Effect of Thiamin-induced Vitamin B₆ Deficiency on NAD- and NADP-linked Glutamate Dehydrogenases in Yeast

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Thiamin-grown cells of Saccharomyces carlsbergensis exhibited decreased activity of NAD-linked glutamate dehydrogenase (NAD-GDH) and, in contrast, increased activity of NADP-linked glutamate dehydrogenase (NADP-GDH), as compared with control cells. Normal levels of glutamate dehydrogenase (GDH) activities were restored by adding pyridoxine. δ-Aminolaevulinate also prevented the thiamin-induced decrease in NAD-GDH activity, but had no effect on the increase in NADP-GDH activity. NAD-GDH activity was decreased similarly under anaerobic conditions, but NADP-GDH activity was little affected. High concentrations of glucose brought about a decrease in NAD-GDH activity and an increase in NADP-GDH activity, as observed in the thiamin-grown cells. When glycerol was used as carbon source in place of glucose, the thiamin effect on GDH activities was not marked. These results suggest that GDH enzymes are independently controlled by thiamin. NAD-GDH activity is decreased mainly through the thiamin-induced respiratory deficiency, and the increase in NADP-GDH activity is due to the thiamin-enhanced glucose effect. Amino acid metabolism seemed not to be involved in the thiamin effect on GDH activities since the intracellular pools of NH₄⁺ and glutamate were not altered by thiamin. The activities of glutamate-oxaloacetate transaminase and glutamate-pyruvate transaminase were not influenced greatly by thiamin, unlike the activity of δ-aminolaevulinate synthase.

INTRODUCTION

Thiamin added to a vitamin B₆-free medium causes severe reduction of respiratory activity and depression of aerobic growth of Saccharomyces yeasts (Nakamura et al., 1974, 1981, 1982). The thiamin-induced respiratory deficiency is due to the block of haem biosynthesis at the δ-aminolaevulinate synthase-catalysing step (Nakamura et al., 1981). The decrease in the enzyme activity is caused by vitamin B₆ deficiency which is thought to be the primary event in the thiamin effect. The thiamin-grown cells have a markedly altered lipid composition (Nishikawa et al., 1974, 1978), probably due to the haem deficiency. Stimulation of glycolysis and ethanol fermentation has also been observed in the thiamin-grown respiratory deficient cells (Kamihara et al., 1981).

It is reasonable to suppose that the metabolism of amino acids is also influenced by respiratory deficiency and/or vitamin B₆ deficiency. Glutamate dehydrogenase (GDH), which occupies a strategic position in an important branch point between nitrogen and carbon metabolism, is known to be metabolically regulated according to the environmental conditions such as glucose concentration, extent of aeration, and nitrogen source (De Castro et al., 1970, 1974; Thomulka &
Moat, 1972; Roon & Even, 1973; Marzluf, 1981). NAD-linked GDH (NAD-GDH) catalyses the dehydrogenation of glutamate to 2-oxoglutarate under aerobic conditions and is repressed by glucose and NH₄⁺. NADP-linked GDH (NADP-GDH), in contrast, catalyses the reduction of 2-oxoglutarate to glutamate in the presence of a relatively high concentration of glucose. Under conditions affecting the levels of the two GDH enzymes, their formation is regulated in a reciprocal manner (see above for references). One of the most interesting problems concerning GDH regulation is whether there is any direct connection between the synthesis of these enzymes.

In this paper, we describe changes in the activities of the two GDH enzymes in yeast cells growing under the influence of thiamin. The purpose of this study was, on the one hand, to investigate the cause of the thiamin-induced changes, and, on the other hand, to clarify whether some close relationship actually exists in the cellular activities of GDH enzymes. Intracellular pools of amino acids and NH₄⁺ were estimated in relation to GDH activities. Cellular activities of glutamate-oxaloacetate transaminase (GOT) and glutamate-pyruvate transaminase (GPT) were also determined since these typical pyridoxal phosphate-linked enzymes are involved in governing the size of the glutamate pool and their activities may be influenced by the thiamin-induced vitamin B₆ deficiency.

