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Metabolism of Poly-β-Hydroxybutyrate: Effect of Mild Alkaline Extraction on Native Poly-β-Hydroxybutyrate Granules[1]

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Mild alkaline extraction of native poly-β-hydroxybutyrate (PHB) granules results in the solubilization of a protein fraction. Both the solubilized protein fraction and the extracted granules are essentially devoid of PHB synthetase activity unless recombined. The protein fraction has been separated by chromatography into two components (A-I and A-II). A-I but not A-II can be recombined with extracted granules to give rise to PHB synthetase activity. Extracted granules no longer require pretreatment with activator or trypsin but are directly susceptible to hydrolysis by *Rhodospirillum rubrum* depolymerase. Addition of A-II or A-I prevents the direct hydrolysis by depolymerase. The inhibition is reversed by activator or trypsin. We conclude that native granules are associated with a protein inhibitor which prevents the hydrolysis of PHB by depolymerase unless the protein is destroyed by trypsin, removed by alkaline extraction, or modified by activator.

In earlier reports (5, 8, 11-13) we described the properties of poly-β-hydroxybutyrate (PHB) granules isolated from *Bacillus megaterium* KM. In brief, PHB granules are composed of a core of PHB surrounded by a membranous coat. Isolated granules are associated with the PHB synthetase system as well as a labile factor involved in PHB degradation. In addition to the labile factor, the enzymatic depolymerization of PHB consists of at least two soluble components (activator and depolymerase) whose successive action results in the breakdown of PHB granules to soluble products. Trypsin treatment of granules simulates the effect of the activator; however, the mechanism of activator action remains to be elucidated. In this communication we describe the solubilization of a protein fraction from PHB granules by mild alkaline extraction of granules. Both the solubilized protein fraction and the extracted granules are required for PHB synthesis. In the degradative system, the extracted granules no longer require the pretreatment with activator or trypsin but are susceptible to direct hydrolysis by depolymerase. The solubilized protein, however, when added back to the system prevents the hydrolysis of the polymer. This inhibition can be reversed by activator or by trypsin. These studies suggest that an inhibitor associated with PHB granules prevents the hydrolysis of PHB by depolymerase. Its partial removal by the alkaline extraction, or its modification by activator and presumably its proteolytic destruction by trypsin, results in granule preparations in which the polymer is directly susceptible to hydrolysis by the depolymerase.

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MATERIALS AND METHODS

Native PHB granules were prepared from *B. megaterium* KM as described by Griebel et al. (5). D(−)-β-hydroxybutyryl-coenzyme A (CoA) was prepared by the mixed anhydride method of Wieland and Rueff (20). Radioactive D(−)-β-hydroxybutyrate was obtained as described by Merrick and Doudoroff (11). The preparation of activator and depolymerase fractions from *Rhodospirillum rubrum* extracts was carried out by a modification of the procedure described by Merrick and Doudoroff (13). The extracellular PHB depolymerase of *Pseudomonas lemoignei* was isolated by procedures described by Lusty and Doudoroff (9).

Enzyme assays. PHB synthesis in the reconstituted system was assayed in reaction mixtures containing 2.5 μmoles of tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (*p*H 7.5), 0.5 μmole of $MgCl_2$, 0.5 μmole of dithiothreitol, 0.01 mg of albumin, and appropriate amounts of extracted protein and granules.

Dilutions of the extracted protein were carried out in 0.02 M Tris-hydrochloride buffer (*p*H 8.0) containing 5 mM mercaptoethanol, 1 mM $MnCl_2$, and 20% (v/v) glycerol (GMM buffer). The above components were allowed to preincubate for 20 min at 30 C before initiating the reactions with 16.7 nmoles of D(−)-β-hydroxybutyryl-^{14}C-CoA (5.63×10^5 counts per min per μmole). Final volume was 0.05 ml. After incubation for 3 min at 30 C, reactions were terminated, by the addition of 1.0 ml of 5% trichloroacetic acid, and filtered on membrane filters (HA; Millipore Corp., Bedford, Mass.) After being washed with at least 50 ml of water, the filters were glued to planchets, dried, and counted in a Nuclear-Chicago gas-flow counter. PHB synthetase activity associated with native granules was measured as described by Griebel et al. (5).

