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Irreversible Inhibition of Biotin Transport in Yeast by Biotinyl-*p*-nitrophenyl Ester

(affinity label/biotinyl-*p*-nitroanilide/acetyl-*p*-nitrophenyl ester/sorbose transport/amino-acid transport)

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ABSTRACT Biotinyl-*p*-nitrophenyl ester (BNP), an active-ester derivative of biotin, irreversibly inactivates biotin transport in the yeast *Saccharomyces cerevisiae*. Transport inactivation is progressive with time and occurs at concentrations of the ester as low as 10^{-7} M. In the presence of sodium azide, a reagent known to block biotin accumulation in yeast, the derivative is still effective. The specificity of inactivation by the ester is revealed by the following findings: (*a*) Biotinyl-*p*-nitroanilide and acetyl-*p*-nitrophenyl ester do not affect biotin transport; (*b*) the nitrophenyl ester does not affect the transport of lysine and aspartic acid, or that of L-sorbose; (*c*) inactivation of biotin transport by the ester is partially prevented when the cells are incubated with it in the presence of relatively high concentrations of biotin.

Biotin enters the yeast *Saccharomyces cerevisiae* by a highly specific mechanism, (apparent K_M of 10^{-7} M) (1). The transport process is pH, temperature-, and energy-dependent. Under optimal conditions, yeast cells accumulate the vitamin to an intracellular concentration that exceeds by several orders of magnitude the extracellular biotin concentration (1, 2). Several biotin derivatives are taken up by yeast similarly to biotin, and appear to act as competitive inhibitors (1). It was thus plausible to assume that a chemically active derivative of biotin might be prepared that would serve as a specific inactivating reagent of the biotin permease system.

In this paper, we present data demonstrating that an active-ester derivative of biotin, biotinyl-*p*-nitrophenyl ester (BNP), specifically and irreversibly inactivates biotin transport by whole cells of yeast. The results suggest that BNP acts as an affinity label for the biotin transport component(s).

EXPERIMENTAL

Preparation of Biotin Derivatives. Biotin (244 mg, 1.0 mmol) was dissolved in 3 ml of hot dimethylformamide. *p*-Nitrophenol (139 mg, 1 mmol) and dicyclohexylcarbodiimide (206 mg, 1.0 mmol) were added, and the resultant mixture was stirred at room temperature for 2 hr. The precipitate formed was filtered off, and the filtrate was concentrated to dryness under reduced pressure. Ether was added, and the resulting solid was recrystallized from water to give 120 mg (50%) of a yellowish crystalline product, mp 198–202°C. The structure of the biotinyl-*p*-nitrophenyl ester thus obtained was verified by UV and IR spectra.

Abbreviation: BNP, biotinyl-*p*-nitrophenyl ester.

Anal: Calcd. for $C_{16}H_{20}N_3O_5S \cdot 3H_2O$: C, 45.7; H, 6.2; N, 10.0. Found: C, 46.0; H, 5.8; N, 9.8.

Biotinyl-*N*-hydroxysuccinimide ester was prepared from biotin and *N*-hydroxysuccinimide by a procedure similar to the one described for the preparation of BNP. A colorless solid was obtained, which was recrystallized from isopropanol, mp 196–200°C.

Anal: calcd. for $C_{14}H_{17}N_3O_5S$: N, 12.3. Found: N, 11.8.

Biotinyl-*p*-nitroanilide was obtained from biotin and *p*-nitroaniline by a mixed-anhydride method (3).

Anal: calcd. for $C_{16}H_{21}N_4O_4S \cdot H_2O$: C, 50.5; H, 6.0; N, 14.5. Found: C, 50.5; H, 6.0; N, 13.7.

