleate, pH 6.80, 1 g/l bovine serum albumin, 0.002 M $MgCl_2$, 0.001 M KCl, 0.005 M KH_2PO_4, and finally suspended in this washing medium and kept in ice.

Protein determination was carried out according to Lowry et al. [10]. The yield was about 16 to 22 mg mitochondrial protein per g fresh cells. Oxygen consumption was measured in a 2 ml cuvette at 25°C with an oxygen sensor (Beckman Instruments, Munich, F.R.G.). ATP was measured enzymatically with the hexokinase/glucose-6-phosphate dehydrogenase method. pH changes were monitored by a commercial pH electrode, amplifier and recorder (Radiometer, Copenhagen, Denmark) in unbuffered solutions containing 0.1 M KCl to increase the ionic charge of the suspension. Nystatin (mycostatin), antimycin A and bovine serum albumin, fractin V, were obtained from Serva (Heidelberg, F.R.G.), and rotenone from Sigma Chemicals (Munich, F.R.G.). All other chemicals were purchased from Merck (Darmstadt, F.R.G.).

The effect of the polyene antibiotic nystatin on the accumulation of D-xylose into cells of *Rhodotorula glutinis* is depicted in Fig. 1A. Nystatin (concentrations expressed as mol nystatin per mg yeast dry weight) was added 2 min before starting the test with D-xylose. Concomitantly, the pH of the unbuffered suspension was recorded (Fig. 1B). The accumulation of sugar proved to be dependent on the nystatin concentration. Xylose uptake was inhibited by an antibiotic concentration of 10^{-9} mol/mg yeast dry wt., varying somewhat with the particular yeast batch. The same concentration of nystatin caused a collapse of the pH gradient across the plasma membrane.

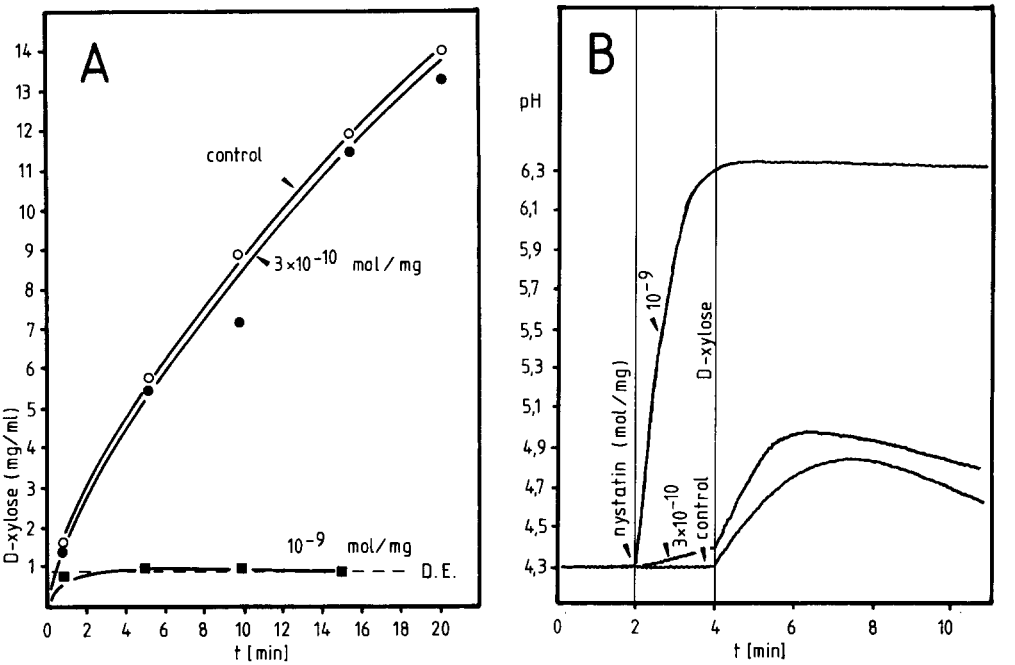

Fig. 1. Effect of nystatin on the uptake of D-xylose in *Rhodotorula glutinis* (unbuffered yeast suspensions). (A) Intracellular accumulation of sugar in the presence of various concentrations of nystatin, expressed in mol nystatin/mg yeast dry wt. D.E. = diffusion equilibrium. Intracellular accumulation of D-xylose is expressed in mg D-xylose/ml intracellular water. (B) pH changes of the yeast suspension during sugar uptake under conditions as in A. Nystatin was added 2 min before D-xylose (10^{-2} M).