Other assays. Protein was determined by the method of Lowry et al. (7) by using crystalline bovine serum albumin as standard. The protein content of PHB granules was determined after solubilization of the granules in 0.14 N NaOH at 100 C for 20 min. Total β-hydroxybutyrate was determined by the method of Slepecky and Law (19). D(−)-β-hydroxybutyrate was determined with the specific D(−)-β-hydroxybutyrate dehydrogenase purified as described by Delafield et al. (1). The reaction was allowed to proceed to equilibrium, and the monomer concentration was calculated with the use of the partial equilibrium constant Keq = 0.13 (18).

Extraction of native PHB granules. Granule suspensions, containing 500 mg of PHB and 25 mg of protein in a total volume of 15 to 30 ml of 0.02 M Tris-hydrochloride buffer (*p*H 8.0), were dialyzed at 4 C for 3 hr against 1.0 liter of 0.01 N NaOH with three changes of the alkali. The dialyzed suspension was layered on glycerol and centrifuged at 4,000 × *g* in a Sorvall HB-4 swinging-bucket rotor for 15 min. The supernatant fluid was removed, centrifuged at 30,000 × *g* for 10 min to remove traces of residual polymer, and dialyzed against 0.02 M Tris-hydrochloride buffer (*p*H 8.0) for 2 hr. It was finally concentrated in an Amicon ultrafiltration apparatus. This extraction procedure results in the removal of approximately 70% of the total protein of the granules. Chromatography of the concentrated extract was carried out on Bio-Gel A-15m (Fig. 1). Fractions 9 to 12 were combined and constitute the A-I fraction; 16 to 22 were combined and constitute the A-II fraction. For the measurement of inhibitory activity in the depolymerizing system, A-I and A-II were dialyzed overnight against 0.02 M Tris-hydrochloride buffer (*p*H 8.0) containing 20% glycerol (v/v) and were finally concentrated by ultrafiltration.

The preparation of extracted granules for studies on the hydrolysis of PHB were carried out by a modification of the above extraction procedure. Granule suspensions containing 35 to 50 mg of PHB in a total volume of 4.0 to 5.0 ml of 0.02 M Tris-hydrochloride buffer (*p*H 8.0) were dialyzed against 1.0 liter of 0.01 N NaOH for 2 hr with one change of the alkali. The dialyzed suspension was layered on glycerol and centrifuged at 3,000 × *g* for 15 min. The polymer suspension was resuspended in half of the original volume of the Tris buffer and utilized as substrate for the soluble depolymerizing factors.

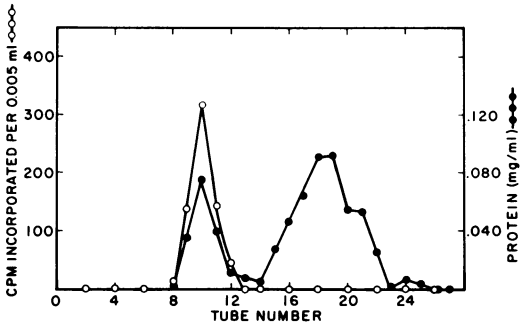

FIG. 1. *Chromatography of protein extract on Bio-Gel A-15m. A 0.3-ml solution of the protein extract (3.04 mg/ml) was applied to a Bio-Gel A-15m column (1.5 by 27 cm) equilibrated with the GMM buffer. The column was eluted with the same buffer. Fractions of 2.0 ml were collected. Assay of the PHB synthetase activity by the reconstituted system was carried out with 5-μl samples as described in the text. Concentration of extracted PHB granules in the reaction mixtures was 7.7 μg (determined as PHB).*

