Pergamon 0031-9422(95)00285-S

Phytochemistry, Vol. 40, No. 2, pp. 377–381, 1995

0031-9422/95 $9.50 + 0.00

PROPERTIES OF FARNESOL PHOSPHOKINASE OF *BOTRYOCOCCUS BRAUNII*

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(*Received in revised form 7 March* 1995)

Key Word Index—*Botryococcus braunii*; alga; farnesol; phosphokinase; farnesyl monophosphate; farnesyl diphosphate.

Abstract—A 100 000 *g* pellet fraction of a strain of *Botryococcus braunii* B race had the capability of phosphorylating farnesol in the presence of CTP to give its mono- and diphosphate esters, but the same fraction did not phosphorylate other isoprenoid alcohols, including geraniol, geranylgeraniol, *Z,E*-polyprenols and dolichols. Exogenously added farnesyl monophosphate was not phosphorylated by this fraction. The K_m values in the formation of farnesyl monophosphate were 0.5 μM and 0.2 mM for farnesol and CTP, respectively. Iodoacetamide strongly inhibited the formation of farnesyl diphosphate, but had no effect on that of farnesyl monophosphate.

INTRODUCTION

The green colonial microalga *Botryococcus braunii*, B race, is well known for its ability to accumulate substantial amounts of triterpenoid hydrocarbons termed botryococcenes [1, 2]. Various feeding experiments with cultures of this organism have been made in order to elucidate the biosynthetic route of botryococcenes. L-Leucine has been shown to be incorporated efficiently into the hydrocarbons [3]. Pulse-chase experiments with sodium [^{14}C]bicarbonate have shown that C_{30} botryococcene is the precursor of C_{31}, C_{32} and C_{34} botryococcenes [4]. It has also been demonstrated that L-[Me-^{14}C]methionine acts as the methyl donor in the methylation process [5]. Based on the results of feeding experiments with stereospecifically ^{2}H- or ^{3}H-labelled farnesol, Huang and Poulter [6] have suggested that botryococcene is synthesized through presqualene diphosphate.

Our previous report [7] indicated that farnesol was incorporated into squalene as well as botryococcenes in feeding experiments and that CTP-dependent phosphorylation of farnesol to the mono- and diphosphate esters was observed in *in vitro* experiments. This paper reports characterization of the farnesol phosphorylation system of this alga.

RESULTS AND DISCUSSION

As previously described [7], when [1-^{14}C]farnesol was incubated with a 100 000 *g* pellet fraction of cell-free extracts of *Botryococcus braunii* B race, farnesyl mono- and diphosphate esters of the alcohol were produced in the presence of CTP and Mg^{2+} or Mn^{2+}.

In order to further confirm these observations, we conducted experiments using ^{32}P-labelled nucleoside triphosphates. When unlabelled farnesol, [γ-^{32}P]ATP, CDP and nucleoside-5′-diphosphate kinase were incubated with the cell-free extracts, [^{32}P]labelled compounds which co-chromatographed with farnesyl mono- and diphosphate esters were produced. The formation of the former was predominant over that of the latter. No significant phosphorylation was observed when farnesol was replaced with geraniol, geranylgeraniol, *Z,E*-polyprenols or dolichols.

2-Mercaptoethanol stimulated the formation of farnesyl monophosphate from farnesol but depressed that of farnesyl diphosphate (Fig. 1). The formation of farnesyl diphosphate was markedly inhibited by iodoacetamide, but that of farnesyl monophosphate was not significantly affected (Fig. 2). These facts suggest that the enzymes involved in the formations of farnesyl monophosphate and of farnesyl diphosphate differ from each other.

