Amphiphilic lipids can form effective barriers between a cell and the outside world. However, they obstruct the movements of ions into and out of the cell, which are essential to cellular life. The earliest cells, called protocells, had 'leaky' monolayers or bilayers composed of single-chain amphiphilic lipids that would have permitted the passage of ions. However, as these lipid barriers evolved and became more impenetrable, nanopores known as ion channels must have formed to provide selective pathways for ions across the hydrophobic regions. The channels had to be able to discriminate among very similar ions and respond to changes in the membrane potential. When ions flow through the channels in response to stimuli, they create an electrical current that changes the voltage across the membrane by altering the distribution of charge, and a chemical gradient due to the differential concentration of ions across the bilayer. The potential energy stored in this electrochemical gradient can then be used to fuel cellular reactions.

Such ion channels must have appeared before the evolution of polypeptides, which were relatively late arrivals in the history of life. It is unlikely that single amino acids or short peptides, such as those found in meteorites or produced in prebiotic experiments, had this proficiency [1,2]. Auspiciously, two simple polymers - inorganic polyphosphates (PolyP) and poly-(R)-3-hydroxybutyrates (PHB) - were available in the primordial environment that could join together to provide the energy for evolving cells.

Abstract

Ion channels are pore-forming structures across lipid bilayers that control the movement of selected ions into and out of cells along their electro-chemical gradients. The flow of ions through the channels establish voltage and ion concentration gradients across the lipid bilayer that are essential to cell survival. It follows that ion channels were components of the earliest cells and existed before the emergence of polypeptides of sufficient complexity to control transbilayer ion movement. Here we examine the formation of ion-selective channels from two primordial biological polymers - inorganic polyphosphates (PolyP) and poly-(R)-3-hydroxybutyrates (PHB) - and follow their evolution into modern proteinaceous ion channels.

Introduction

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It is unlikely that single amino acids or short peptides, such as those found in meteorites or produced in prebiotic experiments, had this proficiency [1,2]. Auspiciously, two simple polymers - inorganic polyphosphate (PolyP) and poly-(R)-3-hydroxybutyrates (PHB) - were available in the primordial environment that could join together to provide the energy for evolving cells.

Inorganic polyphosphate (PolyP) - the first biopolymer

Before the emergence of organic biopolymers – poly-(R)-3-hydroxybutyrates (PHB), carbohydrates, polynucleotides, polypeptides – inorganic polymers of phosphate (PolyP) were formed at sites where phosphate rocks were exposed to dehydrating conditions, such as those that existed in deep-sea thermal vents and volcanic residues [3-5].

\[
\text{n(H}_2\text{PO}_4^-) \rightarrow \text{(PO}_3^-)_n + \text{nH}_2\text{O}
\]

PolyP are constituents of all biological cells - archaeal, eubacterial and eukaryotic [6-9]. The ubiquity of PolyP, their physical properties, and their presence in the primordial environment make them likely constituents of the earliest protocells.

Linear PolyP are structurally very simple. They consist of flexible chains of few to hundreds of tetrahedral orthophosphate residues linked together through shared oxygen atoms [5,9] (Figure 1). The first pK of phosphates is ~ 2.2 and successive ionization constants differ by factors of 10^5; accordingly, at physiological pH, each of the internal phosphate units is strongly acidic and bears a monovalent negative charge, whereas the terminal phosphates are weakly acidic (pK < 7.2) and carry a divalent negative charge at physiological pH (~7.4) [10,11]. However, it is relevant to the selectivity of ion channels that only a small decrease in local pH is required to reduce the charge on the terminal units from divalent to monovalent.
PolyP are stable at neutral pH and room temperatures. Their negative charges protect them from rapid hydrolytic attack by water, making them kinetically stable, in spite of the pronounced thermodynamic instability that makes them effective phosphorylating agents [11-15]. Only half of the phosphoanhydride bonds are hydrolyzed after several years, and this hydrolysis does not occur randomly, but processively from the end of the PolyP chain.