Organism, Growth Conditions, and Preparation of a Resting Cell Suspension of Saccharomyces cerevisiae (ATCC 9896). Fleischmann strain 139 was used throughout. The growth

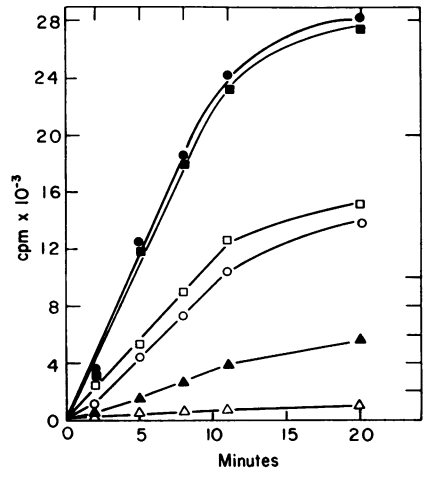

FIG. 1. Inactivation of biotin transport by BNP. A cell suspension (4.5 ml) was mixed with an ethanolic solution of BNP (0.5 ml); the mixture incubated at 30°C for 30 min. The final concentrations of BNP were: △, 80 μM; ▲, 8 μM; ○, 0.8 μM; □, 80 nM; ■, 8 nM; ●, control experiment with ethanol only. Biotin uptake by the BNP-treated cells was as follows: The cells were washed three times with 0.05 M phosphate buffer (pH 4), and suspended in 5.0 ml of the buffer kept at 30°C. 3-ml aliquots were withdrawn and added to 3.0 ml of a reaction mixture, kept at 30°C, that contained glucose (2%), 0.05 M phosphate buffer (pH 4), and radioactive biotin (1.6 μM, 58 Ci/mol).

medium was prepared according to Hertz (4); it contained biotin (0.25 ng/ml) and glucose instead of sucrose. Cells were grown at 30°C with reciprocating shaking (110 strokes/min). The culture was harvested by centrifugation during the late-exponential phase of growth, washed twice with distilled water, and resuspended to 1 mg of dry weight/ml (5×10^7 cells/ml) in 0.05 M potassium phosphate buffer (pH 4).

Measurement of Biotin Uptake. A resting-cell suspension (4.5 ml) was incubated at 30°C with ethanolic solutions of various reagents (0.5 ml), washed three times by centrifugation with 0.05 M phosphate buffer (pH 4), and suspended in buffer, kept at 30°C, to the original volume (4.5 ml). Control experiments involved incubation with ethanol (0.5 ml) only. 3-ml aliquots of the cell suspensions treated as above were added to 3 ml of a reaction mixture, kept at 30°C, that contained glucose (2%), 0.05 M phosphate buffer (pH 4), and radioactive biotin (Radiochemical Center, 1.7 μM, 58 Ci/mol). At various time intervals, aliquots were withdrawn from the incubation mixture and the cells were collected on membrane filters (Millipore Corp.). After they were washed twice with water at room temperature, the cells and filters were placed in scintillation fluid (5) and counted in a scintillation spectrometer.

RESULTS

Irreversible inhibition of biotin uptake by BNP

Biotin uptake after incubation of resting-cell suspensions for 30 min at 30°C with BNP at various concentrations is illustrated in Fig. 1. The inhibitory effect of the BNP cannot be reversed by washing the cells with water, 0.05 M phosphate buffer (pH 4), 0.1 M Tris·HCl (pH 8 or 9), 0.1 M citrate–phosphate buffers (pH 3, 4, 5, 6, or 7), 1 M LiCl, or 0.85% NaCl. Incubation of the activated cells for at least 2 hr in 0.05 M phosphate buffer (pH 4) that contained 1% glucose, at 30°C, also did not reverse the inhibition of biotin transport.

The inactivation of biotin transport by BNP as a function of time is presented in Fig. 2. The finding that the extent of inactivation increases with time supports the assumption that BNP reacts with a transport component(s) to yield a covalently-bound biotinyl derivative.

Protection by biotin from BNP inactivation

In order to determine whether biotin protects yeast cells from transport inactivation by BNP, the following experiment was done. BNP was added to a cell suspension in phosphate buffer (pH 4) that contained a relatively high concentration of biotin (0.5 mM). At different time intervals, aliquots were withdrawn, the cells were washed, and influx of radioactive biotin was determined. The data obtained were compared with the corresponding findings for biotin influx in cells treated with BNP in the absence of biotin. The data given in Fig. 3 indicate that biotin, at the concentration used, partially prevents inactivation of biotin transport by BNP.

