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PHOTOINACTIVATION OF THE THIAMINE TRANSPORT SYSTEM IN *SACCHAROMYCES CEREVISIAE* WITH 4-AZIDO-2-NITROBENZOYLTHIAMINE

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A newly synthesized photoreactive thiamine derivative, 4-azido-2-nitrobenzoylthiamine was found to be a competitive inhibitor of the thiamine transport system in *Saccharomyces cerevisiae*, exhibiting an apparent K_i of 36 nM. When exposed to visible light, 4-azido-2-nitrobenzoylthiamine irreversibly inactivated the thiamine transport. 4-Azido-2-nitrobenzoylthiamine-dependent photoinactivation of thiamine transport was partially protected by thiamine, but not by the nitrene-trapping reagent *p*-aminobenzoate. On the other hand, the irradiation of the yeast cells in the presence of 4-azido-2-nitrobenzoylthiamine did not significantly lead to inactivation of the biotin transport system. The results suggest that 4-azido-2-nitrobenzoylthiamine is a specific irreversible inhibitor of the thiamine transport system in *Saccharomyces cerevisiae*.

It has been recognized for several years that thiamine enters yeast cells by a carrier-mediated active transport process [1,2,3]. However, little is known concerning the molecular constituents of the membrane which are involved in the transport process. In this paper we wish to report that 4-azido-2-nitrobenzoylthiamine, a newly synthesized derivative of thiamine, inactivates the thiamine transport system in *Saccharomyces cerevisiae* when exposed to visible light, suggesting that this compound is an effective irreversible inhibitor of the thiamine transport system in yeast cell membrane.

Materials and Methods

Chemicals. [*thiazole*-2-^{14}C]thiamine hydrochloride (24.3 Ci/mol) and D-[*carbonyl*-^{14}C]biotin (49 Ci/mol) were obtained from the Radiochemical Centre, U.K. 5-Hydroxyethyl-4-methylthiazole [4] and 4-amino-5-bromomethyl-2-methylpyrimidine hydrobromide [5,6] were prepared from thiamine hydrochloride. 4-Azido-2-nitrobenzoic acid was synthesized from 4-amino-2-nitrobenzoic acid essentially by the method of Lewis et al. [7], which applies to the synthesis of 5-azido-2-nitrobenzoic acid from 5-amino-2-nitrobenzoic acid. All other chemicals were purchased from commercial suppliers.

Growth of yeast cells. S. cerevisiae was grown in Wickerham's synthetic medium thiamine was omitted, as described previously [3] After harvesting, yeast cells were washed once with cold water.

Transport assays. The transport of thiamine was determined by the method described previously [2]. The irradiated cell suspensions were centrifuged at $2\,000 \times g$ for 5 min, the cell pellets were washed twice with cold water and finally suspended in 1 ml cold water. The aliquots of the cell suspensions were employed to measure the initial rate of thiamine uptake by the cells; 0.2 ml cell suspension was added to 3.8 ml 50 mM potassium phosphate buffer (pH 5.0) containing 0.1 M glucose and 40 μl 0.1 mM [*thiazole*-2-^{14}C]thiamine which was prewarmed at 37°C. After 2 min at 37°C the radioactivity of 1 ml of the cell suspension was measured as previously reported [2].

Irradiation procedure. Photoinactivation studies were carried out in a 20-ml glass beaker which was kept in ice. The washed yeast cells, suspended in 2 ml 50 mM potassium phosphate buffer (pH 5.0) at the

concentration of 0.5 mg dry weight per ml, were irradiated in the presence of 4-azido-2-nitrobenzoylthiamine and other derivatives of thiamine. Photolysis was carried out for 10 min with a Toshiba black light lamp (40 W), 25 cm from the reaction vessel.

