Thiamine-binding activity of *Saccharomyces cerevisiae* plasma membrane

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Summary. The specific binding activity to [^{14}C]thiamine was found to be located in the plasma membrane of *Saccharomyces cerevisiae*. The activity was inhibited by several thiamine analogs and it was hardly detectable in the plasma membrane from a thiamine transport mutant of *Saccharomyces cerevisiae*. Some properties of the thiamine-binding activity of yeast plasma membrane are discussed in connection with those of the thiamine transport system.
Key words. Thiamine; thiamine-binding; yeast plasma membrane.

Thiamine uptake by microorganisms such as *Escherichia coli*[1], *Lactobacilli*[2,3] and *Saccharomyces cerevisiae*[4,5] has been ascertained to be a carrier-mediated active process. Although some thiamine-binding proteins in the periplasm and cell surface of these microorganisms have been isolated and characterized[3,6-9], membrane-bound carrier proteins directly involved in thiamine transport have been less studied.

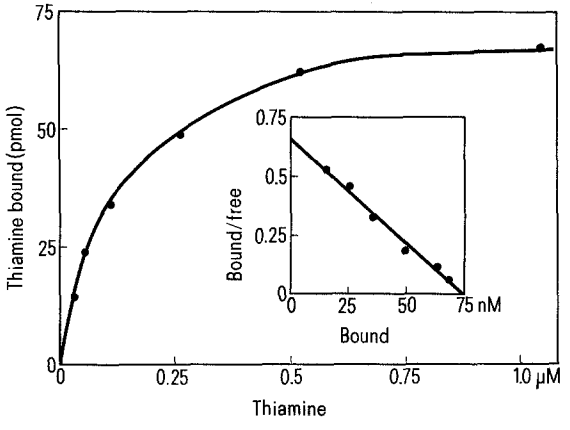
In recent years we have reported that the occurrence of a membrane-bound thiamine-binding protein in *Saccharomyces cerevisiae*, besides a soluble thiamine-binding protein in the periplasm[10], and that several properties of the thiamine transport system of *Saccharomyces cerevisiae* are similar to those of the membrane-bound thiamine-binding protein.

In this paper, we present evidence, obtained by purification of a crude membrane fraction of *Saccharomyces cerevisiae*, showing that the membrane-bound thiamine-binding activity is mainly located in the yeast plasma membrane. Some properties of thiamine binding by the plasma membrane are also described.

Plasma membrane was prepared according to the procedure of Serrano[11], with minor modifications from 33 g (wet wt) of *Saccharomyces cerevisiae* grown at 30 °C for 16 h in Wickerham's synthetic medium with thiamine omitted. Yeast cells were washed with 150 ml of an ice cold solution containing 0.4 M sucrose, 25 mM imidazole, 0.2 mM EDTA and 0.5 mM phenylmethylsulfonyl fluoride, adjusted to pH 7.0 with HCl (SIE medium). The cells were resuspended with chilled SIE medium to a final volume of 20 ml and placed in the 50 ml chamber of a BEAD-BEATER (Biospec Products, Bartlesville, Oklahoma, USA) and 40 ml of glass beads of 0.5 mm diameter were added. The cells were ruptured by 2 min of treatment, repeated five times; they were cooled by ice water around the chamber. The beads were removed using a glass sinter type 17G1 and washed with 50–70 ml of chilled SIE medium. Final volume of filtrate was about 65–85 ml. The pH was adjusted to 7.0 with 1N NaOH. The filtrate was centrifuged twice at 530 × g for 10 min, followed by centrifugation at 123,000 × g for 1 h to obtain a crude membrane fraction. The pellets were resuspended in 10 ml of chilled SIE medium and homogenized using a Dounce homogenizer. The homogenate (9 ml) was layered on top of the gradient with three 10 ml layers of 1.1, 1.65 and 2.25 M sucrose, centrifuged for 14 h at 80,000 × g. The mitochondrial and plasma membrane corresponded to the 1.1/1.65 and 1.65/2.25 interphases, respectively. Both bands were collected with Pasteur pippete, diluted 4 times with an appropriate buffer, cold, and centrifuged at 123,000 × g for 1 h to obtain a plasma membrane and a mitochondrial membrane. The thiamine-binding activity was assessed by an equilibrium dialysis experiment as previously described[10]. [^{14}C]Thiamine ([thiazole-2-^{14}C]thiamine hydrochloride, 24.3 mCi/mmol) was obtained from the Radiochemical Center, England.