RESULTS

Extraction of granules with 0.01 N NaOH resulted in preparations of granules of protein extract which were essentially incapable of carrying out the polymerization of PHB from D(−)-β-hydroxybutyryl-CoA (Table 1). However, when the protein extract was combined with the extracted granules, reconstitution of the synthetase system was observed. The results presented in Table 1 do not give any information regarding recovery of the initial PHB synthetase activity present in the native PHB granules since initial rates were not measured. Under the conditions of this experiment, the reaction is complete at the indicated time, and no further incorporation is observed with longer incubation times. On subsequent analysis, it was noted that the reconstituted system only gave rise to small recoveries of the initial activity of native granules. Part of the reason for low recovery of synthetase activity could be attributed to the extreme lability of the protein extract. This lability was particularly evident when the extract was diluted in buffer (Table 2). The addition of $MnCl_2$, glycerol, and 2-mercaptoethanol to the diluting buffer provided partial stabilization. Even though all further studies of the reconstituted system were carried out with extract that had been diluted in the GMM buffer, the over-all recovery of the initial PHB synthetase activity of native granules was only 5 to 6%. All other attempts to stabilize the protein extract or improve the extraction procedures were unsuccessful in the resolution of the PHB synthetase system.

TABLE 1. *Synthesis of poly-β-hydroxybutyrate (PHB): effect of alkaline extraction*[a]

Expt	Additions	Amt of β-Hydroxybutyrate incorporated (nanomoles)
1	Native PHB granules	44.2
2	Extracted PHB granules	0.4
3	Protein extract	6.3
4	Extracted PHB granules and protein extract	42.3

[a] Reaction mixtures in 0.5 ml contained (in micromoles): tris(hydroxymethyl)aminomethane-hydrochloride (*p*H 7.5), 50; $MgCl_2$, 5; and D(−)-β-hydroxybutyryl-coenzyme A, 0.14 (specific activity, 1.62×10^4 counts per min per μmole). Native PHB granules (0.5 mg of PHB and 14 μg of protein), extracted granules (0.5 mg of PHB and 6.9 μg of protein), and extracted protein (4.0 μg) were added as indicated. The reaction mixtures were allowed to preincubate for 5 min before adding substrate to initiate the reaction. Incubation was for 5 min; temperature was 30 C. Reactions were terminated by the addition of 1.0 ml of ethanol; incorporation was determined as described by Griebel et al. (5).

TABLE 2. *Synthesis of poly-β-hydroxybutyrate (PHB): effect of dilution on protein extract*[a]

Additions	Time from addition to assay (min)	Amt of β-hydroxybutyrate incorporated (10^{-2} nmoles/min)
None	0	24.6
None	15	6.5
2-Mercaptoethanol	5	19.8
	15	10.5
	60	7.3
$MnCl_2$	15	17.3
$MgCl_2$	15	7.8
Glycerol	15	16.2
Albumin	15	7.5
$MnCl_2$ + glycerol + 2-mercaptoethanol	0	21.4
	5	23.7
	15	19.2
	60	15.2

[a] Dilutions were carried out in 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride (*p*H 8.0) containing the added components as indicated. Final concentrations of the added components were: $MnCl_2$ (1 mM); $MgCl_2$ (1 mM); 2-mercaptoethanol (10 mM); glycerol (20% v/v); and albumin (1.4 mg/ml). The protein extract (2.1 mg/ml) was diluted 40-fold in the solutions indicated above and, after the indicated times, 10 μliters was removed and assayed for its ability to restore PHB synthesis in the reconstituted system as described in the text. Concentration of extracted PHB granules in the reaction mixtures was 24 μg (determined as PHB).

