Full Paper

Studies on poly- β -hydroxybutyrate synthase activity of *Nostoc muscorum*

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This study compares the PHB synthase activity of *Nostoc muscorum*, a N_2 -fixing cyanobacterium under control (grown in usual BG-11 medium), nitrogen (N) and phosphorus (P) deprivation and chemoheterotrophic conditions. Specific activity of PHB synthase did not depict significant variations in the latter three types of cultures, except for the control one, where a significantly lower activity was recorded. PHB synthase activity was detected only in the soluble fractions of both the control as well as cells incubated under chemoheterotrophic conditions. A K_m of $80.2 \mu M$ D,L- β -hydroxybutyryl-CoA and V_{max} of $197.5 \text{ nmol thiobenzoate (TNB) mg protein}^{-1} \text{ min}^{-1}$ were observed for the enzyme. PHB synthase remained insensitive to acetyl-CoA, ATP, NADP, NADPH supplementation under in vitro condition. Addition of acetyl phosphate was found to activate the enzyme and the level of activation was dependent on the concentration of acetyl phosphate supplementation. Inhibition of PHB synthase in 2,3-butanedione supplemented cultures and reactivation following acetyl phosphate addition proved the post-translational control of acetyl phosphate over PHB synthase.

Key Words—acetyl phosphate; chemoheterotrophy; *Nostoc muscorum*; PHB synthase; poly- β -hydroxybutyrate

Introduction

Poly- β -hydroxybutyrate (PHB) is a class of naturally occurring polyester which is produced and stored intracellularly in wide varieties of prokaryotes including cyanobacteria under unbalanced growth conditions (Anderson and Dawes, 1990; Stal, 1992; Steinbüchel, 1991). This polymer has attracted the researchers because of its potential to be an alternative to conventional plastics.

The cyanobacterium, *Nostoc muscorum* accumulates PHB profoundly under conditions of nutrient (N

and P) deprivation, mixotrophy, chemoheterotrophy and limitations of gas exchange. Our earlier reports (Sharma and Mallick, 2005a,b) demonstrated accumulation up to 23, 25, 35, 43 and 40% of dry cell weight (dcw), respectively under P-deficient, N-deficient, mixotrophic, chemoheterotrophic and gas exchange limited conditions, as compared to a PHB pool of 8.5% (dcw) in control culture. PHB metabolism has been studied in PHB-accumulating bacteria, especially in *Alcaligenes eutrophus*, now called *Wautersia eutropha*. PHB is synthesized from acetyl-CoA via three enzymatic reactions: 3-ketothiolase (EC 2.3.1.9) converts two acetyl-CoA molecules to an acetoacetyl-CoA molecule, NADPH-dependent acetoacetyl-CoA reductase (EC 1.1.1.36) converts acetoacetyl-CoA to D-3-hydroxybutyryl-CoA with NADPH oxidation, and the last enzyme, PHB synthase (no EC number), catalyzes the linkage of the D-3-hydroxybutyryl moiety to an existing PHB molecule by an ester bond

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Abbreviations: DTNB, 5,5-dithiobis(2-nitrobenzoic acid); PHB, poly- β -hydroxybutyrate; TNB, thiobenzoate.

(Steinbüchel and Schlegel, 1991). In *W. eutropha*, the three enzymes are constitutively synthesized. Minor modification to this biosynthetic pathway, *Rhodospirillum rubrum* requires the additional action of two enoyl-CoA hydratases to form D(-)-hydroxybutyryl-CoA from acetoacetyl-CoA (Moskowitz and Merrick, 1969), without changing the basic principles. The first enzyme of the sequence, 3-ketothiolase, controls the synthesis of PHB with free CoA acting as the key regulatory molecules (Oeding and Schlegel, 1973). In an *Acinetobacter* sp., which shows regulated PHB accumulation, the PHB biosynthesis gene operon is controlled at the transcriptional level (Schembri et al., 1995). However, in the thermophilic cyanobacterium, *Synechococcus* sp. strain MA19 PHB synthesis is controlled by PHB synthase and dependent on C/N ratio (Miyake et al., 1997). In this report, we examined the activity of the key enzyme of the PHB biosynthetic pathway, i.e. PHB synthase of *N. muscorum* subjecting the cultures to nutrient (phosphate and nitrogen) deprivation and chemoheterotrophy.

Materials and Methods

Nostoc muscorum Agardh (Culture collection: Banarus Hindu University, Varanasi, India) was grown axenically in NO_3 -free BG-11 medium (Rippka et al., 1979) at $25 \pm 1^\circ\text{C}$, pH 8.0 under 14 h light ($75 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ PAR): 10 h dark cycles. N deficiency was achieved by transferring the cultures to the NO_3 -free BG-11 medium devoid of Na_2MoO_4 . Further, ferrous ammonium citrate and $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ were also substituted by equimolar concentrations of ferric citrate and $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$. Phosphate deficiency was obtained by replacing K_2HPO_4 with equi-molar concentration of KCl. For chemoheterotrophy stationary phase *N. muscorum* cultures were supplemented with 0.4% acetate and incubated in dark following Sharma and Mallick (2005b).

