

Modification of thiamine pyrophosphate dependent enzyme activity by oxythiamine in *Saccharomyces cerevisiae* cells

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Abstract: Oxythiamine is an antivitamin derivative of thiamine that after phosphorylation to oxythiamine pyrophosphate can bind to the active centres of thiamine-dependent enzymes. In the present study, the effect of oxythiamine on the viability of *Saccharomyces cerevisiae* and the activity of thiamine pyrophosphate dependent enzymes in yeast cells has been investigated. We observed a decrease in pyruvate decarboxylase specific activity on both a control and an oxythiamine medium after the first 6 h of culture. The cytosolic enzymes transketolase and pyruvate decarboxylase decreased their specific activity in the presence of oxythiamine but only during the beginning of the cultivation. However, after 12 h of cultivation, oxythiamine-treated cells showed higher specific activity of cytosolic enzymes. Moreover, it was established by SDS-PAGE that the high specific activity of pyruvate decarboxylase was followed by an increase in the amount of the enzyme protein. In contrast, the mitochondrial enzymes, pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes, were inhibited by oxythiamine during the entire experiment. Our results suggest that the observed strong decrease in growth rate and viability of yeast on medium with oxythiamine may be due to stronger inhibition of mitochondrial pyruvate dehydrogenase than of cytosolic enzymes.

Key words: pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, transketolase, pyruvate decarboxylase, activity, oxythiamine, inhibition.

Résumé : L'oxythiamine est une antivitamin dérivée de la thiamine qui suite à sa phosphorylation en oxythiamine pyrophosphate peut se lier aux centres d'enzymes dépendantes de la thiamine. Dans l'étude présente, nous avons examiné l'impact de l'oxythiamine sur la viabilité des *Saccharomyces cerevisiae* et sur l'activité d'enzymes dépendantes du TPP chez ces levures. Nous avons observé une diminution de l'activité spécifique de la PDC autant avec un milieu témoin et qu'avec un milieu contenant de l'oxythiamine après les premières 6 h de culture. Les enzymes cytosoliques transcétolase et pyruvate décarboxylase ont vu leur activité spécifique diminuée en présence d'oxythiamine mais seulement lors du début de la culture. Toutefois, après 12 h de culture, les cellules traitées à l'oxythiamine ont démontré une activité spécifique supérieure pour les enzymes cytosoliques. De plus, il fut estimé par SDS-PAGE que l'activité spécifique élevée de la PDC était suivie par une augmentation de l'expression de l'enzyme. En revanche, les enzymes mitochondriales, les complexes pyruvate déshydrogénase et 2-oxoglutarate déshydrogénase, furent inhibées par l'oxythiamine tout au cours de l'expérience. Nos résultats indiquent que la diminution prononcée de la prolifération et de la viabilité des levures dans un milieu avec oxythiamine serait causée par une inhibition plus importante de la PDH mitochondriales que des enzymes cytosoliques.

Mots clés : pyruvate déshydrogénase, 2-oxoglutarate déshydrogénase, transcétolase, pyruvate décarboxylase, activité, oxythiamine, inhibition.

[Traduit par la Rédaction]

Introduction

Cell metabolism can be modified by many different factors including vitamins. One of the most important vitamins is thiamine, vitamin B₁ (Hohmann and Meacock 1998;

Sprenger and Pohl 1999). Thiamine is a precursor of thiamine pyrophosphate (TPP), which plays a role as a prosthetic group in the enzymes of many metabolic pathways (Kern et al. 1997). For example, TPP-dependent enzymes play a key role in energy metabolism both in alcoholic fermentation (pyruvate decarboxylase (PDC)) and in aerobic oxidation (pyruvate dehydrogenase (PDH) and 2-oxoglutarate dehydrogenase (OGDH)). On the other hand, TPP is essential in the pentose phosphate pathway as a coenzyme of transketolase (TK) (Hübner et al. 1998). Sufficient TK activity is linked with the synthesis of nucleic acids because it provides an additional dose of pentoses (Shenk et al. 1998; Raïs et al. 1999).