Yeast membrane fractions were tested for the ATPase activities by the method of Serrano[11]. The specific activity of plasma membrane Mg-ATPase was 700–800 nmol of inorganic phosphate released/min/mg of protein at pH 6.8; it was inhibited by 67.2% by 10 μM vanadate, but by 32.4% by oligomycin (10 μg/ml). On the other hand, the ATPase activity in the mitochondrial membrane showed high oligomycin sensitivity (62.7%) and low vanadate sensitivity (17.6%). These relationships are similar to those which have been previously described, suggesting that the plasma membrane preparation was not seriously contaminated by other cellular membranes.

The results concerning the distribution of the thiamine-binding activity to these plasma and mitochondrial membrane fractions obtained from yeast crude membrane are summarized in table 1. The specific thiamine-binding activity of the plasma membrane represents a purification of 3.5-fold from the crude membrane with a recovery of 31.5%. These results show that the binding activity which is present in yeast crude membrane as described



Concentration dependence of thiamine binding to yeast plasma membrane. 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 1.04 μM [^{14}C]thiamine. 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 ($r = 0.993$).

Table 1. Binding of [^{14}C]thiamine to membrane fractions

Membrane fractions	Total protein (mg)	Total activity (pmol thiamine binding)	Specific activity (pmol thiamine bound/mg of protein)	Yield (%)
Crude membrane	278.6	11,714	42.1	100
Mitochondrial membrane	70.8	2,005	28.3	17.1
Plasma membrane	25.4	3,693	145.4	31.5

Table 2. Effect of chloroethylthiamine and O-benzoylthiamine disulfide on the binding of yeast plasma membrane to [^{14}C]thiamine

Addition (concentration)	Thiamine-binding specific activity (pmol/mg of protein)	%
Control	145.4	100
Chloroethylthiamine (10^{-5} M)	23.6	16.2
O-Benzoylthiamine disulfide (10^{-6} M)	40.4	27.8
Thiamine (10^{-5} M)	28.6	19.7

previously was predominantly located in the plasma membrane. The binding of the isotope-labelled thiamine to yeast plasma membrane increased in proportion to protein concentration in the range 0.25 to 2 mg/ml (data not shown). The optimal pH of yeast plasma membrane was between 5.0 and 5.3. This value is comparable to the optimal pH of the thiamine uptake by yeat cells. The binding of [^{14}C]thiamine to the plasma membrane was saturable. The figure shows that the binding activity increased linearly with increasing concentration of thiamine up to 0.1 μM and then began to saturate, attaining a plateau at the concentration of 1 μM. Analysis of the data by the method of Scatchard showed that there was a single class of binding sites which had a dissociation constant (K_d) for thiamine of 0.11 μM and a maximum binding (B_{max}) of 97.9 pmol of [^{14}C]thiamine/mg of protein. The K_d value of yeast plasma membrane to thiamine was close to the apparent K_m (0.18 μM) of thiamine transport in *S. cerevisiae*. As shown in table 2, the binding activity of yeast plasma membrane to [^{14}C]thiamine (1 μM) was inhibited by thiamine analogs, such as chloroethylthiamine (a gift from Sankyo Co. Ltd (Tokyo)) which have been known to be specific inhibitors of yeast thiamine transport. In particular, O-benzoylthiamine disulfide (a gift from Tanabe Chemical Industries Ltd (Osaka)) which is very lipophilic and without effect on the soluble thiamine-binding protein as described previously[10], inhibited thiamine binding by yeast plasma membrane markedly.