**METHODS**

**Growth of yeast.** *Saccharomyces carlsbergensis* 4228 (ATCC 9080) was employed throughout these experiments. Aerobic cultivation of the yeast cells was carried out at 30 °C in a synthetic medium as described in the previous report (Nakamura et al., 1980). The medium contained 0-5 to 10% (w/v) glucose or 2% (w/v) glycerol, and 0-4% (w/v) Casamino acids as carbon and nitrogen sources, respectively. Anaerobic cultivation was carried out under helium in a complex medium of Lindegren (Lindegren et al., 1958) which contained 0-3% glucose and 0-15%(w/v) (NH₄)₂SO₄ as carbon and nitrogen sources, respectively, and was supplemented with 0-002%(w/v) ergosterol and 0-25%(w/v) Tween 80. Cells grown to the stationary phase in each medium were washed three times with ice-cold water and inoculated into freshly prepared medium at 1 μg dry cells ml⁻¹ and cultivated as above. Thiamin. HCl, pyridoxine. HCl and δ-aminolaevulinic acid. HCl were added, when indicated, to the synthetic medium at final concentrations of 1 μg ml⁻¹, 20 ng ml⁻¹ and 84 μg ml⁻¹, respectively. Cells grown with and without added thiamin were termed thiamin-grown cells and control cells, respectively.

**Preparation of cell-free extracts.** Cells were harvested at various growth phases, since cellular activities of enzymes tested changed markedly during cultivation. After being washed three times with ice-cold deionized water, the cells (100 mg dry wt) were disrupted by sonication (20 kc) for 10 min at 0 °C in 10 ml 0-1 M-potassium phosphate buffer (pH 7-5). The resulting homogenate was centrifuged for 20 min at 10000 g and the supernatant dialysed for 6 h against 0-02 M-potassium phosphate buffer (pH 7-5) and used for enzyme assay. For the assay of GOT (EC 2.6.1.1) and GPT (EC 2.6.1.2), the supernatant was used without dialysis to measure the holo- and apoenzyme activities.

**Enzyme assay.** The activities of NAD-GDH (EC 1.4.1.2) and NADP-GDH (EC 1.4.1.4) were determined by measuring spectrophotometrically at 340 nm the rate of NADH oxidation and NADP reduction, respectively. The reaction mixture for NAD-GDH assay contained 100 μmol potassium phosphate buffer (pH 7-7), 100 μmol 2-oxoglutarate, 3 mmol NH₄Cl, 0-9 μmol NADH and the dialysed supernatant, in a final volume of 3 ml. For NADP-GDH assay, 36 μmol sodium glutamate and 1-3 μmol NADP were used instead of 2-oxoglutarate, NH₄Cl and NADH. The reaction was initiated upon addition of the enzyme solution after preincubation of the reaction mixture at 30 °C for 3 min. Values obtained without substrate were subtracted from those with substrate. GOT and GPT were assayed as described by Bergmeyer & Bernt (1974a, b) with slight modification. For GOT assay, the rate of oxaloacetate formation from 2-oxoglutarate and aspartate was determined by measuring the rate of NADH consumption in the presence of excess malate dehydrogenase. Lactate dehydrogenase was also added to the reaction mixture for complete removal of pyruvate present in the sample. GPT was assayed using 2-oxoglutarate and alanine as substrates in a similar way by coupling with lactate dehydrogenase. The rate of NADH consumption due to pyruvate formed in the GPT reaction was measured. One unit of enzyme activity was defined as 1 nmol of substrate utilized min⁻¹. Specific activity was expressed as units (mg protein)⁻¹. Protein was determined by the Lowry method.

**Determination of intracellular pool of amino acids.** Cells in the exponential growth phase were rapidly collected on a membrane filter. Amino acids and NH₄⁺ were extracted from the cells by boiling in water for 10 min (Moat et al., 1969) and determined with a Hitachi KLA-313 amino acid analyser by the method of Moore & Stein (1963). For GPT assay, the rate of oxaloacetate formation from 2-oxoglutarate and aspartate was determined by measuring the rate of NADH consumption in the presence of excess malate dehydrogenase. Lactate dehydrogenase was also added to the reaction mixture for complete removal of pyruvate present in the sample. GPT was assayed using 2-oxoglutarate and alanine as substrates in a similar way by coupling with lactate dehydrogenase. The rate of NADH consumption due to pyruvate formed in the GPT reaction was measured. One unit of enzyme activity was defined as 1 nmol of substrate utilized min⁻¹. Specific activity was expressed as units (mg protein)⁻¹. Protein was determined by the Lowry method.

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Regulation of yeast glutamate dehydrogenase

![Graph showing the effect of thiamin on NAD-GDH and NADP-GDH activities in S. carlsbergensis.](image)

**Fig. 1.** Effect of thiamin on NAD-GDH and NADP-GDH activities in *S. carlsbergensis*. The yeast was grown aerobically in synthetic medium containing 5% glucose with and without added thiamin as described in Methods. Periodically harvested cells were disrupted and used for the assay of NAD-GDH and NADP-GDH activities after dialysis. NADP-GDH activity without thiamin (○), with thiamin (■); NAD-GDH activity without thiamin (▲), with thiamin (◆); growth without thiamin (□), with thiamin (■).