The coenzymes and anticonzyme derivatives of vitamins can increase or decrease enzyme activity after binding in

Received 7 March 2005. Revision received 20 June 2005. Accepted 24 June 2005. Published on the NRC Research Press Web site at <http://cjn.nrc.ca> on 29 October 2005.

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their active centres (Schellenberger 1998). Oxythiamine is an antivitamin derivative of thiamine that after phosphorylation shows a high affinity to thiamine-dependent enzymes and decreases their activity, as was confirmed during an investigation of TK activity in rat adrenal glands (Strumilo et al. 1984). A strong decrease in specific activity was observed during an in vitro study of TPP-dependent enzymes (PDH and OGDH) isolated from European bison heart after the addition of 4'-oxythiamine pyrophosphate to the reaction mixture (Strumilo et al. 1995).

In contrast with earlier obtained data concerning thiamine-dependent enzymes (Strumilo et al. 1984, 1995), our previous experiments carried out with yeast have revealed a strong increase in PDC activity after 3 days of cultivation on a medium containing oxythiamine (Tylicki et al. 2003). These results showed that on the basis of in vitro experiments, conclusions on the character of oxythiamine's action in living cells are sometimes contradictory. These effects have prompted us to attempt an explanation of oxythiamine's action in living cells. Recently, oxythiamine was found to be an inhibitor of the development of in vitro cultured tumor cells (Raïs et al. 1999). This finding opens a new path for further investigation into the influence of oxythiamine in cell metabolism.

Our previous results (Tylicki et al. 2003) excluded PDC as the main factor responsible for the decrease in viability of oxythiamine-treated yeast. Thus, we were forced to widen our investigation to include other thiamine-dependent enzymes to explain the toxic effect of oxythiamine. Taking into consideration the physiological role of TPP and its analogues and the usefulness of yeast in the pharmaceutical, chemical, and food industries (Schörken and Sprenger 1998; Randez-Gil et al. 1999; Turner 2000), any explanation of the action of vitamins and their analogues may be useful.

Materials and methods

Cultivation of yeast and sample collection

For testing the yeast's response to oxythiamine, two types of mineral culture media (according to Rodríguez-Navarro and Ramos (1984) supplemented with 3% glycerol and 2% peptone) were used. The first medium contained 40 mg oxythiamine/L. A second one, without oxythiamine, was used as a control. The media were inoculated by yeast (*Saccharomyces cerevisiae*) (strain S288c obtained from Institute of Biochemistry and Biophysics, Polish Academy of Science) to approximately 10^5 cells/mL that had earlier been cultivated for 24 h on a liquid yeast extract – peptone – D-glucose medium (Rose et al. 1990). Cultures on the control and oxythiamine-containing media were grown on a shaker (150 r/min) in Erlenmeyer flasks containing 250 mL of medium at 30 °C under sterile conditions. The cultures on both types of media were cultivated for 4 weeks.

Material for estimating the rate of growth was taken after 1, 2, 3, and 7 days of cultivation. The specific activity of enzymes was maintained in extracts made from cells taken after 3, 6, and 12 h and 3 days of cultivation. Cell extracts for electrophoresis were made from the 3-day-old culture. The viability of yeast was estimated after 1, 2, 3, and 4 weeks of cultivation. For thiamine and oxythiamine concentration measurements, material was harvested after 3, 6, 12, and

24 h of cultivation. During our investigations, we did three independent experiments, each one with three replications using the control and oxythiamine-containing media.

Rate of growth and viability of the cells

The number of cells in the cultures was counted with a Bürker chamber using an Olympus BX 41 light microscope. The percentage of nonviable cells in the cultures (among 1000 cells) was estimated by means of fluorescein diacetate (Huang et al. 1986) using an Olympus BX41 fluorescent microscope.

