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SOLUBLE AND MEMBRANE-BOUND THIAMINE-BINDING PROTEINS FROM *SACCHAROMYCES CEREVISIAE*

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Summary

Previous communications from this laboratory have indicated that there exists a thiamine-binding protein in the soluble fraction of *Saccharomyces cerevisiae* which may be implicated to participate in the transport system of thiamine in vivo.

In the present paper it is demonstrated that both activities of the soluble thiamine-binding protein and thiamine transport in *S. cerevisiae* are greatest in the early-log phase of the growth and decline sharply with cell growth. The soluble thiamine-binding protein isolated from yeast cells by conventional methods containing osmotic shock treatment appeared to be a glycoprotein with a molecular weight of 140 000 by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The apparent K_d of the binding for thiamine was 29 nM which is about six fold lower than the apparent K_m (0.18 μM) of thiamine transport. The optimal pH for the binding was 5.5, and the binding was inhibited reversibly by 8 M urea but irreversibly by 8 M urea containing 1% 2-mercaptoethanol. Several thiamine derivatives and the analogs such as pyrithiamine and oxythiamine inhibited to similar extent both the binding of thiamine and transport in *S. cerevisiae*, whereas thiamine phosphates, 2-methyl-4-amino-5-hydroxymethylpyrimidine and *O*-benzoylthiamine disulfide did not show similarities in the effect on the binding and transport in vivo. Furthermore, it was demonstrated by gel filtration of sonic extract from the cells that a thiamine transport mutant of *S. cerevisiae* (PT-R_2) contains the soluble binding protein in a comparable amounts to that in the parent strain, suggesting that another protein component is required for the actual translocation of thiamine in the yeast cell membrane. On the other hand, the membrane fraction prepared from *S. cerevisiae* showed a thiamine-binding activity with apparent K_d of 0.17 μM at optimal pH 5.0 which is almost the same with the apparent K_m for the thiamine transport system. The membrane-bound thiamine-

binding activity was not only repressible by exogenous thiamine in the growth medium, but as well as thiamine transport it was markedly inhibited by both pyrithiamine and *O*-benzoylthiamine disulfide. In addition, it was found that membrane fraction prepared from PT-R_2 has the thiamine-binding activity of only 3% of that from the parent strain of *S. cerevisiae*.

These results strongly suggest that membrane-bound thiamine-binding protein may be directly involved in the transport of thiamine in *S. cerevisiae*.

Introduction

Yeast cells contain a highly efficient transport system for the accumulation of thiamine [1]. In previous studies on thiamine transport in *Saccharomyces cerevisiae* we demonstrated that the entry of thiamine inside the cell occurs by means of a carrier-mediated active process and the analysis of mutants unable to transport both thiamine and pyrithiamine indicated that a common carrier specific for thiamine and the analog is involved in the transport system for thiamine in *S. cerevisiae* [2,3].

However, nothing is known concerning the chemical entity of the carrier for thiamine or some other functional components involved in the transport of thiamine in yeast cells. In this connection it is of interest to characterize a thiamine-binding protein found in the soluble fraction of *S. cerevisiae* which appeared to be repressed by exogenous thiamine with a concomitant decrease in the uptake of both thiamine and pyrithiamine by yeast cells [4]. Recently, we isolated the soluble thiamine-binding protein from *S. cerevisiae* by successive procedures of cold osmotic shock treatment, DEAE-cellulose chromatography and ultrafiltration [5]. In addition, we have found another thiamine-binding activity in the membrane fraction prepared from *S. cerevisiae*.

In this paper we describe the relationship between some properties of soluble and membrane-bound thiamine-binding proteins from *S. cerevisiae* and the transport of thiamine in yeast cells. The biochemical evidence obtained strongly suggests that the membrane-bound binding protein play a direct role in some unknown way in the yeast thiamine transport in vivo.