On fractionation of the solubilized protein on Bio-Gel A-15m, two protein fractions were separated (A-I and A-II; Fig. 1). Only the A-I fraction was effective in restoring PHB synthesis in the reconstituted system. A-I eluted in the exclusion volume and was opalescent, suggesting that this protein fraction is of very high molecular weight. A-II did not restore PHB synthesis when added to extracted granules nor did it stimulate PHB synthesis in the reconstituted system. The requirements for PHB synthesis in the reconstituted system are seen in Table 3. Both the extracted granules and the A-I fraction are required for synthesis of PHB. Albumin, dithiothreitol, and $MgCl_2$ are markedly stimulatory. It should be noted that Mn^{2+} which plays a role in the stability of the protein apparently cannot replace Mg^{2+} in the catalyzed reaction. The stimulatory effect of albumin was also observed in earlier studies on the properties of the PHB synthetase of native granules (5). To obtain optimal PHB synthetase activity in the reconstituted system, preincubation of A-I and extracted granules was required. Activity was optimal after 20 min of preincubation of A-I and extracted granules (Fig. 2) and may reflect the time required for reassociation of A-I with the extracted granules. This possibility was supported by the observation that binding of A-I to the extracted granules does indeed occur. After a 20-min preincubation period of A-I and extracted granules, it was found that 79% of the activity of the A-I fraction initially present was found to be associated with the pellet obtained by centrifugation; only 13% was still recoverable in the supernatant fluid. The effects of extracted PHB granule concentration, A-I concentration, and time on the reaction velocity are seen in Fig. 3 and 4, respectively.

The *p*H optimum for synthesis was found to be 7.5 and is similar to that previously reported for the native PHB synthetase (5). The K_m calculated according to Lineweaver and Burk (6) was 3.12×10^{-4} M. The previously reported value was 9.25×10^{-5} M (5). This difference may reflect altered conformation of the synthetase in the reconstituted system with resultant weaker binding of the substrate.

The polymerization of β-hydroxybutyryl residues from D(−)-β-hydroxybutyryl-^{14}C-CoA by the reconstituted system was demonstrated after isolation of the radioactive product from incubation mixtures and its subsequent hydrolysis by the *P. lemoignei* extracellular depolymerase. The extracellular depolymerase hydrolyzes PHB to

TABLE 3. *Synthesis of poly-β-hydroxybutyrate: requirements of the reconstituted system*[a]

System	Amt of β-hydroxybutyrate incorporated (10^{-2} nmoles/min)
Complete	29.1
$-MgCl_2$	13.9
$-MgCl_2 + MnCl_2$ (1 mM)	11.8
$-MgCl_2 + MnCl_2$ (10 mM)	3.37
-Dithiothreitol	5.20
-Dithiothreitol + 2-mercaptoethanol (10 mM)	8.40
-Albumin	4.36
-A-I	1.86
-Extracted granules	2.96
-A-I, -extracted granules	0.00

[a] Assays were carried out as described in the text. A-I fraction (0.35 μg of protein) and extracted granules (15.3 μg of poly-β-hydroxybutyrate) were added except as indicated.

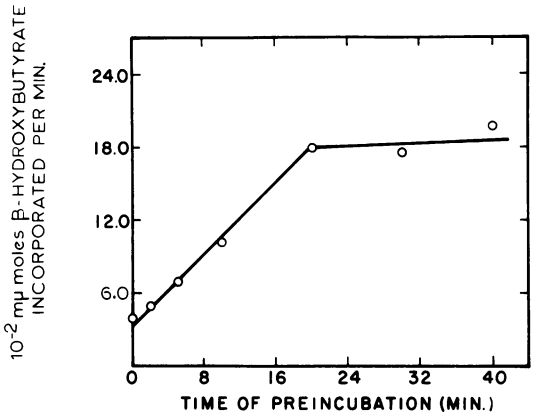

FIG 2. *Effect of time of preincubation of A-I and extracted PHB granules on rate of PHB synthesis. Preincubation was carried out at the indicated time intervals. Assay for PHB synthesis by the reconstituted system was conducted as described in the text. Reaction mixtures contained 0.35 μg of A-I and 15 μg of PHB (extracted granules).*