**PolyP as phosphate stores**

PolyP have been found as phosphate stores in all classes of biological cells. In 1888, Liebermann isolated high molecular weight inorganic phosphates from the cytoplasm of yeast cells, which he called "metaphosphate" [16]. In 1895, Babès observed granules in *Corynebacteria* that were metachromatic, i.e. the granules stained red or purple when treated with blue dyes [17], and called them “Babès” granules or, in reference to his collaborator, “Babès-Ernst” granules. In 1904, Meyer observed metachromatic granules in *Spirillum volutans* and named them “volutin” [18]. In 1947, Wiame identified the phosphate in yeast granules as PolyP and renamed them “polyphosphate” or “polyphosphate granules” [19]. In 1995, Docampo et al. observed acidic electron-dense organelles that were rich in calcium in *Trypanosoma cruzi* and called them “acidocalcisomes” [20]. The acidocalcisomes were later found to be similar to the volutin or PolyP granules in bacteria [21]. They contain long-chain (>500 units), medium-chain (50-100 units), short-chain PolyP (<15 units) and pyrophosphate (PPi), as well as enzymes involved in PolyP metabolism. They are surrounded by membranes, which contain many enzymes including ion pumps, antiporters and channels [22]. The acidocalcisomes have been observed in a wide variety of prokaryotes – archaea, eubacteria, cyanobacteria - and also in diverse eukaryotes – algae protozoa, fungi, plant cells and animal cells, including human cells, indicating that they are ubiquitous components of biological cells [20-24].

**PolyP as energy sources**

PolyP are weak bases. Energy is stored in the covalent bonds between the phosphates of PolyP, with the greatest amount of energy in the bonds between the internal phosphate groups, i.e. the phosphoanhydride bonds [4,5]. The flow of energy in biological cells is governed by the formation and hydrolysis of these phosphoanhydride bonds, which are frequently referred to as 'high energy' bonds. The term ‘high energy’ does not relate to the amount of energy it takes to break the bond but rather to the amount of energy released when a P-O-P phosphoanhydride bond is hydrolyzed. Bond breaking always requires an input of energy; however, the electrostatic repulsion between the negatively charged phosphate oxygens favors separation of the phosphate anions, and the resonance stabilization of the phosphate anions produced by the hydrolysis exceeds the resonance stabilization of PolyP itself. Consequently, the end result of the hydrolysis of phosphoanhydride bonds under physiological conditions is the release of energy [9-12].

The standard free energy of hydrolysis of a P-O-P phosphoanhydride bond in linear PolyP is -40.6 kJ/mol or - 9.7 kcal/mol [25]. The actual free energy of hydrolysis in biological cells depends on many other factors such as Mg$^{2+}$ concentration, pH, temperature and ionic strength. The amount of energy in a phosphoanhydride bond is high relative to the amount of energy released in the organic chemical reactions that occur in living systems. It is within the range of the energy liberated in the hydrolysis of the terminal phosphoanhydride bonds of adenosine triphosphate (ATP) (-30.5 kJ/mol or -7. 3 kcal/mol) or the phosphoanhydride bonds of phosphocreatine (-43.1 kJ/mol or -10.3 kcal/mol) or glucose-1-phosphate (-20.9 kJ/mol or -5.0 kcal/mol) [25,26]. As a result of their intermediate position in the thermodynamic scale of phosphate anhydrides, PolyP have the capacity to accept and/or donate phosphates from many other phosphate anhydrides [6,7,27-29].