When [1-^{14}C]farnesyl monophosphate was substituted for [1-^{14}C]farnesol in the same incubation mixture, the formation of farnesyl diphosphate was not observed even though the conditions of incubation were varied by changing pH from 6 to 8, replacing CTP with ATP, GTP or UTP, and adding the 100 000 *g* supernatant. Possibly both kinases are embedded in membranes which are permeable against farnesol but not against farnesyl monophosphate, so that the farnesyl monophosphate synthesized by the farnesol kinase can be transferred to the active site of the farnesyl monophos-

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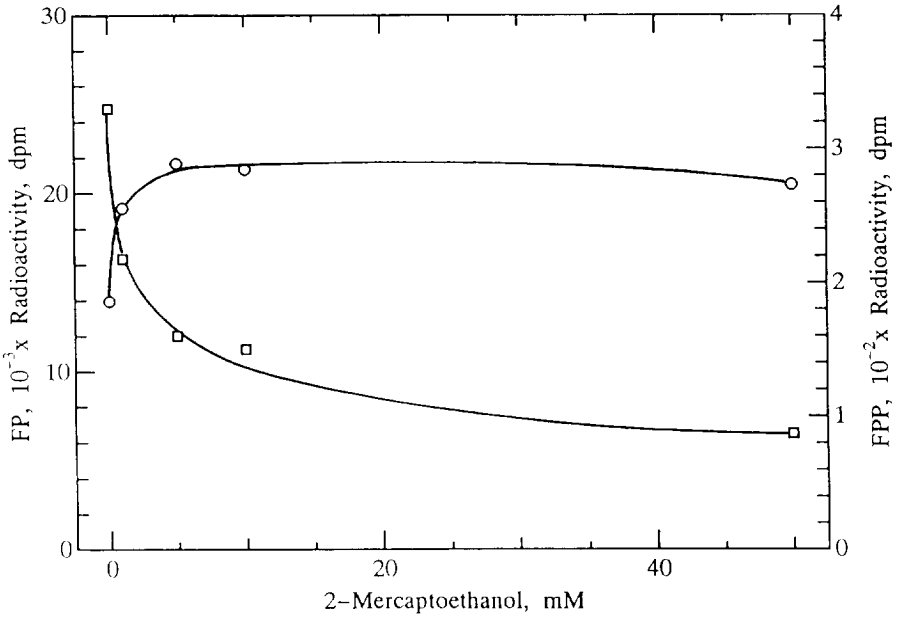

Fig. 1. Effect of 2-mercaptoethanol on the formation of farnesyl mono- and diphosphates. (○) Farnesyl monophosphate; (□) farnesyl diphosphate.

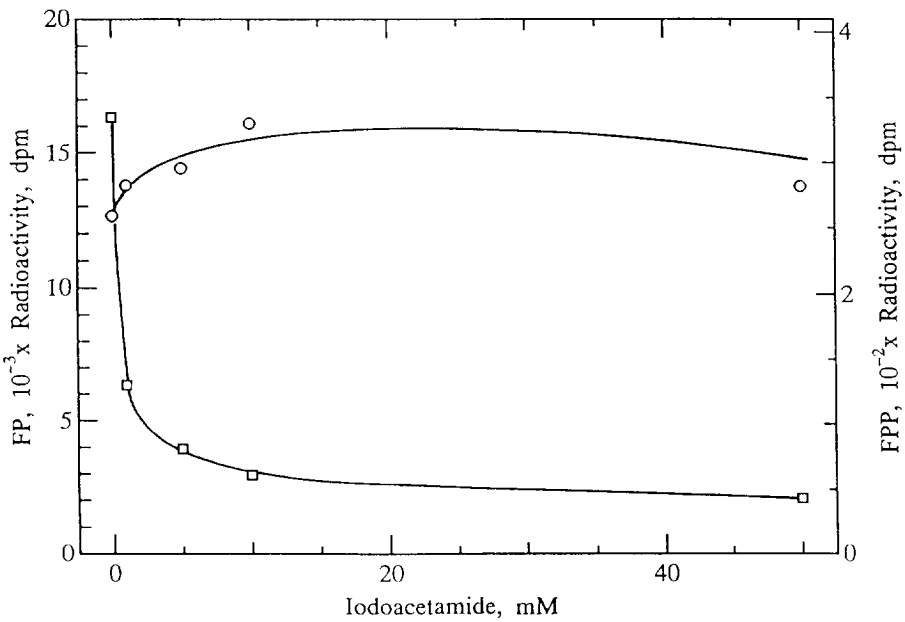

Fig. 2. Effect of iodoacetamide on the formation of farnesyl mono- and diphosphates. (○) Farnesyl monophosphate; (□) farnesyl diphosphate.

phate kinase. Another possibility is that farnesol might be directly diphosphorylated to give farnesyl diphosphate.

