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Photoaffinity labeling of thiamin-binding component in yeast plasma membrane with [^{3}H]4-azido-2-nitrobenzoylthiamin

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When prepared from *Saccharomyces cerevisiae* through an acid precipitation at pH 5.0 for a crude particulate fraction obtained by mechanical agitation of yeast protoplasts with glass beads, the plasma membranes have more remarkable binding quantities of [^{14}C]thiamin (K_d, 51 nM; B_{max}, 263 pmol per mg of protein) compared with our previously prepared membranes [(1986) Experientia 42, 607–608]. Photoaffinity labeling of these yeast plasma membranes with [^{3}H]4-azido-2-nitrobenzoylthiamin resulted in the covalent modification of a membrane component with an apparent molecular mass of 6–8 kDa. The extent of its labeling was markedly decreased by previous addition of thiamin. This result suggests that the small membrane component (6–8 kDa) takes part in the thiamin binding of thiamin carrier protein(s) in yeast plasma membranes.

Thiamin binding; Plasma membrane; Photoaffinity labeling; (*Saccharomyces cerevisiae*)

1. INTRODUCTION

Yeast cells contain a highly efficient transport system for the accumulation of thiamin [1]. In previous studies on thiamin transport in *Saccharomyces cerevisiae* we demonstrated that the entry of thiamin into the cell occurs by means of a carrier-mediated active process [2]. Recently, we have reported the occurence of a membrane-bound thiamin-binding protein in *S. cerevisiae*, besides a soluble thiamin-binding protein in the periplasm [3,4]. Furthermore, it has been disclosed that the thiamin-binding protein located in the plasma membranes of *S. cerevisiae* plays an important role in the thiamin uptake and some properties of thiamin binding by the plasma membranes have also been described [5]. However, the protein components playing the uptake role in the cell membranes have remained unclear until the present time. We have synthesized some affinity labelers such as azidobenzoyl derivatives of thiamin including 4-azido-2-nitrobenzoylthiamin (ANBT) [6,7] and *O*-bromoacetylthiamin [8] which are competitive inhibitors of thiamin transport in *S. cerevisiae*. And then, we have also reported that these derivatives irreversibly inactivate both thiamin-binding activity of the plasma membrane and thiamin-transport activity in yeast cells. In the course of the studies described above, it was realized that these compounds can be used as a potent tool for identification of the membrane components responsible for active transport of thiamin across yeast plasma membranes.

Here, we have developed a novel rapid method for a preparation of yeast plasma membranes having remarkable binding quantities of [^{14}C]thiamin and we have used these plasma membranes to study the binding of [^{3}H]ANBT as a photoaffinity probe for the thiamin-binding component in yeast plasma membranes.

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2. MATERIALS AND METHODS

2.1. *Materials*

[^{14}C]Thiamin([*thiazole*-2-^{14}C]thiamin hydrochloride, 24.3 mCi/mmol) was purchased from Amersham International

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(Buckinghamshire, England). Pyrithiamin hydrobromide and oxythiamin hydrochloride were the products of Sigma. *O*-Benzoylthiamin disulfide was a gift from Tanabe Chemical Industries Ltd (Osaka). Zymolyase-100T was purchased for Seikagaku Kogyo Co., Ltd (Tokyo, Japan). Molecular mass marker proteins for electrophoresis were obtained from Bio-rad Laboratories and BDH Limited (Poole, England). All other chemicals were purchased from commercial suppliers.

2.2. *Plasma membrane preparation*

Plasma membranes were prepared according to the procedure of Ongjoco et al. [9] with some modifications from 10 g (wet wt) of *S. cerevisiae* grown at 30°C for 16 h in Wickerham's synthetic medium without thiamin. After harvesting, the cells were washed once with cold water. The modified procedure is as follows: yeast cells washed in 100 ml of 0.05 M EDTA were suspended in 90 ml of 0.05 M EDTA and 0.25 M 2-mercaptoethanol, and allowed to stand for 30 min at room temperature. The cells were resuspended in 80 ml of a sorbitol-EDTA solution (1 M sorbitol and 0.1 M EDTA) and then Zymolyase-100T (0.1 mg/ml) was added to the cell suspension, followed by 1 h incubation at 37°C. The resultant yeast protoplasts were centrifuged for 10 min at 2000 rpm. The yeast protoplasts washed once in the chilled sorbitol-EDTA solution were suspended in 15 ml of buffer A (250 mM sucrose, 10 mM imidazole, 1 mM EDTA, 2 mM 2-mercaptoethanol, 0.02 % sodium azide [pH 7.5]) containing 1 mM phenylmethylsulfonyl fluoride and homogenized with 30 ml of glass beads, 0.5 mm in diameter, by seven 1-min bursts (alternating with 2-min rests) in a bead-beater (Biospec Products, Bartlesville, OK). After removal of the glass beads by filtration, the homogenate was centrifuged twice for 3 min at 3500 rpm. The supernatant (a crude particulate fraction) was cleared of mitochondria by the procedure of Fuhrmann et al. [10] by acidification to pH 5.0, followed by two centrifugation steps for 5 min each at 8000 rpm leaving precipitates. The precipitates were resuspended in 0.05 M potassium phosphate buffer (pH 5.0) to give the acid precipitates described in table 1. The acidic supernatant was readjusted to pH 7.5 and centrifuged for 60 min at 36 000 rpm. The pellet of the purified membranes was suspended in a cold proper buffer or water by using a Dounce homogenizer and stored at −80°C.