Enzyme specific activity

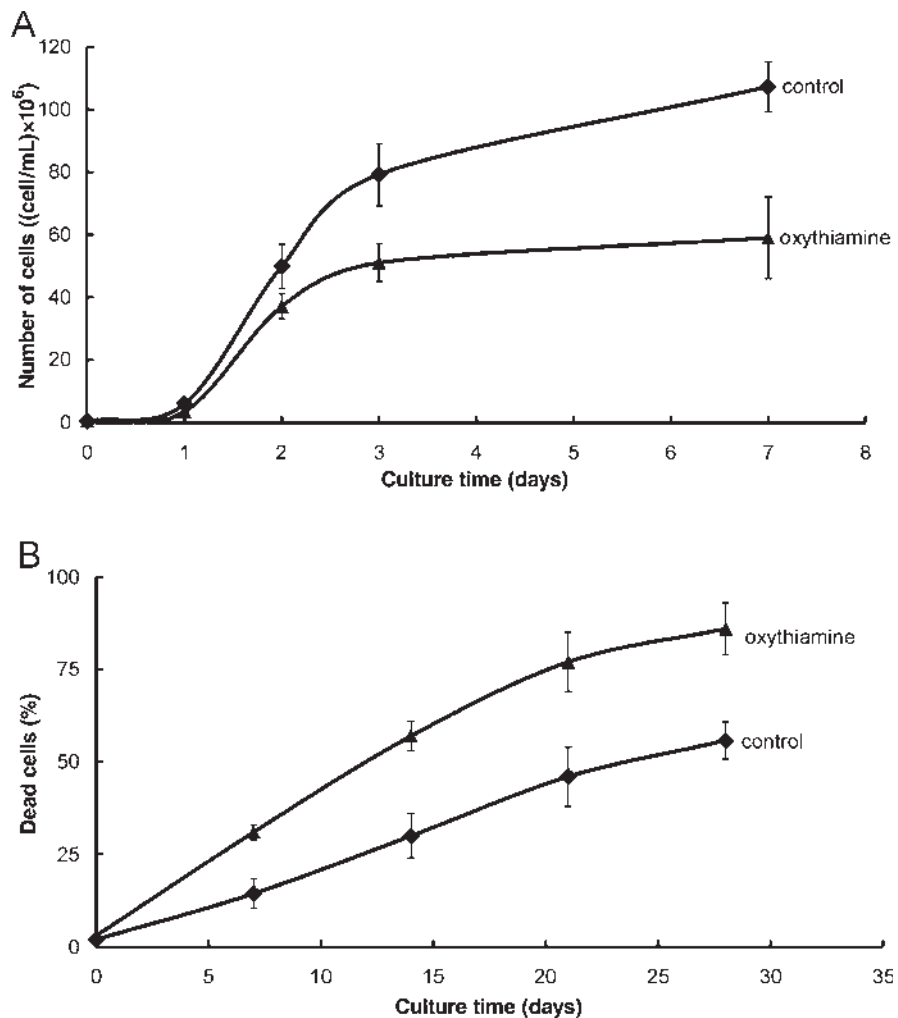
The yeast cells were harvested and homogenized at 4 °C by hand shaking with glass beads (diameter 0.2–0.3 mm) for 10 min in 0.01 mol Tris-HCl/L (pH 6.7) with 650 mmol sorbitol/L, 0.1% BSA, and 0.1 mmol EDTA/L (Guërin et al. 1979). The ratio of glass beads to fresh mass of the yeast and to the buffer was 1:1:1. For separation of the mitochondrial and cytosolic fractions, the homogenates were centrifuged for 10 min at 900g and subsequently, the supernatants were centrifuged for 10 min at 11 000g. Activity of the cytosolic TPP-dependent enzymes (PDC and TK) has been determined in supernatant fractions according to Sieber et al. (1983) and Bruns et al. (1958), respectively. Pellets of the mitochondria were dissolved in a 0.05 mol phosphate/L buffer (pH 7.8) with 0.2% Triton X-100. They were then incubated for 30 min at 4 °C to disrupt the mitochondrial membranes and centrifuged for 30 min at 15 000g. The specific activity of PDH and OGDH complexes was measured using a procedure described in Strumilo et al. (2002). All measurements were done using a Beckman DU 640 spectrophotometer. All measurements were reproduced three times in three independent experiments. The protein concentration was established by the Lowry et al. (1951) method for calculation of the specific activity of the enzymes mentioned.