Materials and Methods

Chemicals

[^{14}C]Thiamine ([*thiazole*-2-^{14}C]thiamine hydrochloride, 18.9 Ci/mol) was obtained from the Radiochemical Centre, England. Pyrithiamine hydrobromide and oxythiamine hydrochloride were the products of Sigma Chemical Co. Chloroethylthiamine (3-2′-methyl-4′-aminopyrimidyl-(5′)-methyl-4-methyl-5-chloroethyl thiazolium chloride hydrochloride) was a gift from Sankyo Co. Ltd (Tokyo) and *O*-benzoylthiamine disulfide was from Tanabe Chemical Industries Ltd (Osaka). Dimethialium (3-2′-methyl-4′-aminopyrimidyl-(5′)-methyl-4,5-dimethyl thiazolium chloride hydrochloride) and 2-methyl-4-amino-5-hydroxymethyl pyrimidine were gifts from Takeda Chemical Industries Ltd (Osaka). Zymolyase 5000 (a mixture of β-1,3-glucanases produced by *Arthro-*

bacter luteus) was purchased from Kirin Brewery Co. Ltd (Tokyo). All other chemicals were purchased from commercial suppliers.

Organisms

The microorganism used was *S. cerevisiae* obtained as a colonal isolate of commercial baker's yeast Company (Orientals). A thiamine transport mutant of *S. cerevisiae* (PT-R_2) was isolated by the procedure previously described [3].

Growth of yeast cells

S. cerevisiae was grown at 30°C for 16 h in Wickerham's synthetic medium [6] except that thiamine was omitted.

Assays of thiamine transport and thiamine-binding

The transport of thiamine and thiamine-binding were determined by the methods previously described, respectively [2,4].

Preparation of membrane fraction

S. cerevisiae cells (2 liters culture) was suspended in 70 ml of 0.05 M potassium phosphate buffer, pH 7.0, washed with distilled water twice. The cell suspensions were then subjected to sonic oscillation (Kubota Insonator model 200 M) for 20 min at 2°C, centrifuged for 10 min at 6000 × *g* and the supernatant was recentrifuged at the same speed. The resultant supernatant was centrifuged for 20 min at 45 000 × *g* and separated into a supernatant (sonic extract) and particulate fraction (membrane fraction). The latter fraction was suspended in 8 ml of 0.05 M potassium phosphate buffer, pH 7.0. Membrane fraction was also prepared from *S. cerevisiae* by differential centrifugation described above the lysate of protoplast prepared from yeast cells using zymolyase 5000 according to the procedure reported by Doi et al. [7].

Purification of soluble thiamine-binding protein

A soluble thiamine-binding protein was purified from *S. cerevisiae* by the procedure as previously reported [5]. *S. cerevisiae* was grown in 12 liters of thiamine-deficient Wickerham's minimal medium at 30°C. They were harvested at the late exponential phase, and the cells were washed twice with distilled water, then subjected to the cold osmotic shock treatment as follows. Washed yeast cells were suspended in 400 ml of 0.1 M Tris-HCl, pH 8.0, containing 0.9 M NaCl, 1 mM 2-mercaptoethanol and 0.5 mM EDTA. The suspension was shaken at 30°C for 20 min, then centrifuged for 10 min at 6500 × *g*. The pellet was suspended in 400 ml of ice-cold 0.5 mM $MgCl_2$ and stirred in the cold for 60 min, centrifuged for 10 min at 6500 × *g* (Step 1). The supernatant fluid containing the released proteins was concentrated to 19 ml using XM 50 Amicon ultrafilter (Step 2). The concentrated shock fluid was adjusted to pH 7.0 with 1 M potassium phosphate buffer, pH 7.0, and applied to DEAE-cellulose column (2.3 × 12 cm) which was previously equilibrated with 0.05 M potassium phosphate buffer, pH 7.0, and washed with 115 ml of the same buffer. All of the thiamine-binding activity from the shock fluid was adsorbed to the column. Elution was carried out with a linear gradient consisting of 140 ml of the buffer in the mixing flask and an equal volume of buffer containing 0.2 M

KCl in the reservoir. The thiamine-binding protein peak was eluted at approximately 0.12 M KCl (Step 3). The DEAE-cellulose fractions were combined, and then concentrated and washed with the buffer on a XM 100A Amicon ultrafilter several times (Step 4). Protein in soluble or membrane preparations was determined by the method of Lowry et al. [8].

Polyacrylamide gel electrophoresis

Analytical polyacrylamide gel electrophoresis was carried out using 7.5% acrylamide at pH 8.9. The sample was run at 7.0 mA per tube for 1 h at room temperature. The protein was detected by staining with Coomassie brilliant blue.