Effect of various compounds on biotin uptake

The effect of acetyl-*p*-nitrophenyl ester, biotinyl *N*-hydroxysuccinimide ester, and biotinyl *p*-nitroanilide, all at a concentration of 0.1 mM, on biotin uptake is shown in Fig. 4. Acetyl-*p*-nitrophenyl ester had no affect on biotin transport. Biotinyl *p*-nitroanilide and the *N*-hydroxysuccinimide ester

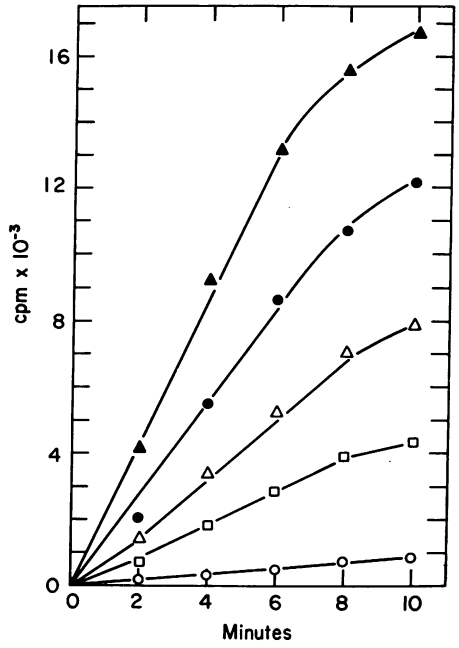

FIG. 2. Inactivation of biotin transport by BNP as a function of time. Resting-cell suspensions (5.0 ml) were incubated for various time intervals in 0.05 M phosphate buffer (pH 4), 12 μM in BNP. The times of incubation with BNP were: ○, 30 min; □, 10 min; △, 5 min; ●, 2 min; ▲, immediate washing after addition of BNP. Biotin uptake by the BNP-treated cells was determined as described in the legend for Fig. 1.

inhibited the initial velocity of biotin uptake, under the experimental conditions used, by 35 and 60%, respectively. At concentrations lower than 10 μM, both reagents had no effect on biotin uptake. Incubation of resting cells with either biotin or *p*-nitrophenol at concentrations up to 1 mM also did not impair biotin transport.

Effect of BNP on amino acid and carbohydrate uptake

Aspartic acid and lysine uptake were determined by a procedure similar to the one used for measuring biotin uptake, except that radioactive amino acids were used in place of biotin. L-sorbose uptake was measured by the method outlined by Cirillo (6). Neither the initial velocities nor the extent of permeation of these compounds is altered after incubation of the yeast with BNP at 0.1 mM.

Inactivation of biotin transport in the presence of sodium azide

Sodium azide, at a concentration of 0.01 M, inhibited 98% of biotin transport under our standard experimental conditions. Biotin uptake can be fully restored when the cells are washed with 0.05 M phosphate buffer (pH 4). The data given in Fig. 5 indicate that BNP, at a concentration of 0.1 mM, inactivates biotin transport as effectively in the absence as in the presence of sodium azide.

DISCUSSION

The molecular components of biological transport systems are, for the most part, unknown. Among the macromolecules involved in the transport process that have been thoroughly studied, one might mention the proteins eluted from bacteria by osmotic shock (7), the M-protein of galactoside transport