Thiamine and oxythiamine concentration

Thiamine and oxythiamine contents of the yeast cytosolic fractions were estimated by the HPLC method (Tallaksen et al. 1997). The cytosolic fractions were obtained by the method described in the "Enzyme specific activity" section. De-proteinization and extraction of thiamine were done by addition of trichloroacetic acid to yeast extracts to a final concentration of 10%. After mixing, incubation for 30 min, neutralization, and centrifugation, the aliquots were separated with a Beckman HPLC system Gold 125 with a Supelcosil LC-NH2 column (250 mm × 4.6 mm). A UV-VIS Detector-166 (Beckman) was used for monitoring absorbance at 272 nm. Beckman Gold 8.1 software was used for system control and data acquisition.

Calculations

Data for statistical evaluation were taken from nine independent measurements. In the case of all collected results (number of cells in the cultures, their viability, enzyme activity, and thiamine and oxythiamine concentration in cell extracts from the oxythiamine-containing medium), the averages and standard deviations are presented in the figures. The averages taken from control and oxythiamine-containing

Fig. 1. Increase in yeast cell (A) number and (B) viability on control and oxythiamine-containing media.

cultures were compared for differences using Student's *t* test. Compared data were normally distributed (Shapiro–Wilk *W* test) and the variances were homoscedastic (Levene *L* test). The *p* values are given in the Results.

Electrophoresis

SDS–PAGE was based on the Laemmli buffer system (Laemmli 1970) with an SE 250 Mini-Vertical Unit (Amersham Biosciences). The separating acrylamide gel concentration was 12%, while that in the stacking gel was 6% with the cross-linking agent 0.1% bisacrylamide. Cells were homogenized and the supernatants were prepared according to the methods previously described in the “Enzyme specific activity” section. Protein concentrations in the supernatants were established according to Lowry et al. (1951). The supernatant fractions were mixed (ratio 1:1) with 2× concentrated sample buffer, Laemmli electrophoresis reagent (Sigma-Aldrich). Samples were boiled in a water bath for 3 min. The volume of samples from the control and oxythiamine-containing cultures was loaded onto a gel containing the same amount of total protein (80 μg). Additionally, the commercial PDC isolated from yeast (Sigma-Aldrich product No. P9474) and 10 μL of Rainbow full-range molecular weight markers (Amersham-Biosciences) were loaded onto the gel. After

electrophoresis (at 15 mA/gel for 90 min with cooling to 4 °C), the protein bands were detected with Coomassie brilliant blue R (Sigma-Aldrich) staining. Densitometry analysis was done using Quantity One Quantification Software (BIO-RAD).

Results

The yeast cultures exhibit a sigmoid type of growth under both examined culture conditions. The lag phase of growth took place for the first 24 h of yeast cultivation and then the exponential phase of growth started in the control as well as the oxythiamine-containing culture and went on until the third day. The oxythiamine-containing culture, in contrast with the control, achieved a stationary state of growth after 3 days (Fig. 1A). The number of cells in the medium with oxythiamine after 7 days of culture was two times lower than in the control (*p* < 0.05) (Fig. 1A). During all of the time of the experiment, cells grown on the medium with oxythiamine showed lower viability in comparison with the control culture (*p* < 0.05) (Fig. 1B).

All investigated TPP-dependent enzymes showed a decrease in their specific activity in cells cultured on the medium with oxythiamine in comparison with the control

Fig. 2. Changes in activity of cytosolic thiamine pyrophosphate (TPP)-dependent enzymes (A) pyruvate decarboxylase (PDC) and (B) transketolase (TK) during 3 days of cultivation on control and oxythiamine-containing media.