The binding activity of the soluble thiamine-binding protein during polyacrylamide gel electrophoresis was detected as follows.

The nonstained part of the gel described above, which was run in duplicate at 4°C, was cut in the parallel pieces numbered from the origin to the anode and homogenized in 2 ml of 0.05 M potassium phosphate buffer, pH 7.0. One ml each of the supernatant from the homogenate by centrifugation at 1000 × *g* for 30 min, was used for the assay of thiamine-binding activity.

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was carried out with the protein which was heated at 80°C for 2 h in 0.1 M sodium phosphate buffer, pH 7.2, 1% in SDS, 25% in glycerol and 5% in 2-mercaptoethanol. It was run in 7.5% polyacrylamide gel contained 0.1 M sodium phosphate buffer, pH 7.2, 0.15% ammonium persulfate and 0.15% tetramethylethylenediamine in the presence of 0.1% SDS.

Results and Discussion

Relation of growth phase to soluble thiamine-binding activity and thiamine transport

Table I shows the dependence of the rate of thiamine transport in aerobically grown cells of *S. cerevisiae* on the growth phase. Activity was greatest in the early log phase and then declined markedly and it reached approximately one fifth of that in the early log phase at the stationary phase. Thiamine-binding activity in the sonic extract from growing yeast cells was also highest in the

TABLE I

RELATION OF GROWTH PHASE TO THIAMINE TRANSPORT AND THIAMINE-BINDING ACTIVITY IN *S. CEREVISIAE*

The yeast cells were grown in 500 ml of thiamine-deficient Wickerham's minimal medium. Samples (10 ml) were removed at the times shown, and thiamine transport, thiamine-binding activity and cell density were determined.

Growth (A_{560} nm)	Thiamine transport (nmol/2 min per mg dry wt.)	Thiamine-binding (pmol/mg protein)
0.140	17.7	63.4
0.250	11.0	37.5
0.650	5.3	22.9
0.900	3.8	7.8

early log phase and then decreased with growth as observed in the transport. These results appeared to indicate that the formation and turnover of the soluble thiamine-binding protein in *S. cerevisiae* may correlate to the rate of thiamine transport in yeast cells.

Properties of purified soluble thiamine-binding protein

In order to investigate the possible role of a soluble thiamine-binding protein on thiamine transport in *S. cerevisiae* the protein was purified as described above. The specific activity of the purified soluble thiamine-binding protein (Step 4) is 10 311.7 pmol thiamine bound per mg protein which represents an overall purification of 7.7-fold from shock fluid with a recovery of 42.2%. It also corresponds to approximately 400-fold purification from sonic extract of *S. cerevisiae*.

As shown in Fig. 1A, polyacrylamide gel electrophoresis at pH 8.9 of a purified soluble thiamine-binding protein revealed a single band and the binding activity toward thiamine was coincident with the stained part of the gel in the

