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FURTHER STUDIES OF PRIMER-INDEPENDENT PHOSPHORYLASE ISOZYMES IN THE ALGAE

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Abstract—Both *Oscillatoria princeps* and *Cyanidium caldarium* contain phosphorylase isozymes that can cause the synthesis of polyglucan from glucose-1-phosphate in the absence of added maltodextrin 'primer' In addition, *O princeps* contains a primer-dependent phosphorylase isozyme When the phosphorylase fractions isolated from extracts of the algae were treated with α-amylase, the primer-independent isozyme became primer-dependent and shifted from the position it was normally found at after polyacrylamide gel electrophoresis This primer-independent isozyme became less mobile towards the anode, and was found at the locus usually occupied by the primer-dependent isozyme It was not possible to restore its mobility towards the anode and its primer-independent properties by preincubation with maltoheptaose The indication is that this isozyme is a glucoprotein and that the glucan component is chemically bonded to the protein

INTRODUCTION

THE BIOSYNTHESIS of storage polyglucans, since the discovery of the enzymes responsible for formation of α-1,4-glucosyl bonds (*phosphorylase*, E C 2 4 1 1, and *synthetase*, E C 2.4 1 11), and α-1,6-glucosyl branched linkages (*branching enzyme*, E C 2 4 1 18), has presented the unique problem of whether 'primer' molecules in the form of straight or branched maltodextrins are necessary in order to initiate polyglucan synthesis Although sporadic attention has been directed to this question in the past,[1,2] it is only recently that primer-independent phosphorylases have been reported in both higher plants[3,4] and in primitive plants [5,6] The *de novo* formation of polyglucoside by a bacterial synthetase has also been reported recently [7]

The question of the necessity for primer has been complicated by the binding or adsorption of polyglucans to both types of α-1,4-glucosyl bond-forming enzymes,[8,9] together with the inability of obtaining non-glucan contaminated phosphorylase protein [10] Another problem has centered around the endogenous glucan which may be present in supposedly pure preparations of the glucose-1-phosphate [11]

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A *glycoprotein* structure for the primer-independent phosphorylase had been proposed for a rabbit muscle enzyme,[1] and has now been suggested for a potato phosphorylase isozyme[4] and an algal isozyme [6] Such a molecular structure has been substantially established for extracellular yeast invertase where the bonding between the protein and carbohydrate moieties is thought to be via a glucosamine linkage [12]

The presence of both types of phosphorylase isozymes in the blue-green alga, *Oscillatoria princeps*,[6] and the presence of primer-independent isozyme alone in *Cyanidium caldarium*[5] presents an unusual opportunity for resolving the problem of the need for exogenous primer These isozymes are readily separated by polyacrylamide gel electrophoresis

RESULTS

Amido-black protein stain revealed two phosphorylase isozymes (a_1 and a_2) after electrophoresis of the untreated phosphorylase fraction from *O princeps* (Fig 1, 1A) When duplicate gels were incubated without the maltoheptaose primer, only the a_2 isozyme was detected after histochemical localization (Fig 1, 1B) Inclusion of the primer caused both bands to be visible after incubation (Fig 1, 1C)

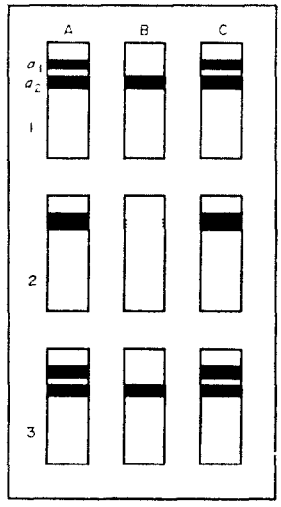

FIG 1 SEPARATION OF PHOSPHORYLASES FROM *Oscillatoria princeps* ON POLYACRYLAMIDE

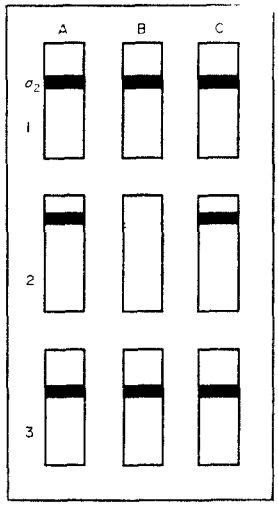

FIG 2 SEPARATION OF PHOSPHORYLASE FROM *Cyanidium caldarium*

Row 1 across Untreated enzyme mixture, a_1, primer-dependent isozyme, a_2, primer-independent isozyme *Row 2 across* Amylase treated before separation *Row 3 across* Preincubated with maltoheptaose before separation *Row A down* Amido-black protein stain *Row B down* Gomori stain after incubation in non-primer substrate mixture *Row C down* Same as B but with maltoheptaose added

After treatment with α-amylase and separation by polyacrylamide electrophoresis, only one protein-stained band at the a_1 site was detected (Fig 1, 2A) Incubation of the gel without the primer showed only a faint phosphorylase activity in this band (Fig 1, 2B) Incubation with primer added to the mixture, revealed intense phosphorylase activity in this same band (Fig 1, 2C)

[12] WAHEED, A and SHALL, S (1971) *Enzymologia* **41**, 291

Incubation of the fractionated phosphorylase mixture with 5% (w/v) maltoheptaose prior to electrophoresis, did not alter the pattern from that obtained with untreated enzyme (see Fig 1, rows 3 and 1)

The results with the fractionated material from *C caldarium* were similar (Fig 2) After treatment with amylase, there appeared to be a shift in the position of the lone a_2 isozyme from its normal position in the untreated enzyme pattern (see Fig 2, rows 2 and 1) It appeared that treatment with α-amylase caused this isozyme to be less mobile towards the anode during subsequent polyacrylamide gel electrophoresis