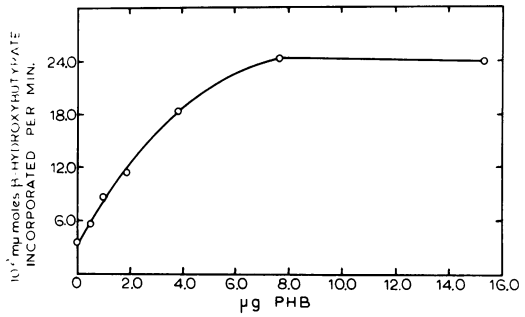

FIG. 3. *Effect of concentration of extracted PHB granules on rate of PHB synthesis. Assays were conducted as described in the text. Concentration of A-I in the reaction mixtures was 0.27 μg. Concentration of extracted granules is expressed as μg of PHB.*

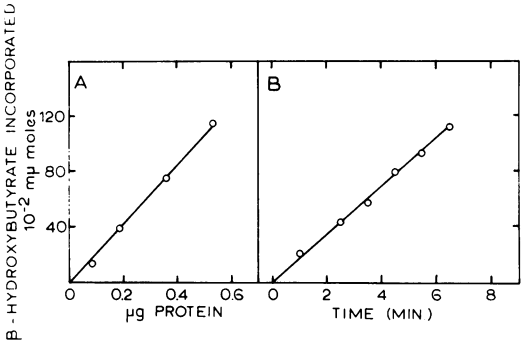

FIG. 4. *Effect of A-I concentration (A) and incubation time (B) on PHB synthetase activity. Assays were conducted as described in the text. In B, reaction mixtures contained 0.27 μg of A-I. In A and B reaction mixtures contained 15.3 μg of PHB (extracted granules).*

oligomers (dimer and trimer) and β-hydroxybutyrate. The radioactive polymer was isolated from reaction mixtures, washed with 0.02 M Tris-sulfate buffer (*p*H 8.0), and subjected to the action of the depolymerase. After acidification of the reaction mixture, the products were isolated by continuous ether extraction and finally subjected to paper chromatography in the following solvent systems: (i) 1-butanol saturated with 1.5 N aqueous ammonia and (ii) ether-benzene-formic acid (50:50:12.5, v/v/v). Only three radioactive substances were detected and these corresponded to authentic β-hydroxybutyrate and its dimer and trimer. On saponification, only one radioactive spot was detected and this corresponded to β-hydroxybutyrate. The results obtained with butanol-NH_3 system are seen in Fig. 5.

Disc-gel electrophoresis of the purified A-I fraction was carried out in the standard 7.5% gels at *p*H 9.3 according to the directions of Buchler Instruments (Fort Lee, N.J.). It was observed that the A-I fraction did not enter the gels, presumably because of its large size. Electrophoresis carried out in gels containing 9 M urea at *p*H 2.7 (15) resulted in the appearance of at least 10 bands. In the presence of 0.5% sodium lauryl sulfate (17), two bands were seen: a minor component which moved very close to the tracking dye and a major component which we believe to be the subunit of the active factor in the A-I fraction. The molecular weight of this

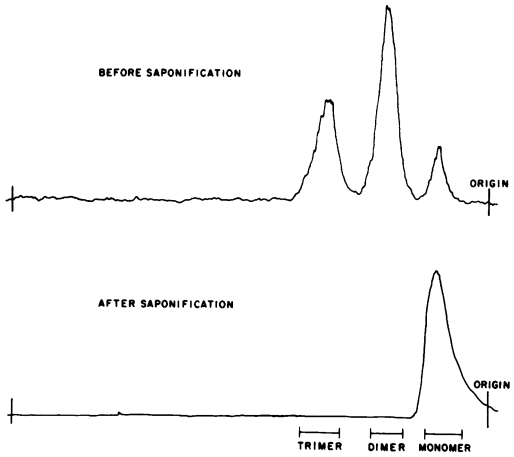

FIG. 5. *Digestion by depolymerase of PHB synthesized by reconstituted system. Radioactive PHB synthesized by the reconstituted system was isolated from reaction mixtures, washed with 0.02 M Tris-sulfate buffer (pH 8.0), and subjected to action of the extracellular P. lemoignei depolymerase. The products of the reaction were isolated after acidification by continuous ether extraction. Chromatography shown above was carried out with the butanol-NH_3 system, and examination of radioactive products was carried out with a Nuclear-Chicago Actigraph III.*