**Chemicals.** Vitamin-free Casamino acids, Bacto peptone and Bacto yeast extracts were obtained from Difco. Skeletal muscle lactate dehydrogenase and malate dehydrogenase from pig heart were purchased from Sigma and Boehringer, respectively, and δ-aminolaevulinic acid. HCl was from Sigma. All other chemicals used were those of analytical reagent grade or of the highest purity available commercially.

**RESULTS**

The results in Fig. 1 show the time course of NAD-GDH and NADP-GDH specific activities in cells growing aerobically in a synthetic medium containing 5% glucose with or without added thiamin (see Methods). A growth curve is also given for comparison. NAD-GDH activity was lower and NADP-GDH activity was markedly higher in the cells which were growing at a decreased rate with added thiamin as compared with those in the control cells. Both vitamin B₆ content and respiratory activity were substantially lower in the thiamin-grown cells, as reported previously (Nakamura et al., 1974, 1980, 1981). Addition of pyridoxine to the thiamin-supplemented culture at mid-exponential phase restored the growth rate and respiratory activity to normal levels (Nakamura et al., 1980). This also brought about an increase in NAD-GDH activity and, in contrast, a greater and more rapid decrease in NADP-GDH activity (Fig. 2).

These findings suggest a close correlation between the activities of respiration and GDH enzymes as pointed out by De Castro et al. (1970, 1974) in *Saccharomyces cerevisiae*. The results in Fig. 3 show the activities of the two GDH enzymes in *S. carlsbergensis* growing under anaerobic conditions in Lindegren's medium containing 0.3% glucose. The complex medium was used since cell growth under anaerobic conditions was extremely poor in the synthetic medium even in the presence of added Tween 80 and ergosterol. The activity of NAD-GDH was substantially lower under anaerobic conditions, while NADP-GDH was little affected by the extent of aeration.

The effect of glucose concentration on cellular activities of GDH enzymes was examined under aerobic conditions in the synthetic medium (Fig. 4). In the absence of added thiamin, NADP-GDH activity was increased and NAD-GDH activity was decreased with increasing concentrations of glucose (0.5 to 10%). The increase in NADP-GDH activity brought about by glucose was also observed in the thiamin-supplemented culture. The NADP-GDH activity was markedly higher at 10% glucose than that at 0.5% glucose, especially at early growth phase when most of the added glucose remained. The thiamin-induced decrease in NAD-GDH activity was not affected by the increase in glucose concentration. Furthermore, at a low concentration of glucose (0.5%), NADP-GDH activity was only slightly increased by thiamin, whereas a marked
Fig. 2. Effect of pyridoxine on GDH activities in yeast cells. Pyridoxine was added at the time indicated by the arrows during cultivation in synthetic medium containing 5% glucose and thiamin. Cells were harvested periodically and GDH activities were measured as described in Methods. (a) NADP-GDH activity without pyridoxine (○), with pyridoxine (●); NAD-GDH activity without pyridoxine (△), with pyridoxine (◇). (b) Growth without pyridoxine (○), with pyridoxine (●).

Fig. 3. Effect of anaerobic cultivation on GDH activities in yeast cells. Cells were grown aerobically and anaerobically in Lindgren's medium containing 0.3% glucose. GDH enzymes were assayed as described in Methods. (a) NADP-GDH activity in aerobic culture (○), in anaerobic culture (●); NAD-GDH activity in aerobic culture (△), in anaerobic culture (◇). (b) Growth in aerobic culture (○), in anaerobic culture (●).

Fig. 4. Effect of glucose on GDH activities in yeast cells. Cells were grown aerobically on 0.5 or 10% glucose in synthetic medium with or without added thiamin. (a) NADP-GDH activity in 10% glucose without thiamin (○—○), with thiamin (●—●); NADP-GDH activity in 0.5% glucose without thiamin (△—△), with thiamin (◇—◇); NAD-GDH activity in 10% glucose without thiamin (○—○), with thiamin (●—●); NAD-GDH activity in 0.5% glucose without thiamin (△—△), with thiamin (◇—◇). (b) Growth in 10%, glucose without thiamin (○), with thiamin (●); growth in 0.5% glucose without thiamin (△), with thiamin (◇).
Regulation of yeast glutamate dehydrogenase

