Poly-β-hydroxybutyrate/Calcium Polyphosphate Complexes in Eukaryotic Membranes (42936)

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Abstract. **Poly-β-hydroxybutyrate/calcium polyphosphate (PHB-CaPolyPi) complexes exist as labile quasi-crystalline structures in bacterial plasma membranes. The composition, structure, and distribution of the complex suggest it may play a role in the regulation of intracellular calcium and in calcium signaling. The importance of these functions led to this investigation of the occurrence of PHB-CaPolyPi complexes in eukaryotes. A variety of plant and animal systems were analyzed and all were found to contain PHB associated with CaPolyPi. The intracellular location of the complex in bovine liver was primarily the mitochondria and microsomes, with smaller amounts in the plasma membranes. Eukaryotic PHB had the same narrow range of chain lengths (120–200 subunits) as PHB in bacterial membranes, and was associated with PolyPi of somewhat greater length (170–220) than the bacterial counterpart (130–170).**

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Poly-β-hydroxybutyrate (PHB) is a stereoregular polymer found in a wide variety of bacteria. *In vivo* condensation of R-(−)-β-hydroxybutyryl CoA forms linear polymers of 700–10,000 monomer units which are deposited as crystalline granules in the cytoplasm (1). A low molecular weight PHB, composed of 140–200 monomer units and complexed to calcium polyphosphate (CaPolyPi) of approximately the same length (130–170 units), was isolated recently from bacterial plasma membranes (2, 3). The structure of this complex was deduced by molecular modeling and molecular mechanics studies, using Corey-Pauling-Kendrew space-filling models and the computer program CHARMM (4). We concluded that PHB forms a transmembrane helix, characterized by an outer lipophilic coat of methyl and methylene groups and a polar lining of carbonyl ester oxygens, which surrounds a framework helix of CaPolyPi (Fig. 1). The two polymer helices are bridged by calcium ions which form ionic bonds to the phosphoryl oxygens of PolyPi and ion-dipole bonds to the ester carbonyl oxygens of PHB (Fig. 2). Export of Ca^{2+} and phosphate (Pi) may occur via elongation of the PolyPi chain at the cytoplasmic face by polyphosphate kinase-induced transfer of Pi from ATP (1, 5), combined with polyphosphatase activity at the outer face (5, 6). Furthermore, Ca^{2+} import may result from changes in membrane potential or an increase in the concentration gradients.

Since regulation of calcium transport is an important physiologic function, it was of interest to determine whether PHB-CaPolyPi complexes exist in eukaryotic membranes. Although it has been widely believed that PHB synthesis occurs only in bacteria (1), the immediate precursor of PHB, β-hydroxybutyryl-CoA, is ubiquitous. PolyPi synthesis is also well known to be widespread in eukaryotic systems (5). Therefore, a variety of plant and animal systems were examined for the presence of PHB and associated CaPolyPi. These components were found to be universally present. The intracellular location of the complex was investigated in bovine liver and found to be the membrane fractions, with highest concentrations in the mitochondria and microsomes.

Materials and Methods

Plant samples were obtained from retail stores. Animal samples were from the Michigan State University meat processing laboratory or retail butchers.

Determination of PHB. Total PHB was estimated from 1-g samples (wet weight) by a variation of the procedure of Karr *et al.* (7). Samples were dried at 90°C and extracted repeatedly with hot chloroform (60°C). Chloroform was removed with a stream of nitrogen and the residue was hydrolyzed in concentrated sulfuric