component determined as described by Shapiro et al. (17) was 43,850. These observations leave considerable doubt concerning the homogeneity of the A-I fraction. Difficulties in demonstrating homogeneity with other solubilized membrane associated proteins have been encountered. For example the purified membrane-adenosine triphosphatase of *Micrococcus lysodeikticus* gave at least five bands on disc electrophoresis in urea but only one major component and a minor component which moved with the tracking dye in the presence of sodium lauryl sulfate. The authors suggest that the complex pattern obtained after urea treatment could represent chemical differences between subunits or different states of subunit aggregation (14).

The extracted granules were subjected to a variety of treatments and subsequently were examined for their ability to carry out the synthesis of PHB from D(−)-β-hydroxybutyryl-CoA when reconstituted with the A-I fraction. The results of such experiments indicated that heating for 5 min in a boiling-water bath had no effect on the extracted granules. However, the extracted granules were extremely sensitive to sonic oscillation, repeated centrifugation, or incubation with trypsin. All of these treatments resulted in preparations which lose over 90% of the original activity of the untreated extracted granules when reassociated with the A-I fraction. The lability of the extracted granules to the above treatments might suggest that inactivation is due to an alteration of a conformational state of PHB which is necessary for the reassociation with the A-I fraction. Possibility such a conformational state is maintained by the association of PHB with the residual protein which remains after the extraction. Stability of the extracted granules to the heat treatment might argue that the role of the extracted granules is principally to bind the A-I fraction and to function as a primer, whereas the A-I fraction functions in a catalytic capacity (e.g., PHB synthetase). The possibility that a protein which participates catalytically in PHB synthesis is still associated with the extracted granules but is stable to heat in the presence of PHB is, however, not ruled out.

The ability of extracted granules to function as a substrate in the depolymerizing system was examined. The extracted granules were much more rapidly hydrolyzed by crude *R. rubrum* enzyme than native PHB granules (Table 4). When analyzed with the purified depolymerizing system (Table 4), it was found that extracted granules no longer were dependent on activator pretreatment but could be hydrolyzed directly by depolymerase. The extent of hydrolysis of PHB by depolymerase was determined by measuring the decrease in turbidity of a suspension of the granules as previously described (13), except that

TABLE 4. *Depolymerization of poly-β-hydroxybutyrate (PHB) granules: effect of extraction*[a]

Substrate	Additions	Amt of β-Hydroxybutyrate produced (μmoles)
Native PHB granules	None	0.01
	Crude extract	0.35
	Depolymerase	0.03
	Activator + depolymerase	0.24
Extracted PHB granules	None	0.00
	Crude extract	1.21
	Depolymerase	0.54
	Activator + depolymerase	0.75

[a] Reaction mixtures contained PHB granules (corresponding to 8 μmoles of the monomer) and 5 μmoles of potassium phosphate buffer (pH 7.6) in a total volume of 0.2 ml. The granules were preincubated for 10 min, alone or with activator (0.72 μg). After preincubation, crude *R. rubrum* extract (25 μg) obtained after centrifugation at 78,000 × *g* for 120 min or depolymerase (1.23 μg) was added as indicated, and incubations were continued for 15 min at 30 C. Reactions were terminated by heating in a boiling-water bath for 3 min and, after centrifugation, a suitable sample was removed and assayed for β-hydroxybutyrate with D(−)-β-hydroxybutyrate dehydrogenase as described in the text.

the buffer concentration was decreased to 2.5 mM. Under these conditions, 60 to 75% of the total polymer was hydrolyzed by depolymerase without pretreatment with activator. The total available polymer for hydrolysis was determined with *R. rubrum* crude extract and was 70 to 83%, respectively. The remaining polymer is presumably inactive and denatured and unavailable for enzymatic hydrolysis (13).

