EFFECT OF CHLORAMPHENICOL AND CYCLOHEXIMIDE ON THE SYNTHESIS OF NITRATE REDUCTASE AND NITRITE REDUCTASE IN RICE LEAVES

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SUMMARY

Synthesis of nitrite reductase in rice leaves was inhibited by both cycloheximide and chloramphenicol. This indicated a cooperative action of 70 S and 80 S ribosomes for its synthesis. Nitrate reductase, however, appeared to be exclusively synthesized on the cytoplasmic ribosomes.

INTRODUCTION

It has been reported that in leaves nitrate reductase (E.C.1.6.6.1) is localized in the cytoplasm and nitrite reductase in the chloroplasts (1,2). It has been demonstrated that reduced ferredoxin, generated during the light reactions of photosynthesis, donates electrons for the latter enzyme (3). However, evidence from differential centrifugation of leaf extracts shows that both the enzymes could be in the cytoplasm (4). The site of synthesis of these enzymes in leaves is also not known with certainty. Protein synthesis in the chloroplasts is known to take place on 70 S ribosomes and is specifically inhibited by chloramphenicol (5), while 80 S ribosomes are involved in the cytoplasmic protein synthesis. The latter process is sensitive to cycloheximide (6). On the basis of the differential chloramphenicol inhibition, it was deduced that the first enzyme is synthesized in the cytoplasm but the second is not (7,8). However, investigations with Lemna minor indicated that both these enzymes are synthesized on the cytoplasmic ribosomes (9). In this paper the effect of the two antibiotics on the induced synthesis of these enzymes has been reported. The results indicate that nitrate reductase is synthesized on the cytoplasmic ribosomes but for the formation of nitrite reductase, protein synthesis on both cytoplasmic and chloroplastic ribosomes might be necessary.

MATERIALS AND METHODS

Fifteen day old rice seedlings (variety Taichung Native I), grown in water, were treated with the antibiotic solutions as indicated and 4h later a modified Hoagland nutrient solution containing either nitrate or nitrite was added. After 16h of further growth in the presence of the inducers, the enzymes from the leaves were extracted and assayed as described earlier (8). A unit of nitrate reductase activity represents μmole NO_2^- formed/g tissue in 20 min while a unit of nitrite reductase activity denotes μmoles NO_2^- reduced/g tissue in 20 min.

RESULTS

Chloramphenicol at 1 mg per ml did not affect the formation of nitrate reductase while that of nitrite reductase was inhibited by about 25 per cent. Cycloheximide at different concentrations, however, inhibited the synthesis of both enzymes to the same extent (Table 1). As observed by Schrader et al. (7) also, the seedlings treated with a lower concentration of chloramphenicol (0.5 mg per ml) possessed a distinctly higher nitrate reductase activity than the control seedlings. It has recently been reported that chloramphenicol, which contains a nitro group, is capable of inducing nitrate reductase in rice seedlings but the enzyme is NADPH specific (10).

It is not yet well established whether nitrite reductase can be induced by nitrate ions directly or by nitrite ions formed by the activity of nitrate reductase. We observed that in the leaves of rice seedlings, nitrite reductase activity appeared 2h after that of nitrate reductase (unpublished data). This sequential synthesis probably implied that nitrite reductase was induced by nitrite rather than nitrate ions. If true, the effect of cycloheximide on the formation of nitrite reductase could have been indirect by affecting the endogenous level of nitrite in the tissues. Hence nitrite was used as an inducer, but even then the synthesis of both the enzymes was equally affected by cycloheximide (Table 2, experiment I). The inability of the seedlings to utilize nitrite due to a block in the synthesis of nitrite

Table 1. Effect of chloramphenicol and cycloheximide on the synthesis of enzymes

Inhibitor	Conc. of inhibitor	Activity as % of control	
		Nitrate reductase	Nitrite reductase
Control	-	100	100
Chloramphenicol (mg/ml)	0.5	133	100
	1.0	100	75
Cycloheximide (µg/ml)	3.0	87	83
	6.0	52	44
	12.0	19	18

Rice seedlings were raised in petri plates. On 14th day to each petri plate 25 ml of nitrate-less nutrient solution containing the specified concentrations of the inhibitors was added. After 4h, the solution was replaced by complete Hoagland solution having the requisite amount of the antibiotics. Activities of nitrate reductase and nitrite reductase in the extracts of the leaves of the seedlings were determined after 16 h. In control seedlings the activities of these enzymes were 3.26 and 11.60 units respectively.

Table 2. Effect of cycloheximide on the synthesis of the enzymes in the leaves of the seedlings provided with nitrite in the nutrient solution

µg cycloheximide per ml medium	Experiment I			Experiment II	
	Activity as % of control			Activity as % of control	
	Nitrate reductase	Nitrite reductase	µmoles NO_2^- per g tissue	Nitrate reductase	Nitrite reductase
Control	100	100	0.08	100	100
3	34	36	0.35	72	74
6	13	19	0.40	46	50
12	10	5	0.64	-	-

The experimental conditions were the same as described under Table 1 except that the enzymes were induced by providing the seedlings with Hoagland solution containing nitrite. In Experiment I, the induction medium contained 3mM NO_2^- and the enzyme activities and nitrite content of the leaf extracts were determined after 24 h. In Experiment II, nitrite was included in the nutrient solution at a concentration of 2mM and the enzymes activites were assayed 12 h after providing the induction medium.