The pH optimum for the formation of farnesyl monophosphate appeared to be slightly lower than that for the formation of farnesyl diphosphate (Table 1). The reactions were linear with time up to 5 hr (data not shown). Figure 3 shows the effect of farnesol concentration on the velocity of synthesis of farnesyl monophosphate in the presence of a fixed concentration of CTP (1 mM). The K_m value for farnesol was estimated from Lineweaver–Burk plots to be 0.5 μM. Figure 4 shows the effect of CTP concentration on the velocity of the same reaction with the concentration of farnesol fixed at 5 μM. The K_m value for CTP was similarly estimated to be 0.2 mM.

Table 1. Effect of pH on the formation of farnesyl monophosphate and farnesyl diphosphate*

pH	FP (dpm)	FPP (dpm)
5.6	0930	165
6.2	3350	397
7.0	3590	445
7.2	2680	485
8.4	1950	216

*For pH 5.6, 6.2 and 7.0–8.4, acetate (100 mM), Tris–maleate (30 mM), and Tris–HCl (100 mM) buffers were used, respectively.

As shown in Table 2, the phosphorylation reactions require Mn^{2+} or Mg^{2+}, the former being more effective than the latter. Triton X-100 markedly promoted the reactions, while sodium deoxycholate suppressed the reactions. The reactions were partly inhibited by CHAPS (Table 3).

The phosphorylation abilities of the alga at least account for the formation of squalene from farnesol. However, the finding in this report that the yield of farnesyl monophosphate from farnesol was much higher than that of farnesyl diphosphate suggests that farnesyl monophosphate is not simply the intermediate from farnesol to farnesyl diphosphate but that the monophosphate ester is involved in other metabolic pathways. Huang and Poulter [6] proposed that the botryococcene skeleton is constructed through presqualene diphosphate, which is well established as a key precursor of squalene biosynthesis. However, no direct evidence has been given. Moreover, the formation of botryococcene from farnesyl diphosphate was not observed in our experiments using cell-free extracts of the algae, whereas the formation of squalene from farnesyl diphosphate was observed in experiments using the same cell-free extract [7]. Although the role of farnesyl monophosphate remains to be elucidated, the possibility cannot be eliminated that the monophosphate ester rather than the diphosphate ester is the direct precursor for the unusual coupling reaction leading to the botryococcene skeleton.

EXPERIMENTAL

Biological materials. The Berkeley strain [8] of *Botryococcus braunii* B race was cultured, harvested and

Table 2. Effect of metal ions on the formation of farnesyl monophosphate and farnesyl diphosphate*

Mn^{2+} (mM)	Mg^{2+} (mM)	FP (dpm)	FPP (dpm)
0	0	0	0
1	0	2740	850
10	0	850	660
0	1	320	0
0	10	385	290

*FP, farnesyl monophosphate; FPP, farnesyl diphosphate.