In a previous paper[12] we reported that 4-azido-2-nitrobenzoylthiamine, a photoreactive thiamine derivative, irreversibly and specifically inactivated the yeast thiamine transport system under the irradiation of visible light. It was therefore suggested that this inactivation was due to the binding of 4-azido-2-nitrobenzoylthiamine to some components in the thiamine transport system, and it led us to study the inactivation of the binding activity of yeast plasma membrane to thiamine with 4-azido-2-nitrobenzoylthiamine. 2 ml of membrane fraction (1 mg of protein/ml) in 0.05 M acetate buffer, pH 5.0, containing 0.02% NaN_3, was incubated with 4-azido-2-nitrobenzoylthiamine (5 μM) for 10 min in the dark at 4 °C. The reaction mixture was then photolyzed with a Toshiba black light (40 W) at a distance of 25 cm for 15 min, followed by dilution with the chilled buffer described above (30 ml). The diluted solution was then centrifuged at 123,000 × g for 1 h and the plasma membranes were washed further with 30 ml of the same buffer. The resuspended plasma membranes were used immediately for the binding assay by centrifugation. 0.5–1 mg of membranes were incubated with 1 μM [^{14}C]thiamine in 2.5 ml of binding buffer (0.05 M acetate buffer, pH 5.0, containing 0.02% NaN_3) with or without 1 mM unlabeled thiamine for 60 min at 4 °C, then centrifuged for 10 min at 100,000 × g. This was sufficient to pellet the membrane while giving a minimal redistribution of the free [^{14}C]thiamine. Aliquots before and after centrifugation were taken with the difference in radioactivity equated to thiamine bound to membranes. As shown in table 3, the thiamine-binding activity of yeast plasma membranes was decreased to 47.8% of that in the dark by the treatment with 4-azido-2-nitrobenzoylthiamine under visible light. These results suggested that there is a plasma membrane-bound protein which specifically binds thiamine, and might possibly be a functional component involved in the yeast thiamine transport system.

Furthermore, it was found that the plasma membrane fraction from *Saccharomyces cerevisiae* PT-R_2, a thiamine transport mutant, has a thiamine-binding activity of only 1.1% of that from the parent strain (table 4). Evidence which has been presented above shows that the thiamine-binding protein present in the plasma membrane may participate directly in thiamine transport in *Saccharomyces cerevisiae*.

Table 3. Photoinactivation with 4-azido-2-nitrobenzoylthiamine of thiamine-binding activity in yeast plasma membrane

Addition	Irradiation	Thiamine-binding (%)
4-Azido-2-nitrobenzoyl thiamine (5 μM)	(−)	100
	(+)	47.8

Table 4. Thiamine-binding activity of plasma membrane from a parent strain and a thiamine transport mutant of *S. cerevisiae*

Strain	Thiamine-binding specific activity (pmol/mg of protein)	%
S. cerevisiae (parent strain)	145.4	100
Thiamine transport mutant (PT-R_2)	1.6	1.1

1 Kawasaki, T., Miyata, I., Esaki, K., and Nose, Y., Archs Biochem. Biophys. *131* (1969) 223.
2 Neujahr, H.Y., Acta chem. scand. *17* (1963) 1902.
3 Henderson, G.B., Zevery, E.M., Kadner, R.J., and Huennekens, F.M., J. supramol. Struct. *6* (1977) 239.
4 Iwashima, A., Nishino, H., and Nose, Y., Biochim. biophys. Acta *330* (1973) 222.
5 Iwashima, A., Wakabayashi, Y., and Nose, Y., Biochim. biophys. Acta *413* (1975) 413.
6 Griffith, T.W., and Leach, F.R., Archs Biochem. biophys. *159* (1973) 658.
7 Matsuura, A., Iwashima, A., and Nose, Y., Biochem. biophys. Res. Commun. *51* (1973) 241.
8 Nishimune, T., and Hayashi, R., Biochim. biophys. Acta *328* (1973) 124.
9 Iwashima, A., and Nishimura, H., Biochim. biophys. Acta *577* (1979) 217.
10 Iwashima, A., Nishimura, H., and Nose, Y., Biochim. biophys. Acta *557* (1979) 460.
11 Serrano, R., Molec. cell. Biochem, *22* (1978) 51.
12 Sempuku, K., Nishimura, H., and Iwashima, A., Biochim. biophys. Acta *645* (1981) 226.

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Use of ^{3}H-QNB in the isolation of plasma membrane from smooth muscle of the urinary bladder: Effect of oxalate on calcium uptake by the membrane fractions

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Summary. Specific binding of tritiated quinuclidinyl benzilate (^{3}H-QNB) to surface membrane muscarinic receptors was utilized to identify plasma membrane (PM) fractions from smooth muscle of the rabbit urinary bladder. Accumulation of ^{3}H-QNB in the PM fraction was 4–5-fold higher than that in fractions of endoplasmic reticulum (EM) or mitochondria (M). A similar pattern of