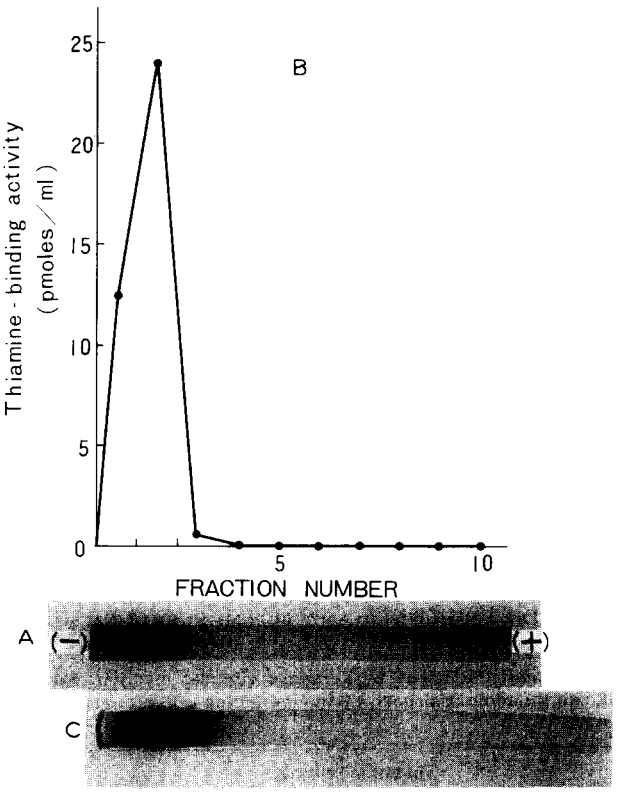

Fig. 1. (A) Polyacrylamide gel electrophoresis of purified soluble thiamine-binding protein of *S. cerevisiae*. Electrophoresis was carried out using 7.5% acrylamide at pH 8.9. 45 μg of protein were run at 7.0 mA per tube for 1 h at room temperature. (B) Binding activity of soluble thiamine-binding protein during polyacrylamide gel electrophoresis. (C) Periodic acid-Schiff staining of soluble thiamine-binding protein. The amount of protein applied was 99 μg, and the electrophoresis was run at 6 mA per tube for 3 h, and the protein was stained by the method of Zacharius et al. [9].

duplicate runs of the purified protein (Fig. 1B).

The molecular weight of the thiamine-binding protein was estimated to be 140 000 by SDS polyacrylamide gel electrophoresis, and the protein was also stainable with periodic acid-Schiff suggesting that the yeast soluble thiamine-binding protein is a glycoprotein (Fig. 1C). The thiamine-binding activity of the purified protein was sensitive to trypsin, whereas it was not to α-mannosidase (data not shown). The optimal pH of thiamine-binding was pH 5.5 when assayed in 0.1 M citrate phosphate buffer, which was slightly higher than that of thiamine transport in yeast cells.

Lineweaver-Burk plots of the binding as function of thiamine concentrations gave a value for the apparent K_d for thiamine of 29 nM which is about one sixth smaller than the apparent K_m of thiamine transport. This may be accounted for by the effect of unstirred layers at the cell surface.

Thiamine-binding reaction by the soluble thiamine-binding protein was inhibited 90.2% in the presence of 8 M urea, and it was fully restored by removal of urea during equilibrium dialysis. However, the presence of 1% 2-mercaptoethanol with 8 M urea caused an irreversible inactivation of the binding protein. From these results it may be concluded that urea alone reversibly inactivates the soluble thiamine-binding protein accompanying by its conformational change, but the cleavage of disulfide linkage in the protein molecule could result in the irreversible inactivation.

Effect of thiamine derivatives and the analogs on soluble thiamine-binding protein and thiamine transport

The competition of several thiamine derivatives and the analogs with

TABLE II

EFFECT OF THIAMINE DERIVATIVES AND ANALOGS ON BINDING OF THIAMINE TO SOLUBLE THIAMINE-BINDING PROTEIN AND THIAMINE TRANSPORT IN *S. CEREVISIAE*

Each thiamine derivative or analog was added to the dialyzing buffer simultaneously with [^{14}C]thiamine at molar ratios as indicated, then the thiamine-binding activity was assayed by an equilibrium dialysis.

Addition	Thiamine derivative / Thiamine	Thiamine-binding (%)	Thiamine transport (%)
Control	0	100	100
Pyrithiamine	2	26.2	26.3
	10	9.0	6.0
Chloroethylthiamine	2	34.1	35.5
	10	5.5	7.9
Dimethialium	2	51.5	44.4
	10	7.3	9.2
Oxythiamine	10	100	100
Thiamine monophosphate	10	14.0	62.2
Thiamine pyrophosphate	10	5.4	80.8
2-Methyl-4-amino-5-hydroxymethylpyrimidine	10	94.3	12.4
O-Benzoylthiamine disulfide	0.1	100	7.1

thiamine for both the binding and transport is illustrated in Table II. The results show that pyrithiamine, chloroethylthiamine and dimethialium are good inhibitors of the binding and transport of thiamine, and as the concentrations of the compounds were varied the binding and transport of thiamine were each

