8-AZIDOADENINE: A PHOTOAFFINITY LABEL FOR THE PURINE TRANSPORT SYSTEM IN *SACCHAROMYCES CEREVISIAE*

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1. Introduction

In *Saccharomyces cerevisiae*, the uptake of the purine bases hypoxanthine, adenine, guanine and the pyrimidine base cytosine is catalysed by a common energy-dependent transport system [1]. In vivo properties of the transport system such as energy coupling and the mechanism of substrate binding to the purine transport system are described in [2–4]. To learn more about the transport protein itself, it is necessary to isolate it from the plasma membrane for further biochemical characterization. Photoaffinity labeling [5–7] has become a very useful tool for the identification and isolation of specific receptor and transport proteins in the minute amounts usually found in membranes.

This paper shows that 8-azidoadenine is a suitable photoaffinity label for the purine transport system in *Saccharomyces cerevisiae*. Kinetic studies demonstrate the required high affinity of the label for the receptor site, as well as its irreversible binding to the transport protein upon irradiation.

2. Materials and methods

2.1. *Organism and culture conditions*

Saccharomyces cerevisiae strain R XII (a kind gift of Dr A. Kotyk, Prague) was cultivated to the early stationary phase (~10^8 cells/ml) in GYNP (2% glucose, 1% Difco yeast nitrogen base, 0.5% peptone) at 30°C with agitation. Cells of a 50 ml culture were washed and preincubated in 250 ml of glucose–citrate buffer (0.05 M sodium citrate (pH 5.5), 2% glucose) at 30°C under aeration for 60 min.

2.2. *Synthesis of 8-azidoadenine*

The synthesis follows with slight modifications the method in [8]. 8-Bromoadenine (0.29 mmol) and sodium azide (0.65 mmol) were dissolved in 12 ml dimethylformamide and stirred for 15 h at 80°C in complete darkness. Dimethylformamide was removed in vacuo onto Cellite. The residue was dissolved in 0.4 ml 0.5 M NH_4OH and the pH adjusted to <2 by the addition of 0.2 ml 2 N HCl. The resulting precipitate was collected on a Whatman paper filter. To remove the residual NaCl, the precipitate was washed with ice-cold water and dried under vacuum.

2.3. *Confirmation of synthesis*

Ultraviolet-visible spectra were determined with a Beckman 25 spectrophotometer, and infrared spectroscopy with a Perkin-Elmer 257 spectrophotometer. Proton NMR spectra were taken on a Varian XL-200 Fourier transform (200 MHz) spectrometer. At least 10 000 transients were accumulated. Thin-layer chromatography was performed on cellulose-coated sheets (Macherey-Nagel, Polygram Cel 300 UV_{254}) (0.1 mm cellulose layer).

2.4. *Photoinactivation by 8-azidoadenine*

After glucose preincubation, ~4×10^8 cells were resuspended in 4 ml ice cold H_2O in a glass petri dish (9 cm) placed on ice on a rotary shaker. After addition of 8-azidoadenine, the cells were immediately irradiated with a UVS-54 lamp (maximum wavelength at 254 nm, 1.4 mW/cm^2) mounted 3 cm above the surface of the solution. For irradiation in the frozen state, 15 ml liquid nitrogen was poured over the cell suspension immediately after the addition of 8-azidoadenine. Once the suspension was frozen (5–6 s) the

excess liquid nitrogen was poured off and the petri dish placed on dry ice for irradiation.

2.5. *Measurement of initial uptake rate for hypoxanthine*

Initial uptake velocities were measured as in [4]. Preincubated cells were incubated for 1 min with ^{14}C-labelled hypoxanthine. The cells were separated from the reaction mixture by filtration over glassfiber filters. The filters were assayed for radioactivity in a Beckman LS-233 liquid scintillation counter. The scintillation fluid contained 5 g 2,5-diphenyloxazole (PPO) and 10 g naphthalene/1 dioxane.

2.6. *Chemicals*

^{14}C-Labelled hypoxanthine was obtained from New England Nuclear. 8-Bromoadenine was purchased from K and K Labs (New York). All other reagents were the best commercial grades available. Irradiation was performed with a Mineralight Lamp UVS-54, Ultra Violet Prod. (San Gabriel CA).