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acid at 90°C for 30 min to convert PHB to crotonic acid. After cooling, a 3-fold volume of saturated sodium sulfate was added, and crotonic acid was extracted with methylene chloride. A solution of 0.4 mg/ml adipic acid (500 μl) was added to the extract to serve as an internal standard and methylene chloride was removed with nitrogen as above. The aqueous remainder was chromatographed isocratically on an Aminex HPX-87H ion exclusion organic acid analysis column (300 × 7.8 mm) (Bio-Rad Laboratories) with 0.014 N H_2SO_4 (1 ml/min) as eluant. PHB was estimated from the peak area of crotonic acid; standards were purified PHB from *Azotobacter vinelandii* similarly treated. Values given are the average of two determinations; deviation was 5–20%. The identity of crotonic acid was confirmed by GC-MS as described previously (4). ^{1}H NMR spectra of PHB were measured in $CDCl_3$ solution containing tetramethylsilane as an internal standard ($\delta = 0$) with a Bruker WM 250 spectrometer. Spectra were recorded at ambient temperature with a spectrum width of 2500 Hz, 2- to 5-μsec pulse, and 100–1000 transients. Chemical shifts (μ) are given in ppm and coupling constantsj (J) in Hz: ^{1}H NMR, δ 1.26 (d, 3H, J = 6.5); δ 2.45 (dd, 1H, J = 5.8, 14.8); δ 2.60 (dd, 1H, J = 7.2, 14.8); δ 5.25 (m, 1H).

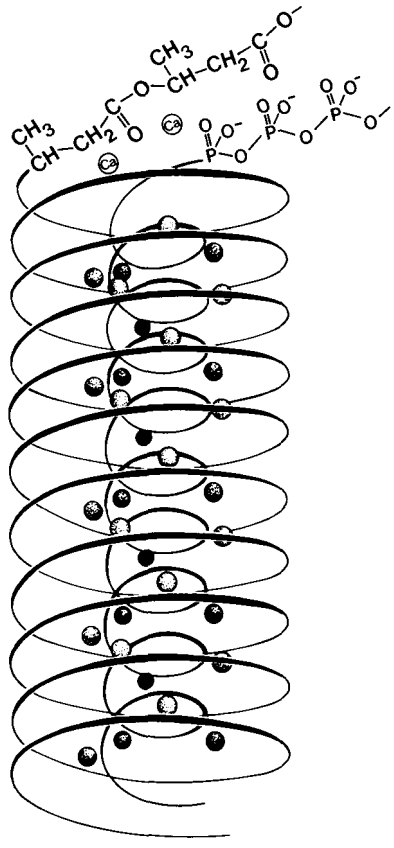

Figure 1. Drawing depicting the relationship between PHB, Ca^{2+} and PolyPi in the complex as indicated by studies with Corey-Pauling-Kendrew space-filling models and energy minimization studies using CHARMM. PHB forms an outer helical channel around a core helix of $PolyPi^-$ with Ca^{2+} bridging the two polymers

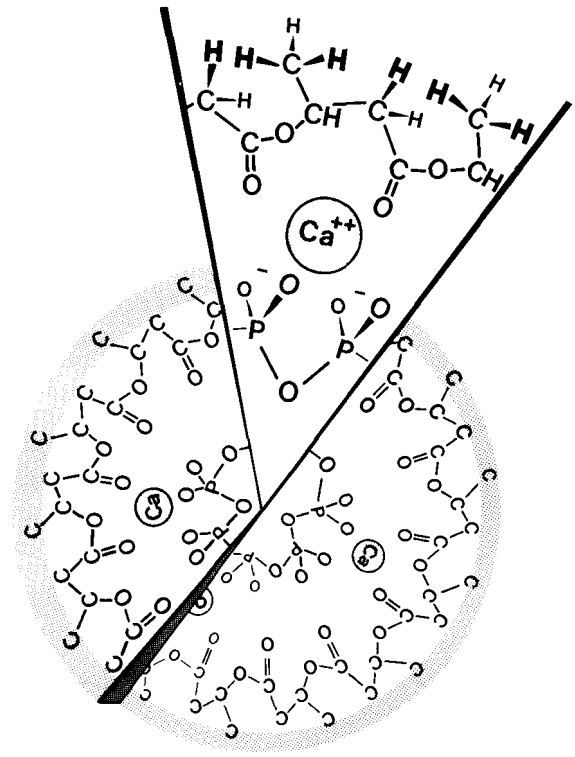

Figure 2. A view looking down the channel structure. The PHB outer helix has 14 monomer units per turn. The methyl and methylene groups form a lipophilic shell (stippling represents the hydrogens), and the carbonyl ester oxygens form a polar lined cavity. The PolyPi helix has 7 monomer units per turn with the phosphoryl oxygens facing outward and the chain oxygens alternately facing in and out. There are 3½ Ca^{2+} per turn, each is coordinated to four phosphoryl oxygens and four ester carbonyl oxygens (two of these are from the turn below and are not shown).