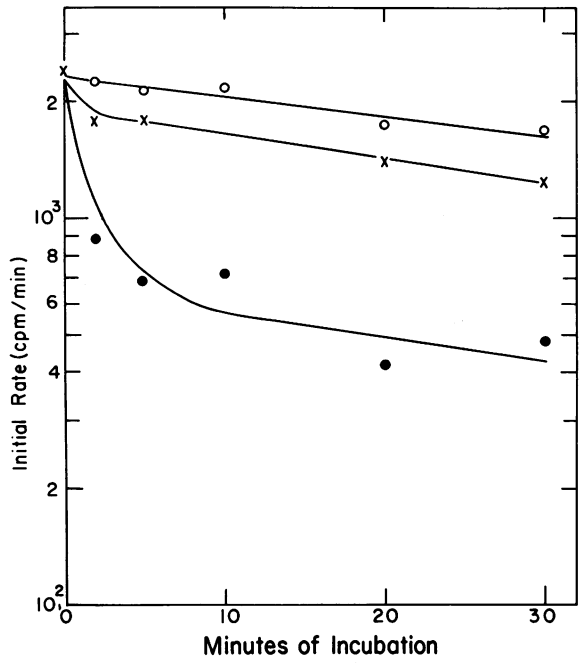

FIG. 3. Inactivation of biotin transport by BNP in the presence of biotin. Biotin uptake was determined as described in the legend for Fig. 1. Initial rates in cpm/min are recorded. Curve 1 (O) gives the initial rates of biotin uptake, at the times specified, by cells that were treated as follows: yeast cells were suspended in 4.5 ml of 0.05 M phosphate buffer (pH 4) to which 0.25 ml of biotin solution (0.01 M) was added. The mixture was incubated at 30°C for 2 min, whereupon 0.25 ml of ethanol was added. Incubation was continued up to the time at which biotin uptake was measured. Curve 2 (×) gives the initial rates of biotin uptake at the times specified, by cells that were treated as were the cells shown in Curve 1, except that 0.25 ml of an ethanolic BNP solution (0.1 mM) was added instead of 0.25 ml of ethanol. Curve 3 (●) gives the initial rates of biotin uptake, at the times specified, by cells suspended in 4.75 ml of 0.05 M phosphate buffer (pH 4), to which 0.25 ml of ethanolic BNP solution (0.1 mM) was added. Incubation at 30°C was continued until biotin uptake was measured.

in *Escherichia coli*, which is labeled *in vivo* by a sulfhydryl reagent (8), and the phosphotransferase complex isolated from bacteria by classical biochemical techniques (9).

The use of specific inactivating reagents (affinity labels) for transport systems was suggested by Baker (10), and has been attempted by various authors (for a review, see ref. 11). The most important criteria for affinity labeling of transport components, similarly to the affinity labeling of enzymes and other biologically specific macromolecules, include specific inactivation at low concentrations and protection by substrates or inhibitors (12). Because of the complexity in composition and structure of transport systems, *in situ* affinity labeling of these membrane components has not been satisfactory in most cases.

The biotin transport system in yeast provides a good system for affinity labeling because: (*a*) The biotin transport system in yeast recognizes biotin via its ureido ring, so that the valeric-acid side chain can be modified without affecting subsequent transport (1, 2); (*b*) The K_M for biotin transport is 10^{-7} M (1); (*c*) Biotin at a low concentration (about 0.25 ng of biotin/ml) suffices to support maximum growth; and (*d*) Biotin is not catabolized by the yeast strain used in this study (1).

After testing several biotin derivatives (biotinyl-bromoacetyl hydrazide, biotinyl-β-naphthyl ester, and biotinyl-*N*-hydroxysuccinimide ester) that did not inactivate biotin transport to a considerable extent, even at a concentration of 10^{-3} M, we found that biotinyl-*p*-nitrophenyl ester (BNP) specifically inactivates biotin transport in yeast. The inactivation is rapid and occurs at as low a concentration as 80 nM (see Fig. 1).

Our finding that inactivation of transport by BNP, at a concentration of 0.1 mM, also occurs in the presence of sodium azide (see Fig. 5), a reagent known to block active transport (13, 14), indicates that transport inactivation in the yeast is not the result of accumulation of a high intracellular concentration of BNP within the yeast cells.

Biotin transport of the yeast treated with BNP could not be restored by washing with mineral salts (NaCl or LiCl), or with buffers (phosphate or Tris·HCl of different pH values). Reactivation of biotin transport did not occur on incubation of the cells in the presence of glucose.

