THIAMINE-INDUCED REVERSIBLE DEFICIENCY IN RESPIRATORY ACTIVITY OF *SACCHAROMYCES CARLSBERGENSIS*: RESPIRATORY ADAPTATION CAUSED BY PYRIDOXINE

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1. Introduction

As reported in a previous paper [1] from this laboratory, the cells of *Saccharomyces carlsbergensis* 4228 (ATCC 9080), growing at a limited rate after a long lag period in the presence of thiamine and in the absence of pyridoxine, exhibited a markedly low respiration rate, a low activity of cytochrome oxidase and contained few cytochrome pigments. Concomitant addition of pyridoxine with thiamine to the growth medium prevented the growth inhibition and eliminated these phenomena caused by thiamine. Furthermore, immediate restoration of respiratory activity was observed upon the addition of pyridoxine in the course of cultivation with thiamine.

This communication deals with the restoration of the respiratory activity of the thiamine-grown cells by transferring the cells to a defined medium containing pyridoxine and incubating them aerobically without proliferation. Remarkable effects of pyridoxine on the increases in the activities of heme-containing enzymes, in addition to those of other respiratory enzymes, under the same conditions are also described.

2. Materials and methods

S. carlsbergensis 4228 was grown as described previously [1] in a modified Atkin's medium with added thiamine (1 μg/ml) and without pyridoxine. The thiamine-grown cells harvested in the mid log growth phase were washed twice with cold water and transferred to a medium containing 9.5 mM citric acid, 31 mM potassium citrate, 2% ethanol, 0.1% glucose, and 0.02% casamino acids. Incubation was carried out aerobically at 30°C with or without addition of pyridoxine (100 μg/ml). A heavy cell suspension (4–5 mg/ml) was used to render cell proliferation during the incubation as little as possible. Glucose and casamino acids were supplied to the medium every one hour during the incubation. The cells were collected by centrifugation and washed twice with cold water. The respiration of the cells was measured manometrically with a conventional Warburg apparatus. Protoplasts of the cells were prepared by using a lytic enzyme from *Arthrobacter luteus* according to the method of Kitamura et al. [2]. Cycloheximide (3 μg/ml) and chloramphenicol (2.5 mg/ml) were added to the cell suspensions together with the lytic enzyme in order to prevent adaptation of the cells to oxygen during the protoplast preparation. Cell-free extracts were prepared by disrupting washed protoplasts with 30 sec sonication, followed by centrifugation at 1000 *g* for 10 min. Low molecular weight substances were removed by passing the extracts through a Sephadex G-25 column. The activities of the following respiratory enzymes of the cell-free extracts were determined spectrophotometrically as mentioned by Perlman and Mahler [3]: cytochrome oxidase (EC 1.9.3.1), NADH oxidase, NADH–cytochrome *c* oxidoreductase (EC 1.6.2.1) and succinate–cytochrome *c* oxidoreductase (EC 1.3.99.1). Lactate dehydrogenase (EC 1.1.2.3) and catalase (EC 1.11.1.6) were assayed also spectrophoto-

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metrically according to the methods of Morton and Shepley [4] and Roggenkamp et al. [5], respectively.

3. Results

As shown in fig.1, when the thiamine-grown cells of *S. carlsbergensis* 4228 were transferred to a freshly prepared thiamine-free medium (see, Materials and methods) and incubated aerobically, the respiration rate of the cells increased linearly with time. Addition of pyridoxine to the medium brought about 3-fold increase in the respiratory development. Table 1 shows that casamino acids stimulated significantly the increase. However, the magnitude of the effect varied extensively from experiment to experiment, presumably depending on the pool size of amino acids in the cells tested. The stimulation caused by addition of casamino acids suggested that de novo syntheses of the respiratory enzymes are involved in this phenomenon. This possibility was confirmed by experiments