PHB synthase activity was assayed following the method of Valentin and Steinbüchel (1994). *N. muscorum* cells incubated under different conditions were centrifuged, washed 2–3 times with 25 mM Tris-HCl buffer (pH 7.5) and resuspended in 5 ml of the same buffer containing 5% (v/v) glycerol, called Tris-glycerol buffer. Cells were disrupted by sonication with Sonics VC-130 (Sonics and Materials, Inc., USA) at 40 W for 10 min. Cell debris was removed by centrifugation (4°C) at $16,000 \times g$ for 20 min. The supernatant (crude

extract) was diluted with Tris-glycerol buffer to a concentration of 1 mg ml^{-1} protein and was used for enzyme assay. Soluble fraction and sediments (membrane fraction) were separated by ultracentrifugation (Sorvall Ultra-80, USA) of the crude extracts at $120,000 \times g$ for 2 h. Crude extract, soluble fraction and membrane fraction were compared for the PHB synthase activity after adjusting the protein content to 1 mg ml^{-1} .

The assay mixture (1.0 ml) contained 800 μl of enzyme extract, 100 μl of DL- β -hydroxybutyryl-CoA and 100 μl 5,5-dithiobis(2-nitrobenzoic acid) (DTNB). The reaction was started by addition of DL- β -hydroxybutyryl-CoA as the substrate. The optical density of the thiobenzoate anions resulting from the reaction of CoA and DTNB was measured at 30°C for 10 min at 412 nm with a Specord S100 spectrophotometer (Analytic Jena, Germany). Protein estimation was carried out following Lowry et al. (1951).

Various activators were added to the Tris-glycerol buffer and were kept on ice until the enzyme assay. Mixtures containing crude extracts of the control culture and the activators were incubated for 10 min at 30°C . Longer incubation resulted in inactivation of the enzyme. One milliliter of the mixture was used for enzyme assay.

Results

Figure 1 shows the time course of PHB synthase activity of *N. muscorum* incubated under control, P-deficient, N-deficient and chemoheterotrophic conditions. The specific activity of PHB synthase of P-deficient, N-deficient and chemoheterotrophic cultures increased from the day of inoculation and reached the maximum value during day 5–8, after which it depicted a declining trend. It is interesting to note here that the specific activity of PHB synthase did not show any significant variation in all the above types of cultures, except for the control cells, where a significantly lower activity was recorded.

Figure 2 presents the DL- β -hydroxybutyryl-CoA saturation curve of PHB synthase in the crude extracts of *N. muscorum*. The K_m value as calculated from the double-reciprocal plots of velocity versus substrate concentrations, was $80.2 \mu\text{M}$ (Fig. 2, inset) with V_{max} of $197.5 \text{ nmol TNB mg protein}^{-1} \text{ min}^{-1}$.

Table 1 lists the PHB synthase activity of crude extracts, membrane fractions and soluble fractions of *N.*

muscorum incubated under control as well as chemoheterotrophic conditions. The specific activity of PHB synthase in crude extracts of chemoheterotrophic cells was higher than that of control cells, and the activity was detected in soluble fractions rather than in membrane fractions of both types of cells.

Table 2 presents the results of some possible activa-

tors on in vitro PHB synthase activity of the crude extracts of control cultures of *N. muscorum*. The intermediates of PHB metabolism, such as acetyl-CoA and acetoacetyl-CoA remained inactive in stimulating the activity of PHB synthase, so also the addition of ATP, NADP and NADPH. However, an increase in absorbance at 412 nm due to 2-nitro-thiobenzoate anions

Fig. 1. Time-course of specific activity of PHB synthase. (○), control; (▲), chemoheterotrophy; (△), N-deficient and (□), P-deficient. All values are mean $\pm$ SE, $n=3$.

Table 1. Specific activity of PHB synthase in crude and fractionated samples.

Sample type	Specific activity of PHB synthase (nmol TNB mg protein ⁻¹ min ⁻¹)
<u>Control (culture incubated under usual light : dark cycles)</u>	
Crude extract	35 $\pm$ 2.35 ^a
Membrane fraction	nd
Soluble fraction	60 $\pm$ 5.21 ^b
<u>Culture incubated under chemoheterotrophic condition</u>	
Crude extract	209 $\pm$ 13.26 ^c
Membrane fraction	nd
Soluble fraction	311 $\pm$ 18.24 ^d

Values are mean $\pm$ SE, $n=3$, nd: not detected.

Values in the column superscripted by different letters are significantly ($p<0.05$) different from each other (Duncan's new multiple range test).

Fig. 2. Specific activity of PHB synthase at different concentrations of the substrate in crude extracts of *N. muscorum*.

Double reciprocal plot (inset). All values are mean $\pm$ SE, $n=3$.

Table 2. In vitro activation of PHB synthase in *N. muscorum* cells grown under usual light : dark cycles.