Fig. 5. GDH activities in yeast cells grown aerobically on glycerol in synthetic medium with or without added thiamin. Glycerol (2%) was used as carbon source in place of glucose. (a) NADP-GDH activity without thiamin (○), with thiamin (●); NAD-GDH activity without thiamin (△), with thiamin (▲). (b) Growth and respiratory activity (QO₂): growth without thiamin (○), with thiamin (●); QO₂ without thiamin (△), QO₂ with thiamin (▲).

decrease in respiratory activity by thiamin was observed (as at a high concentration range of glucose) (data not shown). These findings indicate that both of the GDH enzymes are influenced by glucose and also suggest that the decrease in NAD-GDH activity in thiamin-grown cells was the result of the thiamin-induced respiratory deficiency and, in contrast, the marked increase in NADP-GDH activity was mainly due to the thiamin-enhanced effect of glucose. These indications were confirmed by the following experiments. When 2% glycerol was used as carbon source in place of glucose in the synthetic medium, NADP-GDH activity was somewhat decreased by added thiamin (Fig. 5), even though the respiratory activity was lowered. NAD-
Table 1. Effect of thiamin on the intracellular pools of amino acids and NH$_4^+$ in *S. carlsbergensis*

Yeast cells were cultivated in synthetic medium in the presence or absence of thiamin and harvested at exponential phase where the thiamin effect on GDH enzymes was most marked. Intracellular amino acids and NH$_4^+$ were extracted and estimated as described in Methods.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Control cells</th>
<th>Thiamin-grown cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartate</td>
<td>1-0</td>
<td>3-4</td>
</tr>
<tr>
<td>Threonine</td>
<td>4-8</td>
<td>7-0</td>
</tr>
<tr>
<td>Serine</td>
<td>3-0</td>
<td>3-0</td>
</tr>
<tr>
<td>Glutamate</td>
<td>28-1</td>
<td>36-7</td>
</tr>
<tr>
<td>Glycine</td>
<td>1-6</td>
<td>1-2</td>
</tr>
<tr>
<td>Alanine</td>
<td>10-3</td>
<td>0-9</td>
</tr>
<tr>
<td>Valine</td>
<td>0-8</td>
<td>2-6</td>
</tr>
<tr>
<td>Methionine</td>
<td>2-1</td>
<td>12-3</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0-6</td>
<td>0-7</td>
</tr>
<tr>
<td>Leucine</td>
<td>0-3</td>
<td>0-5</td>
</tr>
<tr>
<td>Lysine</td>
<td>23-3</td>
<td>34-8</td>
</tr>
<tr>
<td>Histidine</td>
<td>1-5</td>
<td>2-9</td>
</tr>
<tr>
<td>Arginine</td>
<td>5-4</td>
<td>15-0</td>
</tr>
<tr>
<td>NH$_4^+$</td>
<td>3-0</td>
<td>2-7</td>
</tr>
</tbody>
</table>

Table 2. Effect of thiamin on the activities of GPT and GOT in *S. carlsbergensis*

Cells were grown aerobically in synthetic medium containing 5% glucose with or without added thiamin and harvested at early growth phase where the difference of NADP-GDH activities between the control cells and the thiamin-grown cells was explicitly observed (Fig. 1). GPT and GOT were assayed as described in Methods.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Cell growth (mg ml$^{-1}$)</th>
<th>GPT</th>
<th></th>
<th></th>
<th>GOT</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total Holoenzyme*</td>
<td>Total Holoenzyme*</td>
<td>Total Holoenzyme*</td>
<td>Total Holoenzyme*</td>
<td>Total Holoenzyme*</td>
</tr>
<tr>
<td>Control cells</td>
<td>1-19</td>
<td>29-4</td>
<td>167</td>
<td>0-18</td>
<td>131</td>
<td>185</td>
</tr>
<tr>
<td>Thiamin-grown cells</td>
<td>0-86</td>
<td>15-0</td>
<td>142</td>
<td>0-11</td>
<td>59-6</td>
<td>271</td>
</tr>
</tbody>
</table>

* Assayed without pyridoxal phosphate.
† Assayed with pyridoxal phosphate (27 μM).
‡ Ratio of specific activity of the holoenzyme to that of the total enzyme.

GDH activity was also slightly lowered, possibly due to the small decrease in respiratory activity. When δ-aminolaevulinate was added to the synthetic medium containing 5% glucose and thiamin, NAD-GDH activity was markedly increased to a level higher than that of the control cells. The increase in enzyme activity was accompanied by the restoration of respiratory activity (Nakamura *et al.*, 1981). However, the thiamin-induced increase in NADP-GDH activity was not influenced by the addition of δ-aminolaevulinate (Fig. 6).