The specificity of BNP inactivation was investigated by the study of the effect of various compounds on biotin uptake. Acetyl-*p*-nitrophenyl ester did not affect biotin transport. Biotin-*p*-nitroanilide, which lacks an active carbonyl, but which has a similar size, and presumably a similar affinity for the biotin transport system as that of BNP, did not inhibit biotin uptake at concentrations below 0.1 mM (see Fig. 4). As mentioned above, BNP is highly active, at the concentration range of 0.1 μM to 0.1 mM. The slight inactivation by the nitroanilide derivative at 0.1 mM might be due to its high lipid solubility, which could lead to its accumulation within the membrane. The high local concen-

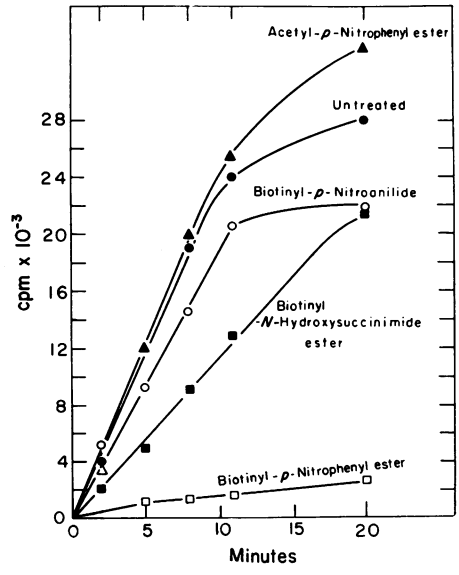

FIG. 4. Effect of various compounds on biotin transport. In each of the experiments shown, a suspension of cells (4.5 ml) was mixed with an ethanolic solution (0.5 ml) of: ■, biotinyl-*N*-hydroxysuccinimide ester; O, biotinyl-*p*-nitroanilide; ▲, acetyl-*p*-nitrophenyl ester; ●, ethanol only. The final concentration of the various compounds was 0.1 mM, and the mixture was incubated at 30°C for 30 min. Biotin uptake by the treated cells was determined as described in the legend for Fig. 1.

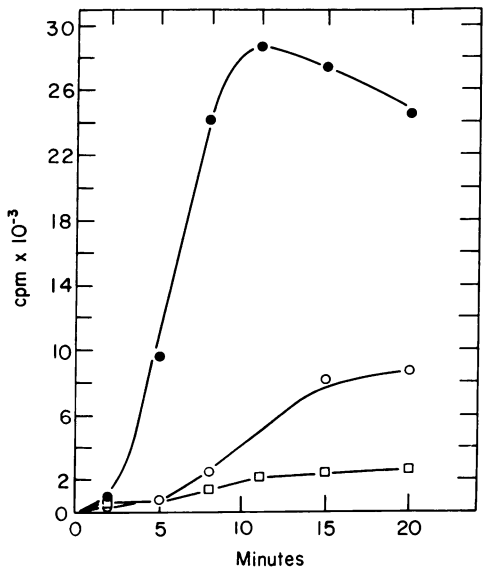

FIG. 5. Effect of BNP on biotin uptake in the presence and absence of sodium azide. Three resting-cell suspensions (5.0-ml each) were incubated at 30°C for 30 min with: □, BNP (final concentration 0.1 mM); ○, BNP and sodium azide (final concentrations 0.1 mM and 10 mM, respectively); and ●, sodium azide (final concentration 10 mM). Biotin uptake by the treated cells was determined as described in the legend for Fig. 1.

tration of the anilide would lead to transport inactivation by competitive inhibition.

Biotinyl-*N*-hydroxysuccinimide ester can also inactivate biotin transport. However, the lowest concentration at which inactivation by the succinimide ester could be noted was considerably higher than that for BNP. The reason for the difference in behavior of the two active esters is unknown.

The mechanism by which BNP inactivates biotin transport has not been elucidated. It is plausible to assume that it proceeds in two stages: specific adsorption followed by covalent binding. If this is the case, one might expect that compounds combining reversibly with the transport system should prevent inactivation by BNP. It was thus gratifying to notice that biotin, at a relatively high concentration, protects the transport system from inactivation by BNP (see Fig. 3).

The specificity of inactivation of biotin transport by BNP is emphasized by the finding that BNP, in the concentrations investigated, does not affect the permeability of aspartic acid and lysine, or of L-sorbose.

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