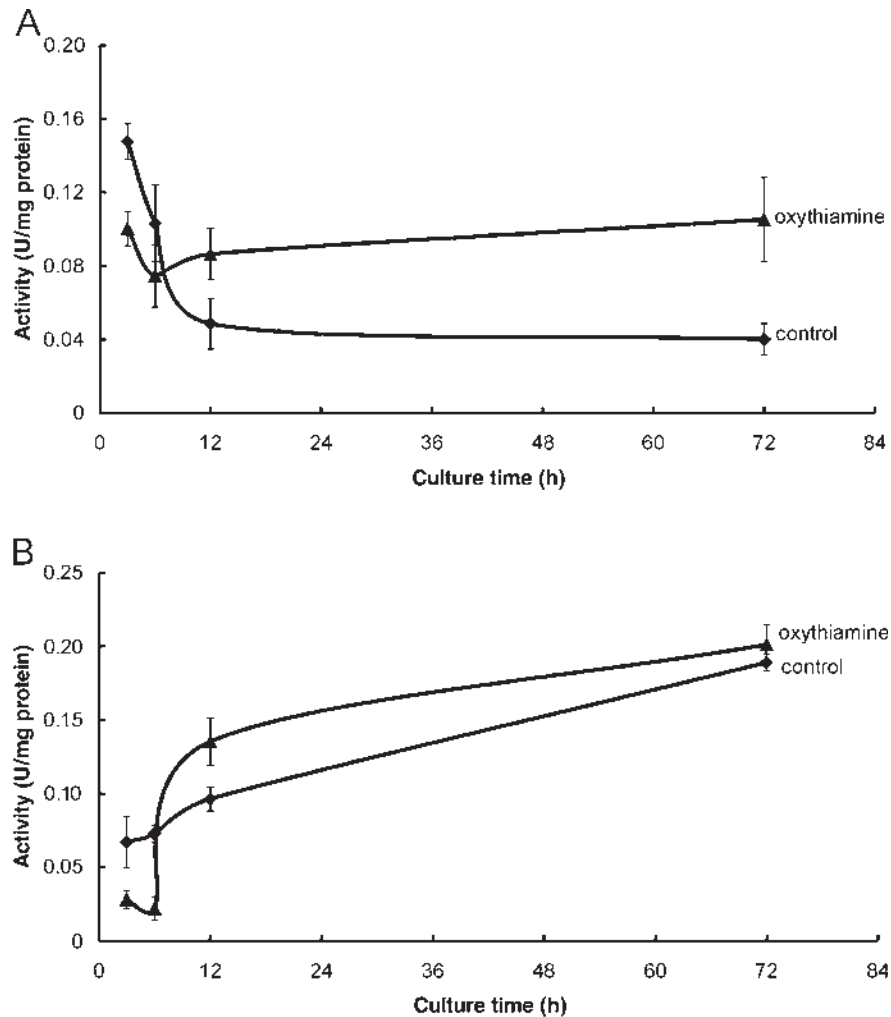


Table 1. Comparison of enzyme specific activity during 72 h of experiment.

Culture time (h)	Enzyme specific activity (% of control)			
	Cytosolic enzymes		Mitochondrial enzymes	
	PDC	TK	PDH complex	OGDH complex
3	68	30.3	77.1	38.9
12	177.6	140.2	40.9	12.9
72	260.9	103.8	49.4	77.3

Note: PDC, pyruvate decarboxylase; TK, transketolase; PDH, pyruvate dehydrogenase; OGDH, 2-oxoglutarate dehydrogenase.

during the first 3 h of cultivation (Figs. 2 and 3). TK and OGDH were the most sensitive to oxythiamine among all tested enzymes after 3 h of cultivation (Table 1). PDC (Fig. 2A), TK (Fig. 2B), and OGDH (Fig. 3A) indicate a statistically significant decrease in their specific activity ($p < 0.01$) in comparison with the control after 3 h of the experiment. In the culture with oxythiamine, we noted a strong,

statistically significant ($p < 0.01$) increase in the specific activity of cytosolic enzymes (TK and PDC) (Fig. 2) and a decrease in the specific activity of mitochondrial enzymes (Fig. 3) after a further 12 h of cultivation in comparison with the control. At the same time, in the case of oxythiamine-treated cells, we found a strong decrease in thiamine concentrations in the cytosolic fraction in comparison with thiamine concentrations after 6 h ($p < 0.05$), which corresponds to the increased import of oxythiamine into the cells (Fig. 4).