Synthesis of 4-azido-2-nitrobenzoylthiamine. 4-Amino-2-nitrobenzoic acid (5.2 g, 28.5 mmol) was dissolved in 12 M HCl (45 ml); $NaNO_2$ (3.19 g, 46.2 mmol) dissolved in water (15 ml) was added portionwise with stirring over 60 min at −5–0°C; acetic acid (42.7 ml) was added at −5–0°C; NaN_3 (3.20 g, 49.2 mmol) dissolved in water (12 ml) was slowly added drop-wise over 1 h at 0–5°C. The reaction mixture was diluted with cold water (56 ml), stirred another 15 min at 0–5°C and further diluted with cold water (200 ml); the resulting precipitate was collected by suction filtration and dried over P_2O_5 in a desiccator to give pale yellow crystals of 4-azido-2-nitrobenzoic acid weighing in total 5 g (yield 84.2%), m.p. 177–179°C (decomposed), Infrared (KBr) 2120 cm^{-1} (azido). In the above reaction sequence the addition of NaN_3 and the succeeding procedures were carried out in subdued light.

4-Azido-2-nitrobenzoic acid (0.9 g, 4.32 mmol) was heated with $SOCl_2$ (3 ml) for 20 min under reflux, concentrated in vacuum at 50°C and traces of $SOCl_2$ were removed by addition of benzene and alternate evaporation in vacuum, to leave crystalline 4-azido-2-nitrobenzoyl chloride, which was then used without purification. 5-Hydroxyethyl-4-methylthiazole (0.65 g, 4.54 mmol) was added to the chloride, the mixture was refluxed in benzene (2 ml) in the presence of pyridine (0.5 ml) for 1 h, concentrated in vacuum at 50°C and the crystalline residue was recrystallized from ethanol to give 4-methyl-5-[2-(4-azido-2-nitrobenzoyl)ethyl]thiazole (1.25 g, yield) 86.8%), m.p. 94–97°C. Further recrystallization from ethanol gave colorless needles, m.p. 98–99°C.

$C_{13}H_{11}N_5O_4S$	(M_r 333.34)
Calculated:	C 46.85, H 3.33, N 21.01
Found:	C 46.94; H 3,21, N 20.80

Infrared (KBr) 2 130 cm^{-1} (azido); 1 715 cm^{-1} (carbonyl); 1 550, 1 375 cm^{-1} (nitro). Ultraviolet (in ethanol) λ_{max} 250 nm (ϵ = 17 800).

A mixture of 4-amino-5-bromomethyl-2-methylpyrimidine hydrobromide (1.7 g, 6.01 mmol) and 4-methyl-5-[2-(4-azido-2-nitrobenzoyl)ethyl] thiazole (2.0 g, 6.00 mmol) in *n*-butanol (5 ml) was heated for 75 min under reflux and filtered while hot; the residue was washed with ethanol and then well-washed with acetone to leave a straw-colored powder of 4-azido-2-nitrobenzoylthiamine bromide hydrobromide (0.94 g, crude yield 25.4%), m.p. 204–205°C (decomposed). Recrystallization from ethanol gave colorless crystals, m.p. 207–208°C (decomposed).

$C_{19}H_{19}BrN_8O_4S \cdot HBr$	(M_r 616.30)
Calculated:	C 37.03, H 3.10, N 18.18
Found:	C 37.01, H 3.25, N 17.74

Infrared (KBr) 2 130, 2 115 cm^{-1} (azido); 1 725 cm^{-1} (carbonyl); 1 550, 1 385 cm^{-1} (nitro). Ultraviolet (in pH 2 aqueous HCl) λ_{max} 251 nm (ϵ = 29 300).

Results and Discussion

From the transport studies [2] 4-azido-2-nitrobenzoylthiamine was found to be a competitive inhibitor of thiamine uptake by yeast cells and an apparent K_i of 36 nM was calculated from a double reciprocal plot relating initial rate of thiamine uptake to thiamine concentration in the presence of 0.1 μM 4-azido-2-nitrobenzoylthiamine. Since 4-azido-2-nitrobenzoylthiamine contains an aryl azido group in the molecule it was expected that the irradiation of the compound with visible light could affect photolysis of the azido moiety, forming a reactive nitrene which could react covalently with the membrane components functionally involved in the thiamine transport system.