The results reported above suggest that perhaps an inhibitor associated with native PHB granules, which prevents direct hydrolysis of PHB by the depolymerase, was removed by the extraction procedures. Indeed, it could be demonstrated that addition of the protein extract to the reaction mixture inhibits the direct hydrolysis of PHB of the extracted granules by depolymerase. Table 5 shows the inhibitory effects of the A-I and A-II fractions. Complete inhibition by A-I was not obtained even at relatively high concentrations. Of considerable interest was the reversal of A-II inhibition by the activator (Table 5, experiment I). Trypsin also caused a similar reversal. Similarly, the activator or trypsin reversed the inhibition caused by the A-I fraction. The mechanism whereby the activator prevents the inhibitory activity of the A-I or A-II fractions is not known. Since trypsin presumably destroys these inhibitors by proteolysis, it might be expected that the activator functions in a like manner. However, no evidence is available which indicates that activator preparations have proteolytic activity.

A number of proteins were tested to see whether the inhibitory effect was due to nonspecific adsorption of protein. Ovomucoid, hexokinase, albumin, and cytochrome *c* at 50 μg were ineffective as inhibitors. Lysozyme, however, did inhibit. For example, under the standard assay conditions 10 and 20 μg inhibited the reaction 30 and 71%, respectively. As with the A-II inhibitor, activator reversed the inhibition by lysozyme.

To be an effective substrate, the extracted granules must be freshly prepared since they lose their capacity to act as a substrate for the depolymerase with age. Our studies were carried out with preparations that were not allowed to age more than 48 hr after the extraction procedures. However, stability of preparations was somewhat variable, some lasting only 24 hr and others stable for more than a week.

The depolymerizing system was also extremely sensitive to the concentration of buffer used in the assay. For example, increasing the buffer concentration from 25 to 50 or 100 mM resulted in a 48 and 80% inhibition of the reaction, respectively. This inhibitory effect is presumably a reflection of the sensitivity of the extracted granules to an increase in salt concentration. A similar argument to that proposed earlier concerning the loss of reconstitutability of extracted granules after treating with certain chemical and physical agents may also be presented here. Thus, it is possible that PHB is hydrolyzable only while it is present in a particular conformational state which is maintained by its close association with protein. Alteration of this association may change the physical state of the polymer and result in preparations no longer suitable for the depolymerase. In support of the possibility are the observations of Ellar et al. (2) which have demonstrated that under certain conditions PHB molecules may assume different morphologies.

TABLE 5. *Depolymerization of extracted poly-β-hydroxybutyrate (PHB) granules: inhibition by A-II and A-I*[a]

No.	Additions during preincubation	Amt of β-hydroxybutyrate produced (μmole)
	Expt 1	
1	None	0.00
2	None	0.60
3	Activator	0.76
4	A-II (10.5 μg)	0.02
5	A-II (5.2 μg)	0.16
6	A-II (2.6 μg)	0.34
7	A-II (10.5 μg) + activator	0.27
8	A-II (5.2 μg) + activator	0.64
	Expt 2	
1	None	0.77
2	A-I (14 μg)	0.42
3	A-I (28 μg)	0.30
4	A-I (56 μg)	0.22

[a] Reaction mixtures contained extracted PHB granules (corresponding to 8 μmoles of the monomer) and 5 μmoles of potassium phosphate buffer (*p*H 7.6) in a total volume of 0.2 ml. The granules were preincubated for 10 min in the presence of activator (0.72 μg), A-I and A-II as indicated. After preincubation, depolymerase (1.23 μg) was added (except for no. 1 in experiment 1), and incubations continued for 15 min at 30 C. Reactions were terminated by heating in a boiling-water bath for 3 min, and, after centrifugation, a suitable sample was removed and assayed for β-hydroxybutyrate with D(−)-β-hydroxybutyrate dehydrogenase as described in the text.