2.3. *[^{14}C]Thiamin-binding assay*

The [^{14}C]thiamin-binding activity was assessed by an equilibrium dialysis experiment as previously described [4].

2.4. *Chemical synthesis*

[^{3}H]4-Azido-2-nitrobenzoylthiamin ([^{3}H]ANBT) was synthesized from [^{3}H]4-amino-5-bromomethyl-2-methylpyrimidine hydrochloride, which has been tritiated by catalytic exchange at the Du Pont-New England Nuclear Research Product Plant, and 4-methyl-5-[2-(4-azido-2-nitrobenzoyl)]-thiazole as reported previously [6].

2.5. *Photoaffinity labeling of the plasma membranes*

For photoaffinity labeling of yeast plasma membranes with [^{3}H]ANBT, suspensions of plasma membrane (1 mg/ml) in 50 mM potassium phosphate buffer (pH 5.0) were incubated in the dark at 4°C with [^{3}H]ANBT for 5 min with or without 1 mM unlabeled thiamin. Irradiation was performed for definite times with a Toshiba black light lamp (40 W) at a distance of 25 cm from the reaction vessel. The irradiated membrane suspensions were diluted with 7 ml of chilled water in the dark, followed by centrifugation at 50 000 rpm for 15 min. The membrane pellets were washed twice with 8 ml of chilled water and dissolved finally in 1 ml of 2% SDS. The radioactivity of the solution was measured on a liquid scintillation spectrometer (Packard Tri-Carb model 460).

Protein concentration was measured by the procudure of Markwell et al. [11] using bovine serum albumin as standard.

2.6. *Photolabeling of the cells*

The cell suspension (8.74×10^6 per ml) in 1 l of 50 mM potassium phosphate buffer (pH 5.0) was irradiated with 1 μM [^{3}H]ANBT for 15 min at 4°C with or without 1 mM thiamin as previously described [6]. Following the photoaffinity labeling of the cells, the plasma membranes were prepared by the procedure as described above. The obtained plasma membranes were kept frozen at −80°C prior to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

2.7. *SDS-PAGE analysis*

Linear gradient 7.5–22.5% polyacrylamide slab gel electrophoresis was performed as in [12]. In order to measure the distribution of radioactivity in the gels, the gels were sliced into 2-mm and digested by overnight incubation in 1 ml of 15% H_2O_2 at 55–60°C and the radioactivities were counted in 10 ml of scintillation fluid.

3. RESULTS AND DISCUSSION

3.1. *Preparation of yeast plasma membranes*

The thiamin-binding activity measured with the crude particulate fraction prepared by the method described in section 2 was increased about 40-fold with a recovery of 54% by an acid-precipitation procedure (adjusting at pH 5) used to enhance the plasma membrane-bound thiamin-binding activity (table 1).

3.2. *Some properties of yeast plasma membranes with respect to [^{14}C]thiamin binding*

The binding of [^{14}C]thiamin to the plasma membranes increased in proportion to protein concentration in the range from 0.5 to 1.5 mg/ml. The optimal pH of the binding reaction was 5.0. This value is compatible with the optimal pH of thiamin uptake by yeast cells [2] and thiamin binding by the previously prepared membrane fraction [5]. The binding activity was decreased more than 50% at a pH value below 4 or above 6 and was lost completely at pH 7.