**PolyP as cation selectors**

PolyP have remarkable ion exchange and ion selection properties. The phosphate group is itself an optimal coordination partner for metal cations in that it exhibits a flexible tetrahedral geometry with variable bond lengths and angles [4,5]. The low energy barrier to rotation about the P-O-P bond of PolyP allows the phosphate tetrahedra to adjust their conformations to correspond to the charge requirements and coordination preferences of approaching cations (Figure 2). Bond angles vary from ~ 120° to 180°, and the orientation of the linked tetrahedra range from eclipsed to staggered conformation [4,5]. Since cations acquire no advantage...
from their size or coordination geometry, cations with multiple charges are selected, owing to their stronger electrostatic interactions and higher binding energies. These physical properties, which enable PolyP to sequester trace amounts of divalent cations from solutions containing monovalent salts, are important attributes for effecting selective binding and permeation of cations in ion channels. However, when divalent cations are not available, PolyP will proficiently bind monovalent cations.

Although PolyP are highly efficient at distinguishing among cations of dissimilar charge, they are less efficient at discriminating among cations of the same charge. Ion exchange relationships for ions of the same charge follow the same trend as their hydration energies and thus follow the sequence \( \text{Mg}^{2+} > \text{Ca}^{2+} > \text{Na}^+ > \text{K}^+ \); accordingly, among divalent cations \( \text{Ca}^{2+} \) is favored over \( \text{Mg}^{2+} \) and among monovalent cations \( \text{K}^+ \) is favored over \( \text{Na}^+ \) [30].

**PolyP as cation conductors**

Whereas PolyP are well known as stores of phosphate anions, and their phosphoanhydride bonds are recognized as important sources of biochemical energy, their unique and essential role as ion conductors has been largely overlooked. Yet PolyP molecules have remarkable ion transport properties. The supple chains of phosphate anions are essentially the ‘electric wires’ of the cell. The PolyP backbone is a flexible ladder of identical, closely-spaced, negatively-charged sites that attract cations, select among cations by charge, partially dehydrate cations, and then may transport those cations in an orderly fashion along its backbone in response to a sufficient potential force. Clearly, a PolyP molecule positioned across a lipid bilayer is as an efficient vehicle for the transbilayer movement of cations (or electrons). The obstacle to such an arrangement however, is the insertion of the polar PolyP salt across the hydrophobic region of the lipid bilayer.

The lipid composition of protocellular membranes is not known; however, it is reasonable to assume that the monolayers and bilayers of the earliest vesicles were composed of simple self-assembling single-chain amphiphilic lipids composed of hydrophilic head groups and hydrophobic chains of methylene or isoprenyl groups that were likely to be present in the prebiotic environment [31]. Such vesicles are known to be permeable to ions, polar molecules and polymers due to their greater lipid dynamics [32-38]. They also tend to display dramatic increases in permeability at the phase transition temperature [35]. These properties suggest that PolyP could interweave across such lipid enclosures through conduits formed by transient pores or packing defects and function as primitive ion channels (Figure 3A).

As simple single-chain lipids were gradually replaced by glycerolipids and then by glycerophospholipids to allow the developing cells to retain essential metabolites and improve their control over import and export functions, lipid bilayers would have become increasingly more impenetrable. Modifications of lipid bilayer structure must have been very gradual processes to allow for the coevolution of bilayer transport systems [39-41]. During this extended period, there would likely have been intermediate evolutionary steps, before the development of polypeptides, in which simple lipid-soluble molecules compensated for the increasing bilayer impermeability. This period in bilayer evolution may well have corresponded with the emergence of the polyesters, poly-(R)-3-hydroxybutyrates (PHB).
PHB are linear polymers of R-3-hydroxybutyrate (R-3HB) (Figure 4). The biosynthesis of PHB requires only acetate and reducing potential. In bacterial cells, PHB are formed from acetyl-CoA in three steps: condensation of two acetyl-CoAs to form acetoacetyl-CoA (catalyzed by β-ketothiolase (phaA)), reduction of acetoacetyl-CoA to form the monomer (R)-3-hydroxybutyryl-CoA (R3HB-CoA) (catalyzed by acetoacetyl CoA reductase (phaB)), and polymerization of R-3HB-CoA with loss of CoA to form PHB (catalyzed by PHB polymerase (phaC)) (Figure 5) [42].