3. Results and discussion

The conversion of 8-bromoadenine to 8-azidoadenine is confirmed by data from NMR and IR spectra. The NMR spectrum of 8-bromoadenine showed proton resonance at δ 7.63 ppm (H-2), and 8-azidoadenine at δ 7.65 ppm (H-2), with 9-NH at δ 5.00 ppm which was partly buried under the H_2O peak. The appearance of δ 4.31 ppm with 8-bromoadenine confirmed the presence of an exchangeable proton at the 8 position. Infared spectroscopy in KBr discs conclusively verified the presence of the azido group (N_3, 2120 cm^{-1}; 8-Br, 532 cm^{-1}). Thin layer chromatography in *N*-butanol/acetone/acetic acid/5% NH_4OH/H_2O (45:15:10:10:20, by vol.) showed 8-azidoadenine, R_F = 0.54, and 8-bromoadenine R_F = 0.71. After photolysis on cellulose-coated plates, the 8-azidoadenine remained at the origin upon chromatography. The UV spectra of 8-azidoadenine before and after 2 min photolysis with UV light (fig.1) illustrate the sensitivity of the photoactive compound to UV irradiation. Upon photolysis the λ_{max} of 8-azidoadenine shifts ~10 nm to the blue and $\Delta E_{270} = 4 \times 10^3\ M^{-1}.cm^{-1}$.

It should be mentioned that the spectroscopy and chromatography data indicated the presence of residual 8-bromoadenine (<6%) in the preparations of

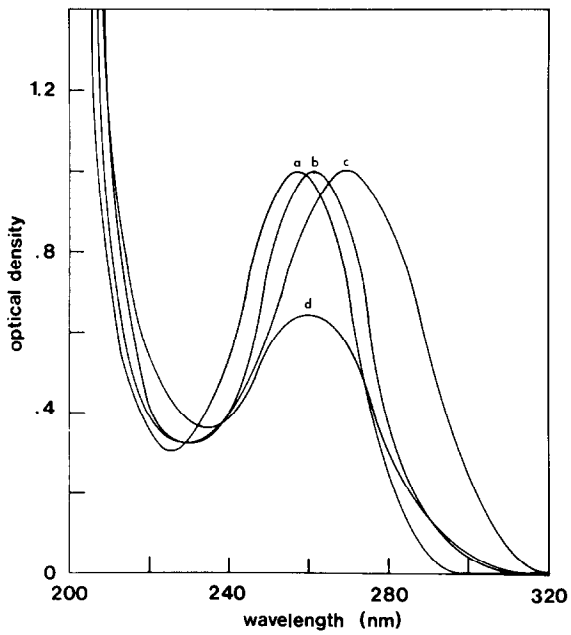

Fig.1. Ultraviolet spectra of adenine (a), 8-bromoadenine (b), 8-azidoadenine before (c) and after (d) 2 minutes of photolysis through quartz at 1 cm distance using UVS-54 Mineralight. Each compound was 0.1 mM in 0.1 N HCl.

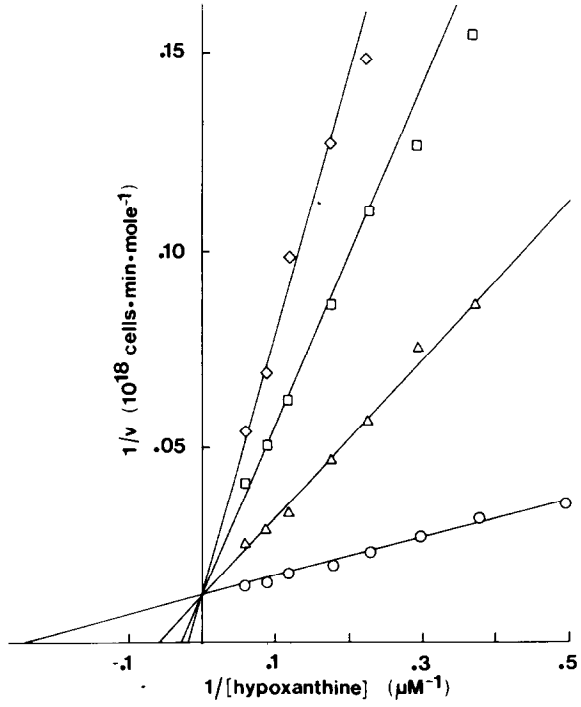

Fig.2. Lineweaver-Burk plot of hypoxanthine uptake in the absence (○) or presence of 33 μM (△), 100 μM (□) and 170 μM (◇) 8-azidoadenine.

8-azidoadenine. This does not invalidate the present study, but means that the effectiveness of 8-azidoadenine as a photoaffinity label will be somewhat underestimated.

The competitive inhibitory effect of 8-azidoadenine on hypoxanthine uptake is shown in fig.2. Data derived from a secondary plot (not shown) show a K_i of 9×10^{-6} M for 8-azidoadenine. This value is ~10-times the K_m of adenine [1] but is still low enough to be considered good for a photoaffinity label. It is noteworthy that kinetic studies of hypoxanthine uptake in the presence of 8-bromoadenine revealed a K_i of 2.5×10^{-4} M, and the addition of 8-bromoadenine during UV irradiation had no detectable effect on the subsequent uptake kinetics.