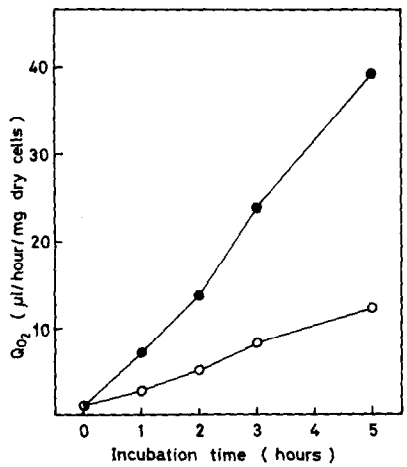

Fig.1. Increase in the respiration rate by pyridoxine in the course of incubation of the thiamine-grown cells of *S. carlsbergensis*. The cells which had been cultivated with thiamine to the middle logarithmic growth phase were collected, washed and transferred to the incubation medium (see text). The cell concentration in the medium was 4.3 mg dry cells/ml. Five-ml aliquots were withdrawn from the incubation mixture (50 ml) at indicated time, cooled rapidly in an ice-bath, and washed twice with cold water. The respiration rate of these cells was measured as described in Materials and methods. (-o——o-) control; (-●——●-) with added pyridoxine.

Table 1
Enhancing effect of casamino acids on the increase in the respiration rate of *S. carlsbergensis* 4228 during respiratory adaptation

Addition to the incubation medium[a]		$Q_{O_2}^{b}$
Casamino acids	Pyridoxine	(µl/h/mg cells)
–	–	17
	+	60
+	–	18
	+	110

[a] Incubation was carried out for 5 h under the same conditions described in Materials and methods except that casamino acids were omitted from the incubation medium when indicated.

[b] The Q_{O_2} values before incubation were subtracted.

with antibiotics. Both chloramphenicol and cycloheximide inhibited completely the increase in the respiration rate (table 2).

Involvement of pyridoxine in the respiratory adaptation was clearly demonstrated in table 3, Exp. 1. That is, the increases in the specific activities of cytochrome-linked respiratory enzymes during the incubation were markedly enhanced by pyridoxine. Thus, the enzyme levels were restored to those in normal cells which had been grown without thiamine and pyridoxine. The pyridoxine-dependent increases in the enzyme activities were inhibited by addition of chloramphenicol or cycloheximide to the incubation medium. Even in the absence of pyridoxine, small but obvious increases in the enzyme activities were observed. However, these increments were much less than those brought about by pyridoxine except for the case of succinate–cytochrome *c* oxidoreductase.

Pyridoxine caused marked increases in the activities of not only respiratory enzymes but also other heme-containing enzymes such as lactate dehydrogenase and catalase (table 3, Exp. 2). The activities of these enzymes were also low in the thiamine-grown cells of *S. carlsbergensis* 4228 as compared with those in the normal cells. The incubation of the thiamine-grown cells with pyridoxine for 6 h restored the enzyme levels to those in the normal cells. The increases in the activities of these enzymes were also inhibited by cycloheximide. In contrast to the case

Table 2
Effects of chloramphenicol and cycloheximide on the increase in the respiration rate of the thiamine-grown cells of *S. carlsbergensis* during the respiratory adaptation experiments with or without added pyridoxine

Experimental conditions	Additions	Q_{O_2} (μl/h/mg cells)
Before incubation	——	6.4
After incubation	None	23.7
	Chloramphenicol	13.9
	Cycloheximide	7.8
	Pyridoxine	109
	Pyridoxine plus chloramphenicol	15.0
	Pyridoxine plus cycloheximide	8.3

Incubation was carried out for 5 h under the same conditions as those in fig.1. Chloramphenicol and cycloheximide were added to the incubation medium in a final concentration of 3 mg/ml and 3 μg/ml, respectively.