Isolation of the PHB-CaPolyPi Complex. Samples (100 g wet wt) were extracted sequentially with alcohol, 1:1 alcohol:acetone and acetone. The dried residue was extracted overnight with chloroform at 4°C and the chloroform solution was filtered. Chloroform was evaporated and this residue was digested with 5% sodium hypochlorite at room temperature, washed with alcohol and acetone, and redissolved in cold chloroform (4).

Separation and Determination of Complex Components. The chloroform solution containing PHB-CaPolyPi complex was extracted with water. Ca^{2+} and PolyPi partitioned into the water layer and PHB remained in the chloroform layer. PHB was purified by chromatography on a nonaqueous size exclusion column (Altex μ-Spherogel 10^3 Å, 8 mm × 30 cm, eluant $CHCl_3$) as described previously (4), then hydrolyzed in concentrated sulfuric acid and estimated as above. Ca^{2+} in the aqueous fraction was determined by graphite furnace AAS (4) and/or with the fluorescent Ca^{2+} indicator, fura-2 (8). PolyPi was purified by chromatography on an aqueous size exclusion column (Showdex OHpak B804/s, 8 mm × 25 cm, eluant 0.1 M NaCl) and determined by assay as described previously (4). Values are the average of two determinations; deviation was 10–30%.

Determination of Polymer Chain Lengths. Chain lengths were estimated by comparison of elution times on size exclusion columns (above) with those of standards as described previously (4). Polystyrene standards (Polysciences) were used for PHB and polyethylene glycols (Polysciences) for PolyPi.

Subcellular Fractionation. Bovine liver was disrupted in 0.25 *M* sucrose, 10 m*M* Hepes (pH 7.5), and subcellular fractions were prepared and purified as described by Fleischer and Kervina (9).

Enzyme Activities. Glucose-6-phosphatase and 5′-nucleotidase (5′-AMPase) were determined according to the method of Aronson and Touster (10). Succinate-cytochrome *c* reductase and rotenone insensitive NADH-cytochrome *c* reductase were determined according to the method of Fleischer and Fleischer (11).

Results

The occurrence and distribution of PHB in eukaryotes was investigated by examining a variety of plant and animal systems for the presence of the lipid polymer using a variation of the method of Karr *et al.* (7) which reliably measures PHB in samples containing levels > 0.01 μg. PHB was found in all samples examined in amounts ranging from 0.15 to 9.2 μg/g wet wt, thereby establishing its wide distribution in eukaryotic organisms (Table I). The amounts were comparable to or greater than those found in log phase cells of *Escherichia coli*, but considerably less than in genetically transformable cells of this organism (Table I). The chain lengths of the PHB were estimated by size exclusion chromatography as described previously (4). In every case, PHB eluted as a single narrow peak with a chain length of 120–200 subunits. The elution profiles of the eukaryotic polymers were indistinguishable from each other and from that of PHB isolated from *E. coli* plasma membranes which had been originally complexed to CaPolyPi (4). The chemical identity of the PHB was confirmed by ^{1}H NMR spectroscopy. The ^{1}H NMR spectrum was identical to that of bacterial PHB (*Azotobacter vinelandii*) and to published spectra (12, 13) and lends itself to straightforward interpretation. Thus, the secondary methyl group appears as a doublet at μ 1.26, the diastereotopic protons of the methylene group appear as a multiplet of eight sharp lines centered at δ 2.52 which is the AB portion of an ABX system with the parameters given in Materials and Methods. Finally, the methine proton is a multiplet at δ 5.25.