The statistically significant differences in enzyme specific activity disappeared in the case of TK (Fig. 2B) and OGDH (Fig. 3A) after 72 h of culture. At the same time, PDH still exhibited a statistically significant ($p < 0.05$) decrease in specific activity (Fig. 3B) in contrast with PDC (Fig. 2A), which showed a significant ($p < 0.05$) increase in its specific activity in the oxythiamine-containing culture compared with the control.

SDS-PAGE shows that the increased amount of the protein corresponded to purified commercial yeast PDC taken from Sigma-Aldrich in extracts from yeast cultured on the medium with oxythiamine in comparison with the control

Fig. 3. Changes in activity of mitochondrial thiamine pyrophosphate (TPP)-dependent enzymes (A) 2-oxoglutarate dehydrogenase (OGDC) and (B) pyruvate dehydrogenase (PDH) during 3 days of cultivation on control and oxythiamine-containing media.

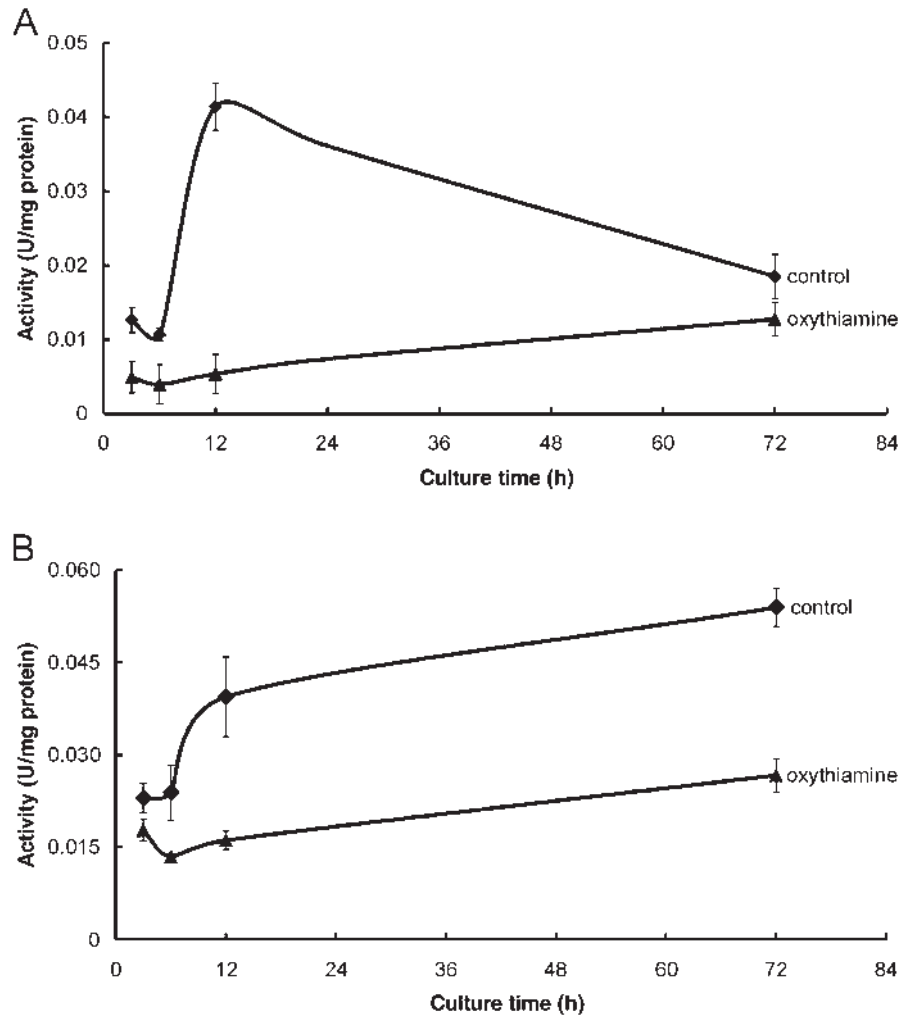
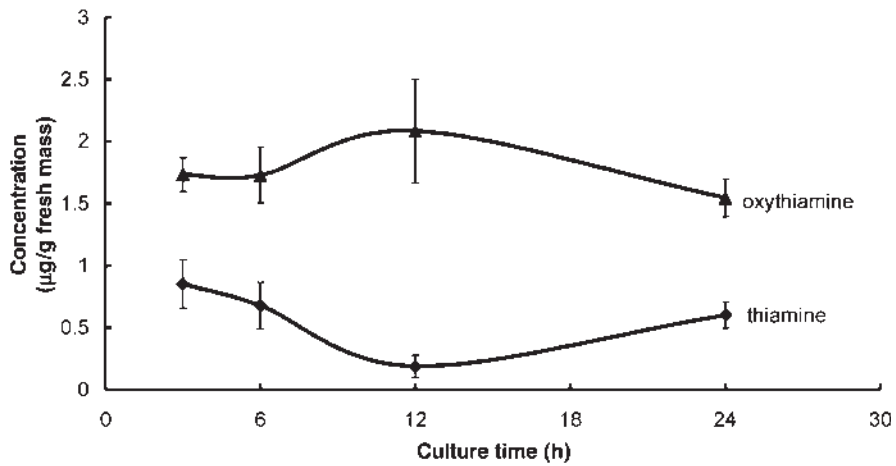