There remained a possibility that the thiamin-induced vitamin B$_6$ deficiency exerted some effect on amino acid metabolism and that GDH enzymes were affected by a resulting alteration in amino acid pool in the cells. We examined the intracellular pools of some amino acids and NH$_4^+$ (Table 1), and the activities of typical vitamin B$_6$-dependent enzymes, GOT and GPT (Table 2). Thiamin exerted remarkable effects on the pools of methionine, arginine, aspartate,
valine, and alanine in the cells harvested in the exponential phase where the effects of thiamin on GDH enzymes were most marked. In contrast, the concentrations of glutamate and NH₄⁺, which are known to regulate directly the NAD-GDH and NADP-GDH activities in yeasts (Roon & Even, 1973; Thomulka & Moat, 1972), were little affected by thiamin. Holoenzyme activities of both GPT and GOT were reduced by thiamin to half of those in the control cells. The ratio of holoenzyme activity to total enzyme activity was also reduced by thiamin. This would reflect the vitamin B₆ deficiency caused by thiamin. However, the decreases in the holoenzyme activities were not marked compared with the decrease in δ-aminolaevulinate synthase activity (Nakamura et al., 1981).

**DISCUSSION**

Cells of *S. carlsbergensis* undergoing thiamin-induced respiratory deficiency exhibited a lowered activity of NAD-GDH and an elevated NADP-GDH activity. The increase in NADP-GDH activity was not due to the respiratory deficiency but to some effect of glucose which was enhanced by thiamin, while the decrease in NAD-GDH activity was due entirely to the lowered respiratory activity. This conclusion is based on the following observations. Firstly, anaerobic cultivation caused a decrease in NAD-GDH activity. On the other hand, there was little difference in NADP-GDH activity between aerobically and anaerobically grown cells. De Castro et al. (1970) also reported that NADP-GDH activity was increased under anaerobic conditions, but their data indicate that the increase was very slight. Secondly, the thiamin-induced increase in NADP-GDH activity was not affected by the addition of δ-aminolaevulinate which was found to eliminate the thiamin effect on NAD-GDH activity as well as that on respiratory activity (Nakamura et al., 1981). Finally, in glycerol medium, thiamin caused a decrease rather than an increase in the activity of NADP-GDH. NAD-GDH activity was also diminished as was respiratory activity, as observed in glucose medium.

In contrast to δ-aminolaevulinate, pyridoxine, which also eliminated the thiamin-induced respiratory deficiency (Nakamura et al., 1980), abolished completely the effect of thiamin on NADP-GDH as well as that on NAD-GDH. On the other hand, the thiamin-induced vitamin B₆ deficiency was found to have no effect on cellular pools of NH₄⁺ and glutamate which are known to regulate the activities of GDH enzymes. Therefore, the effect of pyridoxine, in giving a normal level of NADP-GDH activity, cannot be ascribed to an increased amino acid content. It would be very difficult to elucidate the mechanism of the pyridoxine effect on NADP-GDH activity since the glucose effect is involved in the regulation of this GDH enzyme as mentioned above.

The activities of the two GDH enzymes in yeast cells have invariably been reported to change in opposing directions under the environmental conditions employed, as if there is some mechanism which regulates these enzymes in a reciprocal manner. We have now shown that the two GDH enzymes can be controlled independently of each other. NAD-GDH is undoubtedly repressed also by glucose, but this may be caused by glucose-induced respiratory repression. It is evident that NAD-GDH activity is independent of respiratory activity under the conditions employed in this study. De Castro et al. (1974) reported that *S. cerevisiae* showed increased activity of NADP-GDH when cultivated in the presence of chloramphenicol or other antibiotics that inhibit mitochondrial protein synthesis, although only a very slight increase was observed in anaerobically-grown cells (De Castro et al., 1970), as described above. However, chloramphenicol had no effect on NADP-GDH activity in *S. carlsbergensis* and *S. cerevisiae* cells grown in the synthetic medium; moreover, a marked increase in NAD-GDH activity was observed in these cells (data not shown). These facts suggest that chloramphenicol has some subtle effect on GDH activities in yeast cells, which cannot be explained entirely by respiratory inhibition. It is evident that NADP-GDH is regulated mainly by glucose in the medium as judged from our data and other observations (Mazon, 1978; Mazon & Hemmings, 1979) which show the rapid degradation and biosynthesis of this enzyme under glucose starvation and refeeding, respectively.
REFERENCES