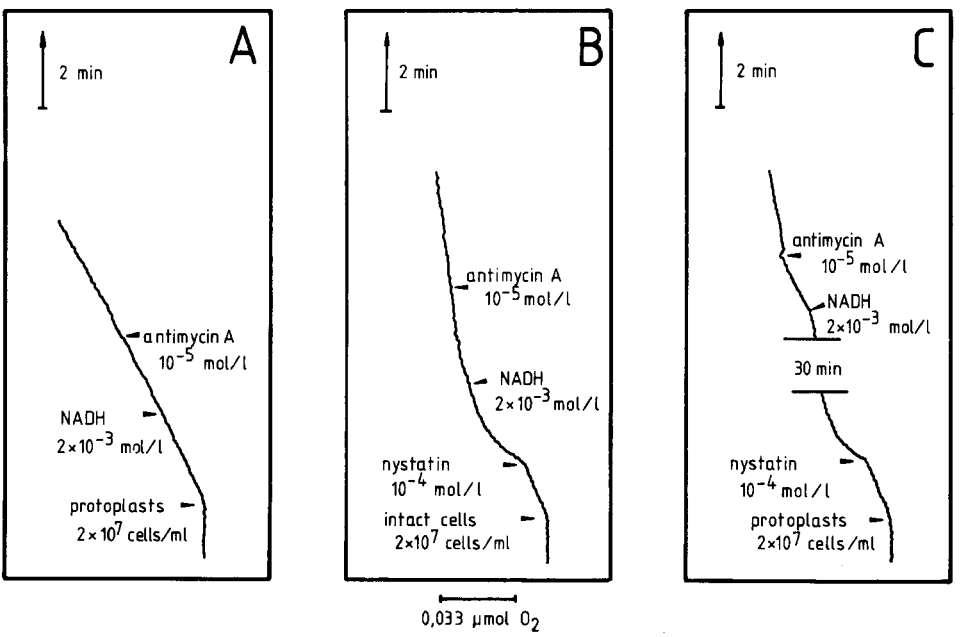

Fig. 2. Oxygen consumption of whole cells and protoplasts from *Rhodotorula glutinis* (yeast suspensions in isolation medium). (A) Addition of NADH and antimycin A to protoplasts, (B) effect of nystatin on respiration of whole cells, (C) effect of nystatin on protoplasts.

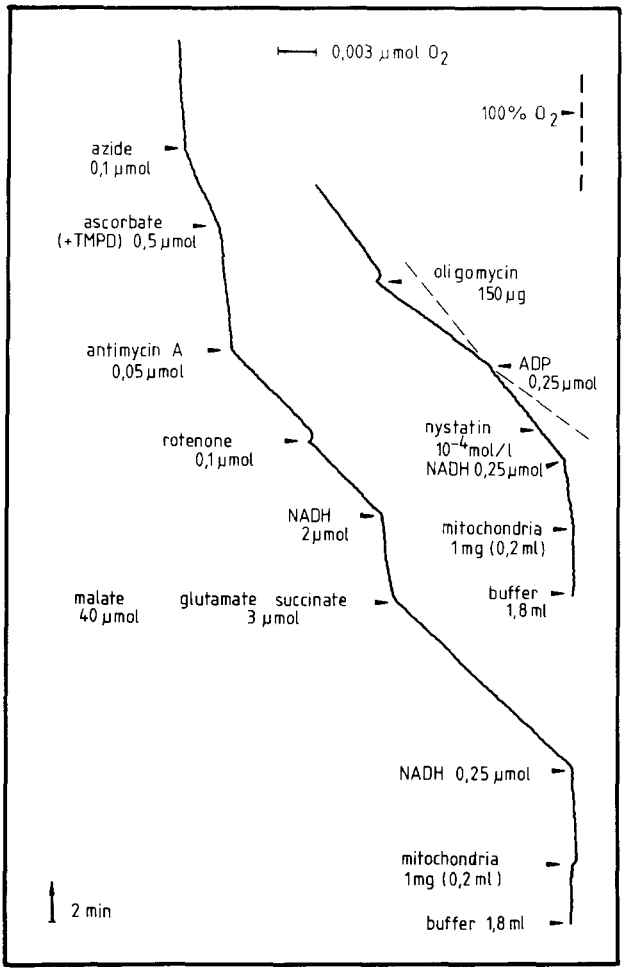

Fig. 3. Oxygen consumption by mitochondria from *Rhodotorula glutinis* with different substrates and inhibitors (lower curve) and effect of nystatin and oligomycin on mitochondrial respiration in state 4 and 3 (upper curve).

Nystatin at $3 \cdot 10^{-10}$ mol/mg yeast dry wt. was without considerable effect both on xylose transport and on pH gradient.