Table 3. Synthesis of nitrite reductase in the leaves of rice seedlings in the presence of both chloramphenicol and cycloheximide.

Treatment	Nitrite reductase Units of activity	% inhibition
Control	15.66	-
Chloramphenicl (2mg/ml)	6.85	56.3
Cycloheximide (4 μg/ml)	11.66	25.6
Chloramphenicol + cycloheximide	7.00	55.4

The experimental conditions were the same as described under Table 1. One set of the seedlings was treated with both the antibiotics. Nitrite reductase activity in the extracts of the leaves was assayed 12 h after providing the seedlings with Hoagland solution containing nitrate.

reductase was evident from the large accumulation of nitrite in the tissues of the cycloheximide-treated seedlings. In the subsequent experiment (Table 2, experiment II) the concentration of nitrite in the medium and the period of induction were reduced in order to avoid toxic effects, if any, but still the magnitude of inhibition of synthesis of both the enzymes was about the same.

In order to see whether the two antibiotics inhibit one and the same enzyme, their combined effect was studied (Table 3). Cycloheximide did not increase the extent of inhibition obtained with chloramphenicol alone, showing thereby that different species of nitrite reductase are not involved.

One of the possible reasons for the observed inhibition of nitrite reductase synthesis by both the antibiotics could be that a part of the enzyme molecule might be formed in the cytoplasm and the other part in the chloroplast. Under such a situation, it is likely that the seedlings treated with chloramphenicol and cycloheximide would contain the components of the enzyme molecule which are synthesized in cytoplasm and chloroplasts respectively. Under optimum conditions these components of the enzyme could possibly be made to associate *in vitro* to form a functional enzyme. However, maceration of the leaves of

Table 4. Effect of mixing the extracts of the seedlings treated separately with chloramphenicol and cycloheximide on the activity of nitrite reductase.

Treatment	μmoles of NO_2 formed/ 0.3 ml extract in 20 min.
1. Control seedlings	1.12
2. Chloramphenicol-treated seedlings	0.55
3. Cycloheximide-treated seedlings	0.56
4. Tissues of treatments 2 and 3 macerated together	0.55
5. 0.15 ml of extracts from treatments 2 and 3 mixed and incubated at 20°C for 1 hr	0.56

15 days old rice seedlings were treated with 2 mg of chloramphenicol per ml or 6 μg of cycloheximide per ml of the medium, as indicated in the table, 4 h before providing them with complete Hoagland solution. 16 h later, 1 g of leaf material was extracted in 4 ml of phosphate buffer (0.1M, pH 7.5) containing 10^{-3}M cysteine. In case of treatment 4, 500 mg of leaf tissue from chloramphenicol and cycloheximide treated seedlings each was macerated together in 4 ml of the extraction buffer. For treatment 5, equal volumes of the extracts of the seedlings treated with these antibiotics were mixed and incubated at 20°C for 1 h or at 4°C for 24 h before assaying the enzyme activity.

the seedlings treated with chloramphenicol and cycloheximide together or mixing the leaf extracts prepared separately followed by an incubation period of 24 h at 4°C or 2 h at 20°C did not show any increase in the activity of nitrite reductase in these extracts (Table 4).

DISCUSSION

Inhibition of the synthesis of nitrite reductase by cycloheximide as well as by chloramphenicol indicates that protein synthesis on both 70 S and 80 S ribosomes might be necessary. It is conceivable that some protein(s) or subunits synthesized in the cytoplasm might be required for the formation of active nitrite reductase in the chloroplasts. Thus a cooperative protein synthesis on both 70 S and 80 S ribosomes might be involved. In the case of ribulose-1,

5-diphosphate carboxylase enzyme, it has been demonstrated that the lighter subunits are synthesized on the cytoplasmic ribosomes while the heavier subunits are formed on chloroplastic ribosomes (11). Failure to observe any enhancement in nitrite reductase activity after incubation of the mixed extracts of the seedlings treated with chloramphenicol and cycloheximide probably indicates that combined effect of 70 S and 80 S ribosomes in vivo is necessary for the synthesis of nitrite reductase.

In the scutellum or other nonchlorophyllous tissues of maize, the presence of two nitrite reductases has been demonstrated by using ion exchange chromatography (12). Although their properties are generally similar, they exhibit marked differences in their thermal stability, ionic charge and behaviour during isoelectric focussing. However, it is interesting to note that green tissues of the same plant contained only one species of nitrite reductase (12). Our results show that only one enzyme is synthesized by the cooperative functioning of cytoplasmic and chloroplastic ribosomes. In case chloramphenicol and cycloheximide individually inhibited formation of two different species of enzymes, being synthesized on 70 S and 80 S ribosomes respectively, then the combined effect of the two antibiotics would have been considerably higher than the effect of each one separately. However, this was not the case (Table 4).

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