When yeast cells were irradiated with visible light in the presence of 1 μM 4-azido-2-nitrobenzoylthiamine, their ability to transport thiamine was rapidly lost (Table I). As shown, the initial rate of thiamine transport after irradiation for 10 min decreased to approx. 35% of the original activity. When yeast cells were irradiated in the absence of 4-azido-2-nitrobenzoylthiamine or were kept in the dark with 4-azido-2-nitrobenzoylthiamine for 10 min, their transport activities were almost the same as that of untreated control cells (Table II), indicating that the inactivation by 4-azido-2-nitrobenzoylthiamine is dependent upon irradiation. It should be stressed that the photoinactivation under these conditions is irreversible

TABLE I

TIME COURSE OF PHOTOINACTIVATION OF THIAMINE TRANSPORT IN *SACCHAROMYCES CEREVISIAE* WITH 4-AZIDO-2-NITROBENZOYLTHIAMINE

Photoinactivation studies were carried out for the indicated time period as described in Materials and Methods.

Minutes of light	Thiamine uptake ($nmol \cdot mg^{-1} \cdot min^{-1}$)
0	6.0
1	4.3
2	3.1
5	2.3
10	2.1

since the inactivation could not be reversed by washing the cells free of inactivator. 4-Methyl-5-[2-(4-azido-2-nitrobenzoyl)ethyl]thiazole, an intermediary product with an aryl azido group for the chemical synthesis of 4-azido-2-nitrobenzoylthiamine, was ineffective in inactivating the transport system. This was consistent with previous findings showing that the thiamine transport system in *S. cerevisiae* is specific for the pyrimidine moiety of the thiamine molecule [8,9].

As regards the specificity of 4-azido-2-nitrobenzoylthiamine photoinactivation, the addition of 10 μM thiamine to inactivation mixture containing 0.5 μM 4-azido-2-nitrobenzoylthiamine caused at 64.0% increase in thiamine uptake, whereas 10 mM *p*-aminobenzoate, a nitrene-trapping reagent [10], produced no protection on 4-azido-2-nitrobenzoylthiamine-dependent photoinactivation of the thiamine transport. These results suggest that nitrene formed from 4-azido-2-nitrobenzoylthiamine bound to the thiamine-specific components (probably thiamine carrier protein) inactivates directly and is not trapped by *p*-aminobenzoate.

It has been reported that biotin transport in *S. cerevisiae* occurs by carrier-mediated active transport, which is specifically inactivated with *p*-nitrophenyl esters of biotin [11]. In contrast to thiamine transport, biotin transport was inactivated only 17.9% by 5 μM 4-azido-2-nitrobenzoylthiamine, whereas thiamine transport was almost completely inactivated.

The data presented here indicate that 4-azido-2-nitrobenzoylthiamine, a structural analog of thiamine which contains a reactive aryl azido group, specifically inactivites the yeast thiamine transport system under the irradiation of visible light. This compound

TABLE II

REQUIREMENT FOR 4-AZIDO-2-NITROBENZOYLTHIAMINE-DEPENDENT PHOTOINACTIVATION OF THIAMINE TRANSPORT IN *SACCHAROMYCES CEREVISIAE*

Photoinactivation studies were carried out as described in Materials and Methods, with or without thiamine derivatives as indicated.

Addition	Concn. (μM)	Irradiation	Thiamine uptake (%)
None		–	100
None		+	99.0
4-Azido-2-nitrobenzoylthiamine	1	–	103.2
	0.5	+	59.9
	1	+	21.7
4-Methyl-5-[2-(4-azido-2-nitrobenzoyl)ethyl]-thiazole	1	+	92.6

may be a potent tool both for identification of the membrane components responsible for active transport of thiamine through the yeast membrane and for elucidating the mechanistic details of the transport process.

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