The oxygen consumption by whole cells and protoplasts under similar conditions as described above is shown in Fig. 2: after a short stimulation nystatin inhibited respiration both in whole cells and in protoplasts (Fig. 2 B and C). Moreover, the data demonstrate that protoplasts treated with nystatin 10^{-4} M (corresponding to $2 \cdot 10^{-8}$ mol/mg yeast dry wt.) became permeable both for antimycin A and for NADH (Fig. 2A and C), thus resembling a mitochondrial preparation (cf. Fig. 3). The addition of nystatin obviously effected the plasma membrane to become permeable to molecules as large as NADH and antimycin A. After adding nystatin in these tests the number of protoplasts was not reduced significantly so that the oxygen con-

sumption observed was not due to a very small amount of liberated mitochondria.

To test whether the effect of nystatin on sugar uptake, pH change and oxygen consumption was due to its effect on plasma membrane or inhibition of energy transfer by mitochondria, the latter were isolated from a protoplast suspension. The consumption of oxygen by a mitochondrial fraction from *R. glutinis* is depicted in Fig. 3. NADH was the only substrate oxidized, whereas the intermediates of the tricarboxylic acid cycle failed to stimulate respiration probably because of damage to the specific acid carrier. However, the electron transport chain from NADH-dehydrogenase to oxygen appeared to be intact since: (1) oxygen consumption was inhibited by antimycin A, which binds to complex III, (2) this inhibition was released by ascorbic acid and *N,N,N',N'*-tetramethylphenylenediamine, and (3) respiration was finally inhibited at complex IV by azide (Fig. 3). The figure demonstrates furthermore that nystatin at concentrations inhibiting completely the uphill sugar transport and the respiration in *R. glutinis* had no effect on mitochondrial respiration both in state 4 and 3.

Since a decrease of respiration upon exhaustion of added ADP (state-3-state-4-transition) could not be observed ATP synthesis was measured by the hexokinase method (Fig. 4). The diagram shows that addition of an oxidizable substrate in the presence of ADP caused a transitory increase in the ATP level, which was insensitive to nystatin (full circles). A nystatin-insensitive ATP

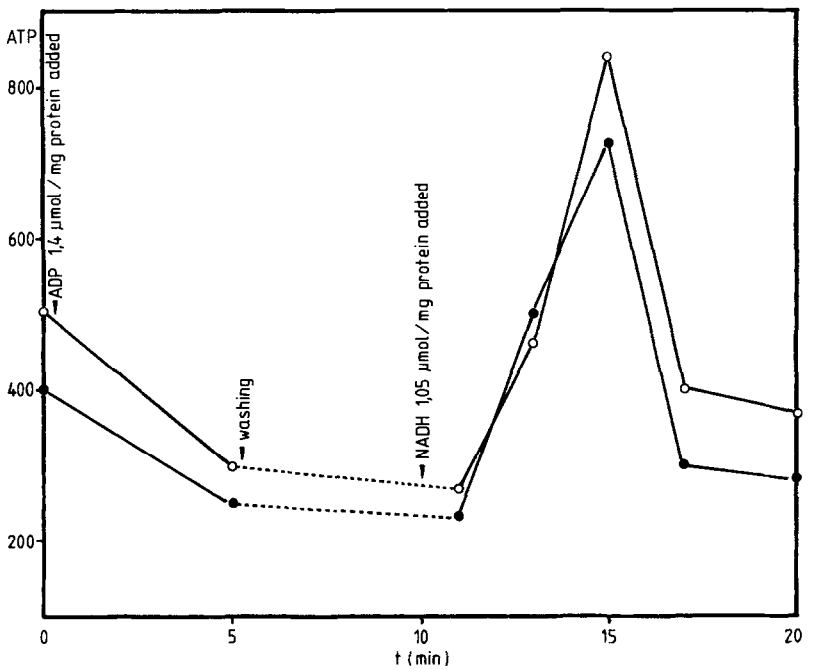

Fig. 4. ATP synthesis (nmol ATP formed/mg mitochondrial protein) in absence (○) and in presence (●) of nystatin (10^{-4} M) measured enzymatically. Conditions: 5 min after adding ADP mitochondria were washed once and resuspended in medium without ADP. Then NADH was added and samples were taken at intervals. Temperature 25°C.

synthesis in mitochondrial preparations was also indicated by the inhibition of the state 3 respiration to the state 4 rate by oligomycin (Fig. 3).

Thus, the inhibitory effect of nystatin on cellular metabolism was restricted only on its interaction with the plasma membrane: oxygen consumption of whole cells and protoplasts was reduced and the proton permeability of plasma membrane enhanced. Consequently, the pH gradient as well as the membrane potential [5] across the plasma membrane collapsed and, in turn, the active sugar transport was suspended.

Our experiments demonstrated that the polyene antibiotic nystatin does not interfere either with the respiratory chain or with the energy-conserving reactions of the mitochondrial inner membrane. It can be concluded generally that in yeast cells the effect of nystatin on energy-dependent processes is caused by its interference with the plasma membrane function and not with the mitochondrial energy metabolism.

This work has been supported by the Deutsche Forschungsgemeinschaft (grant No. Ho/555).

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