Table 3
Increases in the activities of functional respiratory enzymes and other heme-containing enzymes during incubation with pyridoxine

Enzymes	Specific activities (nmoles/min/mg protein)[a]					
	Normal cells	Thiamine-grown cells				
		Before incubation	After incubation[b] with			
			None	PIN[b]	PIN plus CAP[b]	PIN plus CHI[b]
Experiment 1.						
Succinate–cytochrome *c* oxidoreductase	5.5	0.57	2.5	3.5	1.9	0.62
NADH–cytochrome *c* oxidoreductase	73	3.4	11	61	6.0	5.7
Cytochrome oxidase	35	1.8	6.3	28	0.2	1.2
NADH oxidase	30	5.9	6.4	29	4.3	5.8
Experiment 2.						
Lactate dehydrogenase	49	9.8	15	77	62	8.7
Catalase	7.4	0	0	5.6	8.8	0

[a] See text.
[b] The concentrations of CAP (chloramphenicol) and CHI (cycloheximide) were the same as those shown in table 1. Incubation was carried out for 6 h with or without added PIN (pyridoxine) (100 μg/ml).

of the respiratory enzymes, chloramphenicol did not affect the increases in the enzyme activities, indicating that the syntheses of these enzymes occur in the cytoplasmic system of protein synthesis as demonstrated by Pachecka et al. [6] and Kitsutani et al. [7] in the case of catalase.

Furthermore, the pyridoxine-independent increases in the activities were not observed in these enzymes.

4. Discussion

Previous reports of this series [1,8,9] have clarified the following interesting features in thiamine-grown cells of some yeasts such as *S. carlsbergensis* 4228: (a) low vitamin B_6 content, (b) low respiration rate, (c) low cytochrome content, (d) low levels of unsaturated fatty acids, (e) absence of ergosterol and zymosterol. Pyridoxine eliminates all of these abnormal phenomena. From these facts, we postulated tentatively a following mechanism on the action of thiamine: thiamine elicits the severe decrease in the vitamin B_6 content of the cells and the resulting vitamin B_6 deficiency causes a serious lowering in the activity of vitamin B_6 enzyme involved in heme biosynthesis, i.e. δ-aminolevulinate synthetase. The decreased activity of such enzyme(s) would result in a lowering of the contents of heme compounds and consequently a marked reduction of the levels of heme-containing enzymes including respiratory enzymes. Also, syntheses of unsaturated fatty acids and of zymosterol as well as ergosterol would be diminished by the lowering of heme contents, since cytochrome b_5 and P_{450} are known to be involved in fatty acids desaturation [10,11] and lanosterol demethylation [12], respectively.

The results presented here seem to be consistent with this supposition: the activities of the cytochrome-linked respiratory enzymes were increased by the incubation of the thiamine-grown cells with pyridoxine under non-growing conditions; and the activities of heme-containing enzymes other than the respiratory enzymes such as lactate dehydrogenase and catalase were also increased by the incubation with pyridoxine under the same conditions. Furthermore, we have observed that the addition of pyridoxine permits immediate restoration of the lipid profile of the thiamine-grown cells to that of normal cells in parallel to the increase in the activities of the above-mentioned enzymes (to be published elsewhere).

It is particularly noteworthy that de novo syntheses of proteins are involved in the enhancement of the enzyme activities. The results strongly suggest that the levels of the heme-containing enzymes can be regulated by the contents of hemes in the yeast cells as in the case of hemoglobin biosynthesis [13]. Otherwise, an alternative explanation would be possible; that is, apoproteins of the heme-containing enzymes would be present even in the thiamine-grown cells and certain enzymes participating in the syntheses of hemes would be newly synthesized during the incubation of the cells with pyridoxine.

An appreciable increase in the respiration rate of the thiamine-grown cells was observed even in the absence of pyridoxine during the adaptation experiment. The pyridoxine-independent increases occurred in the activities of respiratory enzymes but not in the activities of lactate dehydrogenase and catalase. This would be explained by derepression of glucose effect in the respiratory enzymes, because the cells grown on the modified Atkin's medium containing 5% glucose were washed and incubated in the adaptation medium containing a low glucose concentration (0.1%) as mentioned above. The syntheses of lactate dehydrogenase and catalase might be less sensitive to glucose repression.

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