Fig. 4. Concentration of thiamine and oxythiamine in cytosolic fraction of yeast cultivated on medium with oxythiamine.



(Fig. 5A). Densitometry analysis revealed two times more optical density of the ~60-kDa band in yeast cultivated on the medium with the antivitamin (Fig. 5B).

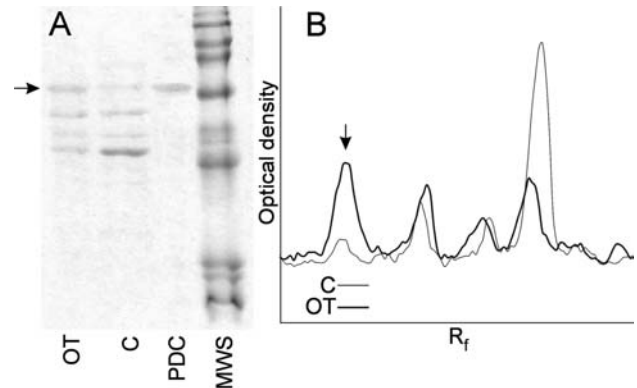
Discussion

In the present work, we indicate the toxic effect of oxythiamine on yeast, which is demonstrated by a decrease in the number of cells and their viability. We are trying to explain this effect at the level of TPP-dependent enzyme activity. Our previous results indicated an increase in PDC specific activity in the presence of oxythiamine in a culture medium (Tylicki et al. 2003). That unexpected effect prompted us to make the hypothesis that oxythiamine causes an accumulation of pyruvate in the early stages of a culture, which was experimentally confirmed in our previous article (Tylicki et al. 2003). An increase in pyruvate concentrations stimulates the synthesis of an additional dose of PDC apoform. That effect is responsible for the increase in total enzyme activity.

In the present work, we observed the same situation under nonfermentable conditions, namely in a culture on a nonfermentative carbon source. Moreover, by electrophoresis and densitometry analysis, we have confirmed the increase in PDC protein synthesis in yeast grown on a medium with oxythiamine. The increased amount of protein corresponding to purified commercial PDC is consistent with our previous hypothesis that an accumulation of pyruvate leads to growth synthesis of the PDC apoform. We also established a decrease in all tested TPP-dependent enzymes activity at the early phase of yeast cultivation on a medium with oxythiamine in comparison with the control. This effect explains the accumulation of pyruvate presented in our previous article (Tylicki et al. 2003). The decrease in the PDC specific activity observed in both examined cultures during the first 6 h is probably caused by the change of carbon source. Inoculum was maintained on the medium with fermentable glucose, but both investigated cultures were grown on a medium with nonfermentable glycerol. Simultaneously for this reason, increased specific activity of mitochondrial enzymes (especially OGDH) was observed after 6 h of culture.