Table I. Occurrence of PHB and PHB-CaPolyPi Complex

Sample	PHB_{total}[a] (μg/g wet wt)	Complex components[b]		
		PHB (ng/g wet wt)	PolyPi (ng/g wet wt)	Ca^{2+} (ng/g wet wt)
Yeast[c]	0.26	22	12	6
Spinach leaves	0.86	163	88	47
Celery leaves	1.62	147	82	40
Celery stalk	1.10	92	45	24
Broccoli flowers	0.37	39	17	8
Broccoli stalks	0.57	64	29	14
Carrot root	0.25	24	11	6
Scallop	0.30	28	13	6
Chicken liver	1.54	111	57	28
Turkey liver	1.75	295	159	87
Beef liver	9.20	662	353	178
Turkey heart	1.20	92	43	22
Lamb heart	0.84	54	29	13
Beef heart	6.50	435	232	126
Pork kidney	0.95	69	36	19
Calf kidney	0.67	55	29	16
Beef brain	0.22	15	8	5
Chicken drumstick	0.15	19	8	4
Lamb shank	1.25	80	37	21
E. coli[d]				
Log phase	0.26			
Competent	156.00	9600	4200	2000

[a] Total PHB was estimated from 1-g samples (wet weight) by a variation of the procedure of Karr *et al.* (7) (see Materials and Methods). Values given are the average of two determinations; deviation was 5–20%.

[b] PHB-CaPolyPi complex was obtained from 100-g samples (wet weight) by anhydrous extraction at low temperatures (see Materials and Methods). PHB was purified by chromatography on a nonaqueous size exclusion column (4), then estimated as above. Ca^{2+} in the aqueous fraction was determined by graphite furnace AAS (4) and/or with the fluorescent Ca^{2+} indicator, fura-2 (8). PolyPi was purified by chromatography on an aqueous size exclusion column and determined by assay as described previously (4). Values are the average of two determinations; deviation was 10–30% (see Materials and Methods).

[c] *Saccharomyces cerevisiae*.

[d] Values for *E. coli* are estimated from previous studies (3, 4).

These samples were also examined for evidence of CaPolyPi complexed to PHB. An isolation procedure was designed to extract any such complex with minimal dissociation and to then separate and purify its constituents. Since PHB-CaPolyPi complexes are highly labile and the anhydrous CaPolyPi component is very hygroscopic, low temperatures and dry solvents were used throughout. The samples were first washed with alcohol and acetone to remove water and most of the lipids, and then extracted with chloroform. Ordinarily Ca^{2+} and PolyPi are very insoluble in chloroform, but when complexed with PHB they coextract with it into this hydrophobic solvent. This clearly distinguishes the CaPolyPi of the membrane complex from the many cytoplasmic polyphosphates. The chloroform-soluble material was purified of proteinaceous material by digestion with sodium hypochlorite, after which CaPolyPi which remained soluble in chloroform was separated from PHB by extraction with water. Finally, the individual polymers were purified and assayed (4, 8) (Materials and Methods).

Only a fraction of the PHB was isolated by this process. Losses were due primarily to coextraction into alcohol and acetone, incomplete extraction at low temperatures, and cochromatography with other lipids; but in every case PHB was found to be associated with CaPolyPi (Table I). Size exclusion chromatography (4) of PolyPi associated with PHB indicated they all were of the same length—170–220 subunits—which is slightly longer than the 130–170 subunits estimated for the corresponding bacterial species. The ratio of Ca^{2+} to PolyPi in all of the samples was roughly 1:2, as expected. However, the proportion of PHB to PolyPi was greater than the 1:1 ratio predicted by the relative lengths of the polymers, probably because dissociation of the labile complex leads to loss of CaPolyPi which partitions into water or precipitates leaving PHB in solution.

The intracellular distribution of the PHB-CaPolyPi complex was determined for bovine liver. Subcellular fractions were isolated and purified from disrupted liver cells by sucrose density gradient centrifugation (9) and characterized by enzymatic activities (10, 11) (Table II). An aliquot from each fraction was analyzed for total PHB content, and the remainder in each case was first digested with hypochlorite and then analyzed by the previous procedures to determine levels of PHB, PolyPi, and Ca^{2+} (Table III). In the event, PHB associated with CaPolyPi was isolated from several organelles with the highest concentrations occurring in the mitochondria and heavy microsomes. Based on total PHB, the complex in these organelles may store 3.2 and 2.8 nmol of Ca^{2+}/mg protein, respectively. Only the nuclear membranes and supernatant had no significant PHB. The relative proportion of CaPolyPi to PHB was smaller than in the whole cell samples, presumably due to its solubility in the sucrose gradient solutions.