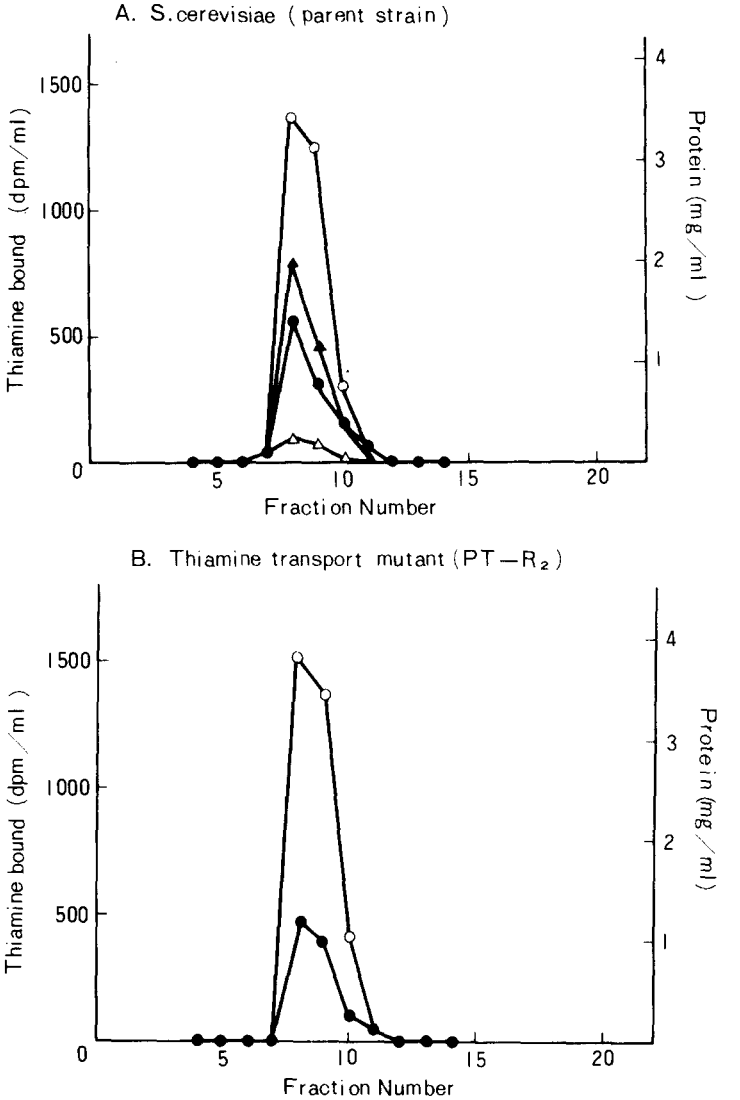

Fig. 2. Sephadex G-75 gel filtration of thiamine-binding protein in the soluble fraction from a parent strain and a thiamine transport mutant of *S. cerevisiae*. The cells grown in 500 ml of Wickerham's minimal medium in the presence or absence of 1 μM thiamine were suspended in 10 ml of 0.05 M potassium phosphate buffer, pH 7.0, sonicated for 20 min and then centrifuged at 45 000 $\times g$ for 20 min. 1 ml of the supernatant was applied on a Sephadex G-75 column (1.0 × 63 cm) at 4°C. Proteins were eluted with 0.05 M potassium phosphate buffer, pH 7.0 at a flow rate of 0.5 ml/min. Fractions (2 ml) were collected. (A) Thiamine-binding activity (○, △) and protein concentration (●, ▲) in 1 ml each of the fractions from *S. cerevisiae* (parent strain) cells grown in the absence (circles) or presence of 1 μM thiamine (triangles) were determined. (B) Thiamine-binding activity (○) and protein concentration (●) in 1 ml each of the fraction from a thiamine transport mutant (PT-R_2) cells were determined.

inhibited to nearly the same degree. Oxythiamine inhibited neither the binding nor the transport under the conditions employed.

On the other hand, thiamine phosphates such as thiamine monophosphate and thiamine pyrophosphate strongly inhibited the thiamine binding and did less thiamine transport, whereas 2-methyl-4-amino-5-hydroxymethylpyrimidine, the pyrimidine moiety of thiamine, and *O*-benzoylthiamine disulfide which is a thiol-form derivative of thiamine with a potent inhibitory activity on yeast thiamine transport system [10], showed adverse effects. These results show that structural specificity of the soluble thiamine-binding protein is not always reflected in specificity of the thiamine transport in vivo, suggesting that the protein is not a sole protein component even if it is involved in thiamine transport of *S. cerevisiae*. As shown in Fig. 2A the soluble thiamine-binding protein was eluted in the void volume by Sephadex G-75 gel filtration and its activity in the cells grown in the presence of 1 μM thiamine was shown to be markedly low as previously described [4]. On the other hand, a thiamine transport mutant of *S. cerevisiae* (PT-R_2) was found to contain the soluble thiamine-binding protein in a comparable amounts to that in the parent strain (Fig. 2B).