The decreased rate of growth and lower viability of the yeast cells observed on the medium with oxythiamine seem to be a result of the inhibition of mitochondrial thiamine-dependent enzymes. Our results show that PDH is mainly responsible for this effect because this enzyme exhibits a strong, continuous decrease in its specific activity. PDH plays a key role in aerobic metabolism (König 1998), especially during growth on glycerol as a single nonfermentative carbon source (Lagunas 1986; Pronk et al. 1996). It is known that PDH is regulated by phosphorylation and TPP decreases the specific PDH kinase activity (Walsh et al. 1976; Czygier and Strumilo 1995). Even if the oxythiamine action (after its phosphorylation) is the same, an increase in PDH activity could be expected. However, the reverse effect was observed. Therefore, we suggest that oxythiamine does not affect the PDH activity by phosphorylation but modifies PDH activity as an anticonzyme molecule that blocks the active sites of TPP-dependent enzymes (Wittorf and Gubler 1971; Strumilo et al. 1995). Our data reveal that OGDH is

Fig. 5. (A) SDS-PAGE of extracts from yeast cells cultivated on control (C) and oxythiamine-containing media (OT) (80 µg of total protein was loaded) and purified pyruvate decarboxylase (PDC) and Rainbow molecular weight standards (MWS). (B) Densitometric analysis of two lanes (C and OT) presented in Fig. 5A. PDC is commercial purified yeast from Sigma-Aldrich. In Fig. 5A, bands of interest are indicated by arrow.



not as sensitive to oxythiamine as PDH. In spite of the inhibition of its activity at the beginning phase of cultivation, the difference in specific activity between the control and oxythiamine cultures had disappeared by the end of the experiment. Even the higher activity of PDC in cells cultivated on an oxythiamine-containing medium is not able to compensate for the inhibition of mitochondrial enzymes owing to the nonfermentative carbon source. TK seems to be most resistant to inhibition by oxythiamine. This property may be explained by its strongest binding of TPP among all of the investigated enzymes (Muller et al. 1993).

Rais et al. (1999) indicated that oxythiamine inhibits growth of Ehrlich's tumor cells. The authors suggested that the mechanism of inhibition is correlated with the decrease in TK activity by oxythiamine and therefore, DNA synthesis is not sufficient because of the decreased amount of pentoses, which are necessary for nucleic acid synthesis. In light of our results, yeast TK is not very sensitive to oxythiamine. Moreover TK, excluding the first hours of the experiment, indicated an even higher mean level of specific activity in an oxythiamine-containing culture. Therefore, we suggest that in the case of yeast, the decreased activity of PDH, which is very important in energy metabolism (Patel and Roche 1990), may be responsible for the inhibition of cell growth even more than inhibition of TK. This situation may suggest different mechanisms of oxythiamine action on unicellular and higher organisms.

Oxythiamine is phosphorylated in the same way as thiamine by specific kinase, and it is transported into the mitochondria (Rindi et al. 1963; Kozik 1996). On the other hand, some recent publications suggest that oxythiamine can disturb thiamine transport in cells (Said et al. 2002; Dudeja et al. 2003). In our experiment, we found a statistically significant decrease in thiamine concentration in the cytosol fraction of cells cultivated on a medium with oxythiamine after the first 12 h of cultivation when compared with only the first 6 h of cultivation. This corresponds to an increase in oxythiamine concentrations. Taking into consideration that thiamine is not the exclusive exogenous vitamin for yeast

cells, this effect can suggest that the uptake of this vitamin (in our case, an antivitamin derivative) from the medium is more favourable than synthesis. It suggests also that the yeast thiamine carrier is not able to distinguish between thiamine proper and oxythiamine. Our results indicate a more distinct response of mitochondrial enzymes. It may suggest a more intensive process of thiamine phosphate derivative (including oxythiamin pyrophosphate) transport from the cytosol to the mitochondrion as an answer to toxic oxythiamine action.

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