Discussion

This survey of plant and animal tissues has shown that PHB is synthesized by a wide variety of eukaryotes (Table I). Indeed, the diversity of the samples examined is sufficient to suggest that PHB is universally present in cells or nearly so. The long chain PHB which form granular deposits in some bacteria were not found in eukaryotes. It was the relatively short chain PHB associated with CaPolyPi which was omnipresent. In bacteria, the PHB-CaPolyPi complex is situated in the plasma membrane (3); the intracellular distribution of the complex in bovine liver indicates that in eukaryotes the complex is also in membrane fractions, with highest concentrations in the mitochondria and microsomes (Table III).

Although the intact complex was not unequivocally demonstrated in this study, there is considerable evidence for its existence. PolyPi and Ca^{2+} are highly polar and very insoluble in chloroform, so that we may infer they are solubilized by complexation with lipophiles, and it seems likely that hydrophobic proteins or nonpolymeric lipids which could serve this purpose would be removed by the isolation protocol. Also, there is a

Table II. Characterization of Purified Subcellular Fractions of Beef Liver

Fraction	Total protein (g)	Glucose-6-phosphatase[a]	Succinate-cytochrome c[b]	5′-AMPase[a]	NADH-cytochrome c red[b]
Homogenate	22.6	112	116	42	311
Supernatant	5.6		3		6
Mitochondria	1.09	5	327	25	283
Microsomes (light)	0.69	235	7	67	355
Microsomes (heavy)	0.48	219	14	52	1170
Plasma membranes	0.11	32	25	891	112
Nuclear membranes	0.28	45	6		75

Note. Bovine liver was disrupted in buffered sucrose solution, and subcellular fractions were prepared and purified as described by Fleischer and Kervina (9). Enzymatic activities are expressed as nmol/min mg protein. Values given are averages of data from two experiments.

[a] Glucose-6-phosphatase and 5′-nucleotidase (5′-AMPase) were determined according to the method of Aronson and Touster (10).

[b] Succinate-cytochrome c reductase and rotenone insensitive NADH-cytochrome c reductase were determined according to the method of Fleischer and Fleischer (11).

Table III. Intracellular Distribution of PHB and PHB-CaPolyPi Complex in Bovine Liver

Fraction	PHB_{total}[a] (ng/mg protein)	Complex Components[b]		
		PHB (ng/mg protein)	PolyPi (ng/mg protein)	Ca^{2+} (ng/mg protein)
Homogenate	287	39	17	9
Supernatant	12			
Mitochondria	560	80	26	12
Microsomes (light)	105	14	5	2
Microsomes (heavy)	480	74	18	8
Plasma membranes	132	19	7	4
Nuclear membranes	18	2	tr	

[a] Fractions were prepared as described in Table II. Total PHB was determined in portions of the homogenate and organelles (2–9 mg of protein) as described in footnote *a* to Table I. Values are the average of two determinations; deviation was 10–20%.
[b] The remainder of the homogenate and organelle fractions (see Table II) were each digested with 5% sodium hypochlorite, and PHB, PolyPi, and Ca^{2+} were purified and analyzed as described in footnote *b* to Table I. Values are the average of two determinations; deviation was 10–35%.

relatively constant ratio between PHB and CaPolyPi recovered despite the lability of the complex.

The sensitivity which the complex in bacteria has demonstrated to intra- and extracellular events (2, 3) indicate that the structure traverses the membrane, and the chain length of PHB, which is the same for prokaryotes and eukaryotes within the accuracy of the measurements, is sufficient for this purpose (4). The chain length of eukaryotic PolyPi is somewhat greater than that of the corresponding bacterial PolyPi, but since this polar polymer extends from the PHB envelope into the cytoplasm and the outer aqueous medium (Figs. 1 and 2), its length may be less critical. The ubiquitous nature of the complex would suggest that PHB-Ca-PolyPi structures have an important physiologic function. The composition, putative structure, and intracellular distribution of the complex suggest it is involved in the storage and transport of Ca^{2+} and PO_4^{2-} which would give it a role in the regulation of intracellular Ca^{2+} and in Ca^{2+} signal transmission.

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