Fig.1 shows that the binding activity was increased linearly with increasing thiamin concentration

Table 1

Purification of plasma membrane-bound thiamin-binding protein

Fraction	Total protein (mg)	Total activity (pmol thiamin-binding)	Specific activity (pmol thiamin bound/ mg of protein)	Yield (%)
Crude particulates	1034	3728	3.6	100
Acid precipitates	712	1498	2.1	40
Plasma membranes	13.2	2013	152.5	54

up to 0.05 μM and then began to saturate, attaining a plateau at 0.25 μM. Scatchard analysis of the data showed that there was a single class of binding sites which had a dissociation constant (K_d) of 51 nM for thiamin and a maximum binding (B_{max}) of 263 pmol of [^{14}C]thiamin per mg of protein. The K_d value or the maximum binding (B_{max}) of the plasma membranes to thiamin was reduced to one half and increased to 3-fold compared to the respective values from the previously prepared membranes [5]. Acquisition of the plasma membrane fraction including many thiamin-binding components seems to result from the preparation of yeast protoplasts, followed by homogenization of the protoplasts with glass beads in a bead-beater. Actually, the thiamin-binding activity of plasma membranes, when prepared by the same method except producing the protoplasts, was decreased to less than one third of its binding activity of the plasma membranes prepared in this text. As described previously [5], the binding activity of yeast plasma membranes to [^{14}C]thiamin was inhibited by several thiamin analogs, such as pyrithiamin and *O*-benzoylthiamin disulfide which have been known to be specific inhibitors of yeast thiamin transport. However, oxythiamin which has no effect on the thiamin transport did not inhibit the thiamin binding by yeast plasma membrane (data not shown). The evidence presented above shows that the plasma membrane-bound thiamin-binding protein of *S. cerevisiae* may participate directly in the thiamin transport.

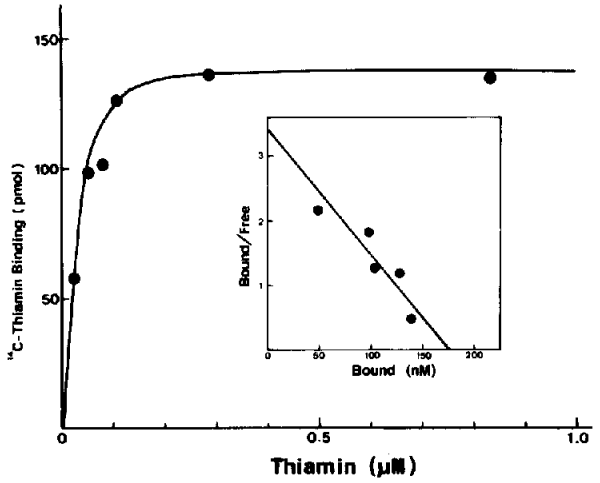

Fig.1. Concentration dependence of thiamin binding to yeast plasma membranes. The binding assay was carried out by equilibrium dialysis at 4°C for 24 h in 0.05 M potassium phosphate buffer ranging in concentration from 0.03 to 0.83 μM [^{14}C]thiamin. Each value is the mean of duplicate determinations. (Inset) Scatchard plot of the binding. The slope of the plot, $-1/K_d$, was determined by linear regression analysis ($r = 0.912$).

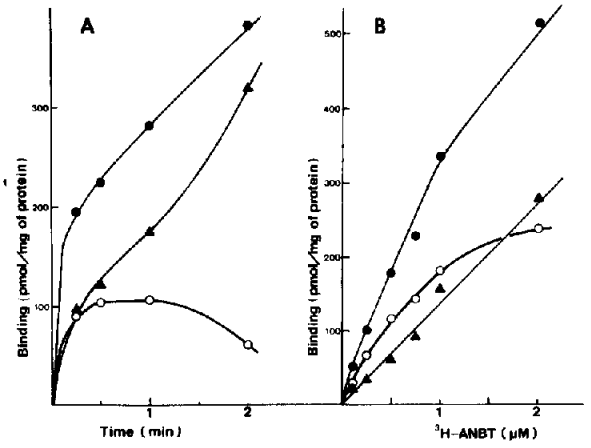

Fig.2. Specific photolabeling of [^{3}H]ANBT to yeast plasma membrane. Effect of irradiation time on the labeling of [^{3}H]ANBT (A) and saturation curve for the photolabeling of [^{3}H]ANBT at various concentrations (B) were investigated. Total bound [^{3}H]ANBT (●), nonspecifically bound [^{3}H]ANBT (▲) and specifically bound [^{3}H]ANBT (○) were measured as described in section 2. The values are means from duplicate determinations.

3.3. *Photoaffinity labeling of yeast plasma membranes with [^{3}H]ANBT*

As reported previously, ANBT inactivated the yeast thiamin-transport system and thiamin binding to plasma membranes when exposed to visible light. In order to investigate the ANBT binding to yeast plasma membranes, [^{3}H]ANBT (spec. act. 6500 dpm/nmol) was synthesized as in [6]. The time course of specific [^{3}H]ANBT photolabeling indicated (fig.2A) that the maximal labeling of the plasma membranes with incubation at 4°C was achieved very rapidly in 1 min. Longer photoirradiation led to a gradual increase in non-specific labeling. Photolabeling specificity of the membrane fraction was defined as labeling which was prevented when 1 mM thiamin was present during the binding assay. As shown in fig.2B, the photolabeling of yeast plasma membranes with [^{3}H]ANBT for 1 min at 4°C is a saturable process. The specific labeling of [^{3}H]ANBT was saturated at 1–2 μM. On the other hand, a specific photoaffinity labeling of plasma membranes prepared from PT-R2 strain (thiamin transport mutant) [13] with [^{3}H]ANBT was entirely unattainable (data not shown).