**Poly-(R)-3-hydroxybutyrates (PHB) - the first organic biopolymer?**

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In eukaryotes, 3-hydroxy-3-methylglutaryl-CoA synthase (HMG synthase) catalyzes the condensation of acetoacetyl-CoA with a third acetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). The enzyme HMG-lyase then catalyzes the decomposition of HMG-CoA to form acetoacetate and acetyl-CoA, and acetoacetate is further reduced with NADH by R-3-HB dehydrogenase to form R-3-HB (Figure 5). Enzymes that polymerize R-3-hydroxybutyrate or its CoA ester in eukaryotes have not yet been identified.

The simple structure and relative ease of formation of PHB suggest they were among the earliest, and likely the first, of the organic biopolymers. In the early earth, there was a reducing atmosphere, and acetate could have been synthesized from methane and carbon monoxide with the aid of catalysts. Studies have shown that an aqueous slurry of co-precipitated NiS and FeS convert CO and CH\textsubscript{3}SH into the thioester CH\textsubscript{3}-CO-SCH\textsubscript{3}, which then hydrolyzes to form acetic acid [43]. When NiS-FeS was modified with catalytic amounts of selenium, acetic acid and CH\textsubscript{3}SH were formed from H\textsubscript{2}S and CO as the only carbon source.

**Long-chain PHB:** PHB were first identified in 1925 in *Bacillus megaterium* by Lemoigne as high-molecular-weight long-chain polymers (100-1000 kD) contained within cytoplasmic granules (inclusion bodies) [44]. Similar granules of long-chain PHB have since been found in a wide variety of euabacteria and archaea. When cell growth is limited in these organisms by the absence of nutrients other than carbon, acetyl-CoA is diverted from synthesis of proteins and nucleotides to the formation of PHB [45-47]. The polymers, which are accumulated in osmotically neutral granules that may constitute up to 80% of the cell dry weight, are presumed to serve as carbon and energy stores. Elustondo et al. [48] found PHB associated with granules in mammalian cells. The PHB-enriched granules did not co-localized with mitochondria, lysosomes, or endoplasmic reticulum, suggesting that PHB can accumulate in the cytoplasm of mammalian cells in granules similar to 'energy storage' carbonosomes found in PHB-accumulating bacteria.

**Short-chain PHB:** While only certain prokaryotes accumulate high molecular weight, long-chain PHB within cytoplasmic granules, short-chain polymers of R-3HB (< 150 residues) are universal cell constituents, having been found in representative organisms of all phyla [49-55]. Short-chain PHB are broadly distributed among all cellular compartments as well as intra- and intercellular fluids where they are usually found in association with other cellular molecules. In some studies, they have been referred to as cPHB (complexed PHB) [49-58] or cOHB (complexed oligo-(R)-3-hydroxybutyrates) [59-63], depending on their chain-length. The short-chain PHB can be further divided into polymers whose association with other molecules is entirely noncovalent (free short-chain PHB), and polymers (usually < 15 residues) in which the carboxy terminus is covalently attached to an amino acid residue (usually serine) of polypeptides.

Despite their universal presence in biological cells, short-chain PHB were undetected until the early1980s. This oversight may be attributed to their low molecular size, lack of signal atoms or moieties, sparse distribution within cells, and the lack of sensitive methods for detection. Moreover, due to the greater lability of ester bonds as compared to amide bonds, the polymers are hydrolyzed during Edman sequencing which exposes them to extremes of pH at elevated temperatures. They also tend to fragment during mass spectroscopy, and their molecular flexibility and disordered conformations make them ‘invisible’ in X-ray structures in which they may be mistaken for lipids or detergents. Free short-chain PHB were first discovered by Reusch and Sadoff in the early1980s in the cytoplasmic membranes of genetically competent bacteria [64]. Covalently attached, short-chain PHB escaped detection until more than a decade later, when they were identified in Western blot assays of *E. coli* proteins by Huang and Reusch [65].