Fig.3 shows the effect of short-wavelength irradiation (254 nm) on hypoxanthine transport after different times of exposure and at different temperatures. Damage to the cells in terms of transport is considerably reduced at lower temperatures. This, and the

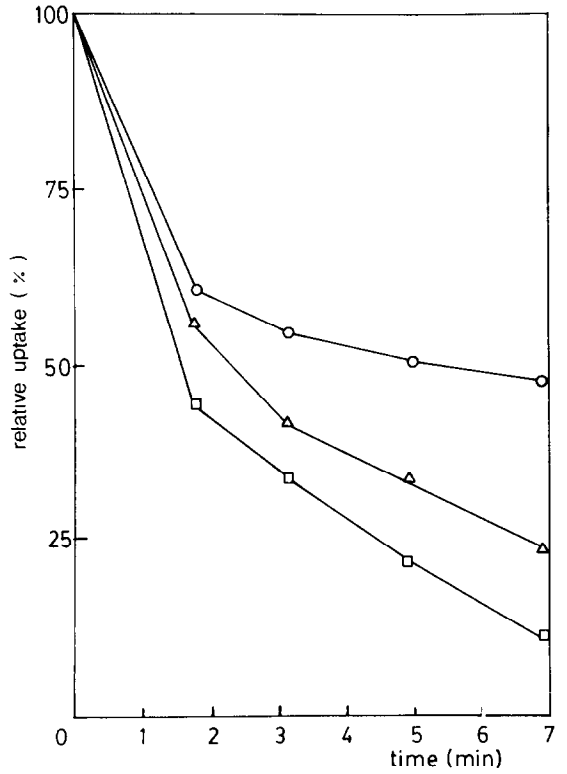

Fig.3. Relative uptake of hypoxanthine after irradiation of yeast cells by UVS-54 Mineralight (3 cm distance) for various times at 25°C (□), in an ice bath (△) and in the frozen state (○). Uptake was measured for a 5 mm period in 100 μM hypoxanthine.

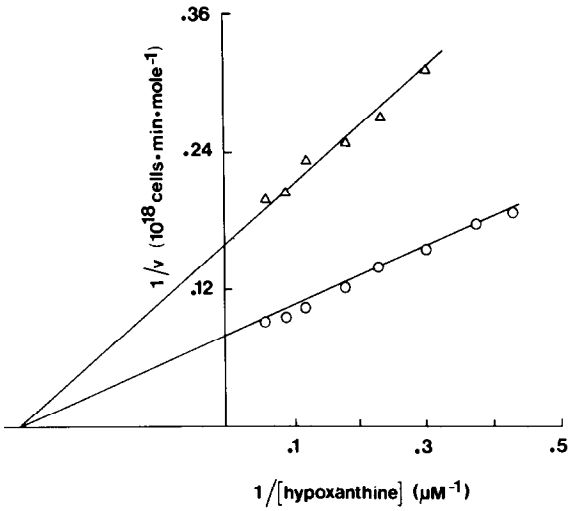

Fig.4. Lineweaver-Burk plot of hypoxanthine uptake in cells exposed to 5 min of UV irradiation (UVS-54 Mineralight, 3 cm distance) while in the frozen state in the absence (○) and presence (□) of 50 μM 8-azidoadenine.

reported result that irradiation of a ligand–receptor mixture in frozen state increased incorporation 10-fold above that achieved in aqueous state [9], led us to perform the photoaffinity labeling experiments in the frozen state.

Kinetic data from fig.4 demonstrate that after irradiation of the cells in the presence of the photoaffinity label and in the frozen state, the K_m-value is unchanged but V_{max} is reduced by ~50% compared with cells irradiated for the same time in the absence of 8-azidoadenine. Irradiation of the cells with the photoaffinity label while in the liquid state (4°C) resulted in a drop of only 35% in V_{max} (not shown). In order to distinguish between direct and indirect effects of the photoaffinity label, we protected the transport system against the inhibitory binding of 8-azidoadenine through simultaneous addition of one of its substrates. The presence of 200 μM adenine during irradiation of the cells with the photoaffinity label completely prevented the reduction in V_{max} (not shown). To further demonstrate the specificity of the interactions, kinetic studies of glycine uptake were performed with cells which had been irradiated in the presence or absence of 8-azidoadenine. Glycine was chosen since its transport, like that of the purines [3], appears to be dependent on the proton and potassium gradients across the cell membrane [10]. No significant differences in glycine uptake were found

between cells irradiated with or without the photoaffinity label (not shown).

It is concluded that the effect of irradiation in the presence of 8-azidoadenine on the V_{max} of hypoxanthine transport (fig.4) indicates a specific irreversible binding of the label to the purine-transport system. Furter studies are in progress to isolate and identify the transport system after binding with a radioactive label.

Acknowledgements

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