Examination of the A-II fraction by disc electrophoresis in standard 7.5% gels at *p*H 9.3 revealed the presence of a single major band, two very minor bands, and a band which moved very close to the tracking dye.

DISCUSSION

The results of experiments reported in this

communication have shown that extraction with dilute alkali has resolved at least two components of the PHB granule: a protein fraction (A-I) whose function resides in PHB synthesis and a second protein fraction (A-II) which is capable of preventing the hydrolysis of PHB by depolymerase. Other types of complexes have also been dissociated by alkaline treatment. For example myosin, dissociated by alkali, has been successfully reconstituted so that it still maintains adenosine triphosphatase activity and actin-binding ability (4). Catalase after dissociation in alkali recovers the same physical and chemical properties as the native protein (16). Alkali has also been used for extraction of an oligomycin sensitivity-conferring protein from an oligomycin-sensitive adenosine triphosphatase complex (10); viruses dissociated by alkali can be reassembled to produce infective particles (3). Several possibilities exist which may explain the overall low recovery of PHB synthetase activity in the reconstituted system compared to the activity obtained with native PHB granules. Thus, the lability of the A-I fraction has been noted. Dissociation of A-I from the granule may result in a conformational change when liberated from the stabilizing matrix of the membrane protein of the native PHB granule. Other reasons for low recovery include the possibility that some component necessary for catalytic activity may have been destroyed. Alternatively the dissociation may result in altered conformation of the protein-PHB complex which remains. Under these conditions certain acceptor sites necessary for A-I binding are no longer available or possibly altered in such a way that reassociation is not optimal.

Although studies have not as yet been carried out to describe the role of A-I and extracted granules, a possible mechanism for PHB synthesis is the following:

$$\text{D}(-)\text{-}\beta\text{-hydroxybutyryl-CoA} + \text{A-I} \rightarrow \beta\text{-hydroxybutyryl-A-I} + \text{CoA} \quad (1)$$

$$\beta\text{-hydroxybutyryl-A-I} + \text{primer (extracted granules)} \rightarrow \beta\text{-hydroxybutyrate-primer} + \text{A-I} \quad (2)$$

The proposed mechanism suggests that PHB synthesis proceeds in two partial reactions with an acyl-enzyme intermediate in the initial reaction. The acyl group is then transferred to a primer acceptor in the second reaction. Some preliminary studies have, however, been obtained which might argue against the above pathway. For example, attempts to isolate β-hydroxybutyryl-A-I after incubation of A-I with D(−)-β-hydroxybutyryl-^{14}C-CoA in absence of extracted granules were unsuccessful. If indeed the A-I fraction functions as the PHB synthetase as indicated above, then it is conceivable that catalytic activity is only expressed after association with the extracted granule. Thus, in addition to its function as primer, the extracted granule may also induce appropriate conformational changes in the A-I protein.

Of particular interest were the properties of the extracted granules when examined in the depolymerizing system. The extraction resulted in preparations of granules which no longer require pretreatment with activator or trypsin. A-II was a potent inhibitor of the direct hydrolysis by depolymerase but whose effects could be reversed by activator or trypsin. We have concluded from these experiments that native PHB granules are associated with an inhibitor, presumably a protein which interferes in the hydrolysis of PHB by depolymerase. The inhibitor can be destroyed by proteolysis by trypsin, can be removed by the alkaline extraction, or its inhibitory effects can be prevented by the activator. The activation of native granules by activator has, however, been poorly understood since it has not been possible to demonstrate any proteolytic activity with this fraction. Presumably, it too interacts with the inhibitor, but no information concerning its mechanism is available. The mechanism of inhibition by the inhibitor and the mechanism of activator-inhibitor interactions are currently being investigated.

ACKNOWLEDGMENTS

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