DISCUSSION

Amylase acts upon the a_2 phosphorylase isozyme to render it primer-dependent However, complete loss of the original primer-independent activity did not occur As can be seen in Fig 1 (2B), some of the activity was retained While these observations did not elucidate the nature of the bond existing between the protein and glucan moieties of the a_2 isozyme, it seemed highly unlikely that endogenous carbohydrate could have been carried along as a physically adsorbed entity on the protein under the conditions of electrophoresis used for the separation [13]

Treatment of the a_2 phosphorylase isozyme with amylase caused it to move more slowly towards the anode during electrophoresis In fact, its mobility was very much like that of the primer-dependent a_1 isozyme of *O princeps* As can be seen in Fig 1 (row 2 across), the area of the a_1 isozyme showed a thicker, more intense protein staining and enzymatically-active band *after* amylase treatment Indeed, there was very little enzyme-active material left in the area normally occupied by the a_2 isozyme (see Fig 1, 2A and 2C) after preincubation with amylase The activity that remained in the a_2 area may be indicative of the internal structure of the glucan component, since α-amylase is blocked by branched points in a polyglucan,[14] it is probable that the glucan moiety contained some α-1,6-glucosyl linkages The same observations were evident for the lone isozyme, a_2, of *C caldarium* (Fig 2, 2A, B and C)

Whatever bonding is involved, and the changes in mobility would suggest that there is a *chemical* bonding rather than a non-specific adsorption,[8] it cannot be re-established by preincubation of the amylase-treated a_2 isozyme with maltoheptaose prior to polyacryl amide gel electrophoresis Likewise, it was not possible to establish a bond between the a_1 isozyme of *O princeps* and maltoheptaose (see Fig 1, 3A, B and C), so that this isozyme would become primer-independent

The possibility is suggested that the bonding in the a_2 isozyme between the protein and glucan moieties might be the result of the action of an, as yet, unknown enzyme Recently, Cardini and his colleagues isolated a system capable of transferring glucosyl units from UDPG to a protein, with the formed glycoprotein then acting as glucose acceptor from glucose-1-phosphate or from ADPG [15]

Disruption of the glucosamine bonding between the protein and carbohydrate components of yeast invertase caused the enzyme to become more electronegative [12] However, in the case of primer-independent phosphorylases, the loss of the glucan moiety results in a *less* electronegative structure. For example, the a_2 isozymes of both *Oscillatoria* and *Cyanidium*

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[15] FRYDMAN, R B (1973) private communication

move rapidly toward the anode *before* amylase treatment, and move much less rapidly after amylase digestion of the glucan component The holo-enzyme appears to be more electronegative Slabnik and Frydman[4] reported that the primer-independent potato phosphorylase isozyme was the most anodic-mobile of the five isozymes Tsai and Nelson[3] had reported that their phosphorylase II of maize was more strongly retained on positively charged DEAE columns than the primer-dependent phosphorylase I

In view of these considerations, it seems unlikely that the a_2 phosphorylase isozyme was contaminated with endogenous glucan The presence of endogenous maltodextrin in the glucose-1-phosphate substrate also seems improbable If endogenous glucan had been present in the glucose-1-phosphate, it would have caused the synthesis of polyglucans by the amylase-treated phosphorylases (Fig 1, 2B and C), and by the normally occurring a_1 primer-dependent isozyme of *Oscillatoria*

It is probable that the a_2 isozymes of both algae are glycoproteins which do not require exogenous primer for polyglucan synthesis This type of *holo*-enzyme appears to be necessary for initiating storage polyglucoside synthesis in the algal cell

EXPERIMENTAL

Growth and extraction Oscillatoria princeps was cultured in Gerloff's modification of Chu No 10 medium [16] *Cyanidium caldarium* was cultured in a modification of Allen's medium with carbon dioxide gassing [17] Extracts were prepared in the cold and fractionated with $(NH_4)_2SO_4$ as described by Frederick [18] The phosphorylase fractions were dialyzed against cold 0 005 M Tris-HCl buffer of pH 7 1 for 4 hrs

Treatment with amylase Aliquots of the fractionated phosphorylase mixture obtained from the extracts of both algae were incubated for 2 hr with crystalline α-amylase (Worthington) or with 5% (w/v) maltoheptaose prepared after Whelan and Roberts [19] 1 0 mg of the amylase was used for approximately every 4 mg of protein in the fractionated and dialyzed material in 0 01 M Tris buffer at pH 6 9, containing 0 006 M NaCl at 25° After incubation, the mixture was dialyzed against 6 changes of the Tris buffer at 5° The mixture was separated by electrophoresis in 7% polyacrylamide gel in an E C Vertical Cell, using the slab method [20] Aliquots of the phosphorylase fractions were also incubated with 5% maltoheptaose in Tris buffer at 25° for 3 hr These too, were dialyzed and separated on polyacrylamide gels

Gel staining After separation, some of the gels were stained with amido-black Duplicates were incubated at 25° for 3 hr in 0 01 M Tris buffer at pH 7 2 containing 0 015 M K_2 glucose-1-phosphate Parallel incubations were carried out in the same mixture to which 3% maltoheptaose was added The incubated gels were treated by a modified Gomori method described by Fredrick[21] in order to localize the area on the gel demonstrating phosphorylase activity

The amylase in the incubated mixtures was very mobile under the conditions of electrophoresis used,[22] and was always at the extreme anodic ends of the gels after separation It did not interfere with the subsequent incubations in the medium described for the Gomori technique

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[22] DOANE, W W (1967) *J Exp Zool* **164,** 363