Activator	Specific activity of PHB synthase (nmol TNB mg protein ⁻¹ min ⁻¹)
Control	38.2±2.05 ^a
Acetyl-CoA (10 mM)	34.7±3.15 ^a
Acetoacetyl-CoA (10 mM)	35.3±3.61 ^a
ATP (20 mM)	37.9±2.16 ^a
NADP (20 mM)	39.8±3.01 ^a
NADPH (20 mM)	40.0±1.89 ^a
Acetyl phosphate (20 mM)	159.5±4.23 ^b

Values are mean±SE, *n*=3.

Values in the column superscripted by different letters are significantly (*p*<0.05) different from each other (Duncan's new multiple range test).

Fig. 3. In vitro activation of PHB synthase by acetyl phosphate in crude extracts of *N. muscorum* cells grown under usual light : dark cycles.

All values are mean±SE, *n*=3.

or activation of the enzyme was observed in presence of acetyl phosphate. The level of activation was, however, dependent on the concentration of acetyl phosphate added (Fig. 3), and at higher concentration (40 mM) deactivation of the enzyme was evident.

Figure 4 shows the impact of various concentrations of 2,3-butanedione on PHB synthase activity of *N. muscorum* culture incubated under chemoheterotrophic condition. It is interesting to note here that supplementation of 2,3-butanedione at the initiation of dark incubation suppressed PHB synthase activity in a concentration dependent manner, and at

Fig. 4. Interactive effects of 2,3-butanedione and acetyl phosphate on PHB synthase activity of *N. muscorum* incubated under chemoheterotrophic condition.

All values are mean±SE, *n*=3.

concentration of 10 mM a complete suppression of the activity was observed. Similar inhibition was also observed in control cultures (data not shown). Activity of the enzyme was, however, found to be restored following acetyl phosphate supplementation (Fig. 4).

Discussion

As presented in Fig. 1 the specific activity of PHB synthase did not vary significantly in *N. muscorum* cells incubated under P-deficient, N-deficient and chemoheterotrophic conditions. However, a great variation in PHB accumulation potential was reported under these conditions, i.e. a PHB pool of 23, 25 and 43% (dcw) were recorded, respectively in P-deficient, N-deficient and cultures incubated under chemoheterotrophic conditions (Sharma and Mallick, 2005a, b). This demonstrates that though activation of the enzyme was similar under all the above conditions, in chemoheterotrophic cells the presence of acetate might play significant role in PHB accumulation. The results of Miyake et al. (1997) also supported the above speculation, where PHB content of *Synechococcus* MA19 grown under nitrogen deprivation in presence of acetate was higher (18% dcw) than its counterpart, grown without acetate (10% dcw), despite a 3-fold higher enzyme activity in the latter.

The *K_m* value of PHB synthase was recorded to be 80.2 μM (Fig. 2). This is much closer to the value reported for *Synechococcus* MA19 (70.7 μM, Miyake et

al., 1997), as compared to 720 μM for *Wautersia eutropha* (Haywood et al., 1989). This low K_m may aid the accumulation of PHB under conditions of low carbon flux through the photoautotrophic mechanism of cyanobacteria (Smith, 1983).

PHB synthase of *N. muscorum* was exclusively detected in soluble fractions of control as well as the cultures incubated under chemoheterotrophic condition (Table 1). This agrees well with the reports of Fukui et al. (1976), Haywood et al. (1989) and Liebergesell et al. (1994), where PHB synthase activity was detected in soluble fractions of *Zoogloea ramigera*, *Chromatium vinosum*, and *Wautersia eutropha*, respectively, but contradicts Miyake et al. (1997) where PHB synthase of *Synechococcus* MA19 was a membrane-bound enzyme. The membrane-bound nature of *Synechococcus* PHB synthase resulted in formation of PHB granules very closely associated and surrounded with thylakoid membrane (Asada et al., 1999). From the point of view of application, close association of PHB granules with thylakoid membranes resulted in great difficulty in down-streaming. Therefore, exploiting *N. muscorum* as a host for PHB production could be advantageous owing to the soluble nature of PHB synthase, as has been observed for the transformants of *Synechococcus* (Asada et al., 1999).

It is evident from Table 2 that addition of acetyl-CoA, acetoacetyl-CoA, ATP, NADP and NADPH remained inactive in stimulating the in vitro PHB synthase activity, except acetyl phosphate. Maximum stimulation of the enzyme was recorded at 30 mM acetyl phosphate supplementation (Fig. 3). Acetyl phosphate is synthesized by another enzyme, phosphotransacetylase, which catalyzes the reversible acetyl group transfer between CoA and orthophosphate, and 2,3-butanedione is a known inhibitor of the enzyme, phosphotransacetylase (Iyer and Ferry, 2001). Thus, inhibition of PHB synthase under 2,3-butanedione supplementation and reactivation following acetyl phosphate addition (Fig. 4) prove the post-translational control of PHB synthase by acetyl phosphate.

In conclusion, the soluble enzyme PHB synthase of *N. muscorum* with a low K_m (80.2 μM DL- β -hydroxybutyryl-CoA) enables it to accumulate PHB under photoautotrophic condition. Acetyl phosphate plays a major role on the post-translational control over the enzyme.

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