Some properties of membrane-bound thiamine-binding protein

Independently of the soluble thiamine-binding protein another thiamine-binding activity was found in the membrane fraction prepared by differential centrifugation after disruption of yeast cells by sonication or after lysis of protoplast prepared by zymolyase treatment. The binding activity was proportional to the amount of protein up to 1.0 mg, and it was lost completely by heating at 100°C for 10 min. The apparent K_d of the binding for thiamine was 0.17 μM at optimal pH 5.0 which is almost the same with the apparent K_m (0.18 μM) of thiamine transport in *S. cerevisiae*. As shown in Tables III and IV both membrane-bound thiamine-binding protein and thiamine transport in *S. cerevisiae* were not only repressible by exogenous thiamine as was found with the soluble protein, but their activities were markedly inhibited by pyrithiamine and also by *O*-benzoylthiamine disulfide which is without effect on the soluble thiamine-binding protein as described above. Furthermore, it was found that the membrane fraction from PT-R_2 contains the thiamine-binding activity of only 3% of that from the parent strain (Table V).

Evidence has been presented above which shows that the membrane-bound thiamine-binding protein of *S. cerevisiae* may participate directly in the thia-

TABLE III

EFFECT OF THIAMINE ADDED TO THE GROWTH MEDIUM ON MEMBRANE-BOUND THIAMINE-BINDING ACTIVITY AND THIAMINE TRANSPORT IN *S. CEREVISIAE*

After 16 h of growth in Wickerham's minimal medium (500 ml) containing the indicated thiamine concentration, the cells were harvested. Membrane fractions were prepared from these cells as described in Materials and Methods and their binding activities to thiamine were assayed.

Addition to the growth medium	Thiamine-binding (%)	Thiamine transport (%)
None	100	100
Thiamine (0.1 μM)	16.8	27.0
Thiamine (0.5 μM)	10.0	7.1

TABLE IV

EFFECT OF PYRITHIAMINE AND *O*-BENZOYLTHIAMINE DISULFIDE ON MEMBRANE-BOUND THIAMINE-BINDING ACTIVITY AND THIAMINE TRANSPORT IN *S. CEREVISIAE*

Thiamine-binding activity in the presence of pyrithiamine or *O*-benzoylthiamine disulfide was assayed as described in the footnote of Table II.

Addition	Thiamine derivative / Thiamine	Thiamine-binding (%)	Thiamine transport (%)
Control	0	100	100
Pyrithiamine	1	74.9	57.8
	5	21.8	15.0
O-Benzoylthiamine disulfide	0.02	42.0	57.0
	0.1	10.4	7.9

TABLE V

THIAMINE-BINDING ACTIVITY OF MEMBRANE PREPARATIONS FROM A PARENT STRAIN AND A THIAMINE TRANSPORT MUTANT OF *S. CEREVISIAE*

Strain	Thiamine binding (pmol/mg protein)
S. cerevisiae (parent strain)	36.7 (59.1 *)
Thiamine transport mutant (PT-R_2)	0.95 (1.80 *)

* The membrane fraction was prepared from protoplast formed by the use of zymolyase 5000 as described in Materials and Methods.

mine transport. These conclusions are based primarily upon the cellular location of the binding protein, similarity of the kinetic constants, parallel regulation of the transport and the activity of binding protein, correlation between the inhibition by thiamine derivatives of the transport and binding and deficiency of the binding protein in a thiamine transport mutant.

The exact role of the soluble thiamine-binding protein in thiamine transport is still unknown, but at least it does not appear to be involved in translocation step of thiamine in the cell membrane of *S. cerevisiae*. Although we have not yet succeeded to solubilize membrane-bound thiamine-binding protein, functional reconstitution by insertion of the purified membrane-bound binding protein into liposome should give further information on the role of both binding proteins in the transport system of thiamine in *S. cerevisiae*.

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