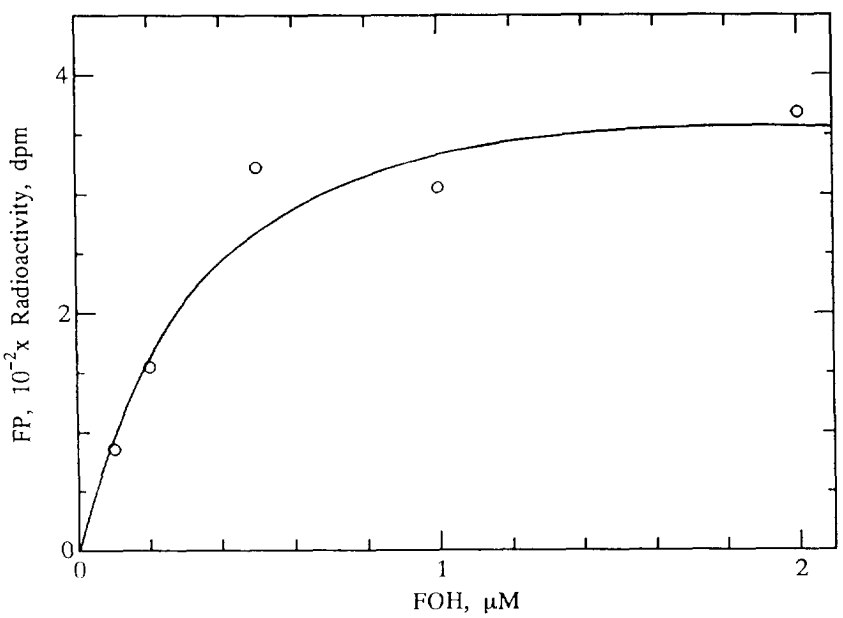

Fig. 3. Dependency of the formation of farnesyl monophosphate on the concentration of farnesol. The reaction velocity was measured under the standard conditions described for phosphokinase assay except that varied concentrations of [1-^{14}C]farnesol were used and that the time of incubation was 180 min. FOH, farnesol; FP, farnesyl monophosphate.

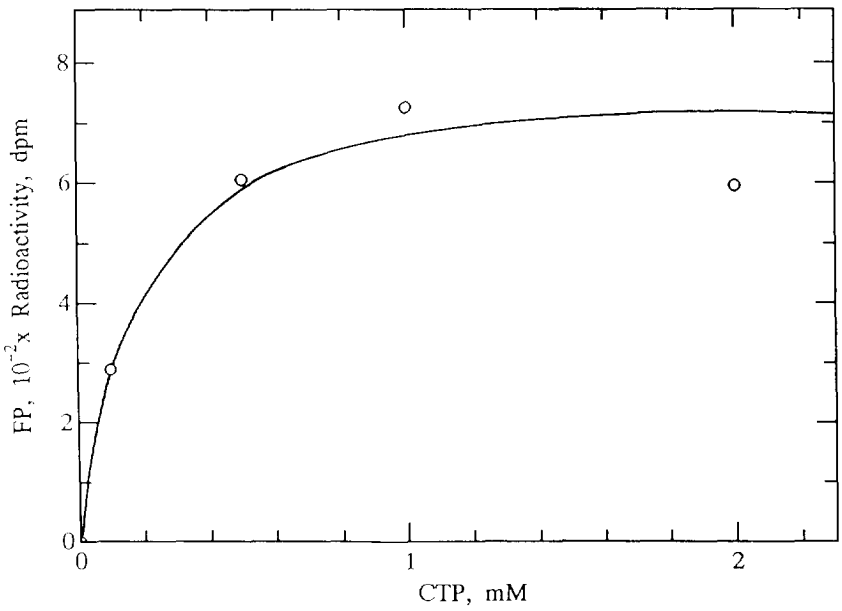

Fig. 4. Dependency of the formation of farnesyl monophosphate on the concentration of CTP. The reaction velocity was measured under the standard conditions except that varied concentrations of CTP were used in the presence of 5 μM of [1-^{14}C]farnesol. The incubation time was 225 min. FP, farnesyl monophosphate.

Table 3. Effect of detergents on the formation of farnesyl mono- and diphosphates*

Detergent concentration	FP (dpm)	FPP (dpm)
Without detergent	1930	250
Triton X-100		
0.05%	6560	1530
0.1%	4260	1440
CHAPS		
15 mM	1070	180
30 mM	640	95
DOC		
10 mM	0	0
20 mM	0	0

*CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulphonate; DOC, sodium deoxycholate; FP, farnesyl monophosphate; FPP, farnesyl diphosphate.

stored as previously described [9]. The cell-free extracts and microsomal fraction were prepared from stored cells according to the same procedure as described previously [7].