Since the photolabeling of [^{3}H]ANBT to the plasma membranes is highly affinitive and specific, it should be plausible to identify the binding component for thiamin in yeast plasma membranes by means of photoaffinity labeling. However, the acquisition of plasma membrane samples with detectable radioactivities in SDS-PAGE analysis was not successful by direct photolabeling of [^{3}H]ANBT to the plasma membranes. Therefore, as described in section 2, the cell suspensions were irradiated with [^{3}H]ANBT, followed by isolation of the plasma membranes. The radioactivities per microgram of protein due to the total binding of [^{3}H]ANBT to this plasma membrane were found to be 77.8 dpm and those due to nonspecific binding were 37.7 dpm. Accordingly, the specific labeling of [^{3}H]ANBT to the membrane was calculated as 6.17 nmol/mg of protein. As shown in fig.3A, photoaffinity labeling of yeast plasma membranes in the cells with [^{3}H]ANBT revealed in SDS-PAGE analysis a clear covalent incorporation of radioactivity into a portion of very small apparent molecular mass (6–8 kDa). This incorporation of radioactivity was remarkably decreased by the addition of 1 mM thiamin. Then, as shown in fig.3B,

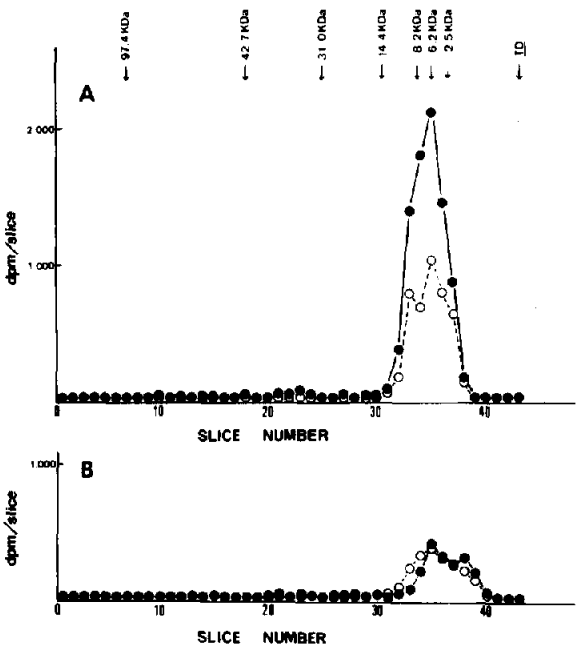

Fig.3. Distribution of radioactivity after SDS gel electrophoresis of plasma membranes from the yeast parent strain [A] and from the thiamin-transport mutant strain (PT-R2) [B] photolabeled with [^{3}H]ANBT. The cell suspension was treated with 1 μM [^{3}H]ANBT in the presence (○) and absence (●) of nonradioactive thiamin (1 mM) as competing ligand as described in section 2. After preparation of the plasma membranes, proteins (150 μg) were separated by 7.5–22.5% SDS-polyacrylamide gel electrophoresis. The radioactivity in the various regions of the gel was determined by slicing the gel into 2-mm fractions. Molecular masses (in kDa; at top figure) of the marker proteins and the position of the tracking dye bromophenol blue (TD) are indicated by arrows.

photoaffinity labeling of the plasma membranes in the cells of the PT-R_2 strain (thiamin transport mutant) with [^{3}H]ANBT remarkably decreased the incorporation of radioactivity compared with its incorporation for the yeast parent strain and the specific binding shown in a region of 6–8 kDa in fig.3A disappeared entirely. These results suggest that the small component (6–8 kDa) is a constituent taking part in the binding to thiamin on thiamin carriers in yeast plasma membrane and that yeast thiamin uptake proceeds by a complex transport system consisting of several essential components. It is not reasonable, however, to rule out the possibility that the band (6–8 kDa) photolabeled with [^{3}H]ANBT could be a proteolytic degradation product of a larger membrane polypeptide.

The exact relationship between the 6–8 kDa [^{3}H]ANBT-labeled component and the thiamin-transport system will require further characterization of the thiamin-binding component by solubilizing the plasma membranes. In this context, an experiment on thiamin transport into vesicles reconstituted from yeast plasma membranes, obtained by the method of preparation described here in the text, will be needed in order to elucidate the precise mechanism of the transport process.

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