**Structure and physical properties of short-chain PHB**

In the solid-state, PHB forms 2\textsubscript{1} helices with a pitch of 5.96 Å that fold in lamellar crystals of ~ 50 Å thickness [66-70]; however, short-chain PHB incorporated within lipid bilayers at physiological temperatures may be considered to be in a state of solution. As polymers lack the stabilizing influence of hydrogen bonding, they have more flexible structures than polypeptides. Akita et al studied the structure of short-chain PHB in dilute solution, using viscometry, light scattering, and optical rotatory dispersion, and concluded that the polyester forms a random coil [71]. Seebach et al confirmed the extremely high conformational flexibility of the PHB backbone by studies of short-chain PHB in solution and in phospholipid bilayers using NMR spectroscopy, circular dichroism, fluorescence microscopy and molecular dynamics simulations [72-75].

PHB have a high intrinsic viscosity [76]. They are water-insoluble and relatively resistant to hydrolytic degradation. PHB are amphiphilic - methyl groups alternate with ester carbonyl oxygens (Figure 4). While the ester groups must be maintained in their preferred antiperiplanar orientation, slight changes in one or more of the three other backbone dihedrals have pronounced effects on the conformation of the molecules [77]. This flexibility and amphiphilicity allow short-chain PHB to engage in multiple noncovalent interactions with other molecules - the methyl groups may form hydrophobic bonds and the ester groups may act as hydrogen bond acceptors and/or form coordinate bonds to cations. Such interactions allow PHB to transform the surface of a polypeptide from hydrophilic to hydrophobic or hydrophobic to hydrophilic [78] (Figure 6). Moreover, PHB may itself be rendered soluble in aqueous media by complexation to water-soluble proteins, such as albumin. These remarkable attributes led Albert et al [79] to refer to PHB as a 'chemical chameleon'.
It is important to realize, however, that the flexibility of the PHB backbone is highly sensitive to changes in temperature below the physiological range. The glass transition temperature of granule PHB is -0.5 - 5 °C [80,81]; hence, though short-chain PHB are malleable, amorphous molecules at physiological temperatures, their flexibility decreases markedly when temperatures are lowered below this range. An important consequence of this effect is that the conformations and thus the functions of PHB-conjugated proteins will be sensitive to temperature. Accordingly, studies of PHB-conjugated proteins should be conducted at physiological temperatures.

Yet another remarkable physical characteristic of PHB is that it possesses the molecular properties found only in a small group of synthetic polymers, known as polymer electrolytes, which are characterized by their capacity to form ion-conducting complexes with salts [82-85]. Noteworthy features of these polymer electrolytes are (1) flexible backbones with low barriers to bond rotation to allow segmental motions of the polymer chain, (2) heteroatoms with sufficient electron donor power to form coordinate bonds with cations, and (3) a suitable distance between these heteroatoms to permit the formation of multiple intra-polymer coordinate bonds to cations.

The most well known member of this class of synthetic polymers is the polyether, polyethylene oxide (PEO), whose complexes with lithium perchlorate have been used commercially in lithium batteries. The solvating power of PEO may be attributed to the optimal spacing of ether oxygens along a flexible backbone, allowing multiple contacts between these electron-donating groups and cations. It is significant that when this spacing is decreased, as in polymethylene oxide (PMO), the chain flexibility is greatly reduced, and that when it is increased, as in 1,3-polypropylene oxide (1,3-PPO), the distance between oxygens is too large for optimal polymer-cation coordination. In either case, the ability of the polyether to solvate cations is greatly reduced or lost. It is also relevant that substituents pendant to the backbone, as in 1,2-polypropylene oxide, diminish but do not eliminate the ability of the polymers to form ion-conducting complexes. Polyesters with flexible backbones and proper spacing between electron-donating ester oxygens may also serve as polymer electrolytes; e.g. poly-3-hydroxypropionate forms ion-conducting salt complexes with lithium perchlorate [85]. Since PHB have flexible backbones and their ester oxygens bear the same relationship to poly-3-hydroxypropionate as 1,2 polypropylene oxide does to polyethylene oxide (Figure 7), it follows that PHB possess the structural features essential to the formation of ion-conducting salt complexes. PHB may be regarded as the polymer electrolytes of the cell.