Phosphokinase assay. The standard incubation mixture contained, in a final vol. of 0.5 ml, 100 mM of Tris–HCl buffer (pH 7), 10 mM of $MgCl_2$, 10 mM of $MnCl_2$, 1 mg of bovine serum albumin, 0.05% of Triton X-100, 1 mM of CTP, 1 μM of farnesol (53 Ci mol^{-1}), 0.15 ml of the microsomal fraction (1.8 mg protein ml^{-1}). After incubation at 25° for an appropriate time, the mixture was extracted with *n*-BuOH, and the *n*-BuOH extracts were analysed by TLC [silica gel-60, *n*-PrOH–NH_4OH–H_2O (6:3:1)]. The radioactivities of farnesyl monophosphate and farnesyl diphosphate on TLC plates were estimated with a Fuji BAS-2000 bioimage analyser.

Substrate specificity of phosphokinase. The incubation mixture contained, in a final vol. of 0.5 ml, 100 mM of Tris HCl buffer (pH 7), 10 mM of $MgCl_2$, 10 mM of $MnCl_2$, 1 mg of bovine serum albumin, 0.05% of Triton X-100, 0.15 μM of [γ-^{32}P]ATP (40.5 Ci mmol^{-1}), 5 mM of CDP, 4 units of nucleoside-5'-diphosphate kinase and 10 μM of farnesol, geraniol, geranylgeraniol, *Z,E*-polyprenols or dolichols. The mixture was kept at 25° for 30 min prior to the addition of 0.15 ml of microsomal fraction (1.8 mg protein ml^{-1}). The mixture was then incubated for 12 hr at 25° with shaking, and the products analysed as described above.

Reaction with [1-^{14}C] *farnesyl monophosphate.* [1-^{14}C] Farnesyl monophosphate (2.65 Ci mol^{-1}) was chemically synthesized from [1-^{14}C]farnesol according to the procedure of ref. [10]. The incubation mixture contained, in a final vol. of 0.5 ml, 100 mM of Tris–HCl buffer (pH 7), 10 mM of $MgCl_2$, 10 mM of $MnCl_2$, 1 mg of bovine serum albumin, 0.05% of Triton X-100, 10 mM of KF, 1 mM of CTP, ATP, GTP or UTP, 10 μM of [1-^{14}C] farnesyl monophosphate and 0.15 ml of the microsomal fraction. After incubation at 25° for 4 hr, the mixture was analysed.

REFERENCES

1. Maxwell, J. R., Douglas, A. G., Eglinton, G. and McCormick, A. (1968) *Phytochemistry* 7, 2157.

2. Metzger, P., Berkaloff, C., Casadevall, E. and Coute, A. (1985) *Phytochemistry* **24**, 2357.
3. Casadevall, E., Metzger, P. and Puech, M. (1984) *Tetrahedron Letters* **25**, 129.
4. Wolf, F. R., Nemethy, E. K., Blanding, J. H. and Bassham, J. A. (1985) *Phytochemistry* **24**, 733.
5. Metzger, P., David, M. and Casadevall, E. (1987) *Phytochemistry* **26**, 129.
6. Huang, Z. and Poulter, C. D. (1989) *J. Am. Chem. Soc.* **111**, 2713.
7. Inoue, H., Korenaga, T., Sagami, H., Koyama, T. and Ogura, K. (1994) *Biochem. Biophys. Res. Commun.* **200**, 1036.
8. Wolf, F. R., Nonomura, A. M. and Bassham, J. A. (1985) *J. Phycol.* **21**, 388.
9. Inoue, H., Korenaga, T., Sagami, H., Koyama, T., Sugiyama, H. and Ogura, K. (1993) *Biochem. Biophys. Res. Commun.* **196**, 1401.
10. Kandutsch, A. A., Paulus, H., Levin, E. and Bloch, K. (1964) *J. Biol. Chem.* **239**, 2507.