The stability of such polymer salt complexes is enhanced by the entropic advantage known as the ‘polymer effect’; i.e. the cooperative effect of neighboring ligands attached to a common backbone. Segmental motions of the polymer backbones transport the cations from one complexation site to the next, moving them along the polymer chain in an orderly manner in response to a sufficient electrochemical gradient. Since this process requires significant fluidity of the polymer chain, the polymers are only conductive in the amorphous state, i.e. above their glass temperatures. Accordingly, PHB would be capable of transporting cations across lipid bilayers at physiological temperatures.
Transbilayer ion transport by short-chain PHB

The molecular flexibility, amphiphilicity and salt-solvating properties of PHB allow them to act as intermediaries between polar molecules and the lipid bilayer. Short-chain PHB can facilitate the passage of a polar molecule across the lipid bilayer or enable it to reside within the bilayer. As the lipid bilayers of primordial cells evolved to become less penetrable to polar molecules, the contemporaneous synthesis and insertion of short-chain PHB into these lipid bilayers would have allowed PolyP salts to continue to penetrate into and subsist within them (Figure 3B).

The physical properties of PHB that are critical to their ability to transport ions across lipid bilayers were established by studies in planar lipid bilayers. A match between polymer length and bilayer width proved to be an important factor. The width of planar lipid bilayers, formed by 1, palmitoyl, 2, oleoyl-phosphatidyl choline (16:0, 18:1 PC), as estimated from electrical capacitance measurements [90], is 48 Å, and the thicknesses of the lamellar crystallites formed by monodisperse oligomers (Mw/Mn = 1.0) of R-3HB of 8, 16, and 32 units, as measured by transmission electron microscopy, X-ray scattering and atomic force microscopy, are ca. 26, 52, and 53 Å, respectively [91,92]. Seebach et al. [88] observed channel-forming activity for PHB of 16, 32, 64, and 96 monomer units, but not for 8mers. It appears that the oligomers must span the bilayer at least once, and oligomers > 16 units fold back on themselves in a hairpin arrangement. Stereo-regularity was not essential; however, end group structure was found to be of importance; oligomers in which the end groups had been derivatized displayed no single-channel activity.

Studies of the structure of PHB in solution and in phospholipid bilayers by Rueping et al. [75], using circular dichroism and fluorescence spectroscopy, were compatible with the inference that longer PHB chains fold in a hairpin fashion in these environments. Considering that high concentrations of the polyesters are required to form channels (≥ 0.1%), it seems probable that the pores are formed by aggregates of several molecules of appropriate size. Seebach et al. [88] propose that the oligomers form islands of lamellar crystallites in the bilayer, and that ion permeability results from areas of mismatch at the interfacial regions between phospholipids and the oligomers.

Further insights into the importance of the relationship between polyester size and bilayer width were provided by the studies of Das et al. [93], who examined the channel-forming ability of synthetic oligomers of R-3-HB prepared synthetically by Jedlinsky et al. [94]. These oligomers had an average of 19 residues by number average molecular weight measurements (Mn) and 23 by weight average molecular weight measurements (Mw) and thus are referred to as PHB19/23. The end groups of these polymers (OH and COOH groups), as shown by NMR and ESI-MS spectroscopy [95], were the same as those present in natural PHB. Solid-state measurements based on a 2, helix conformation of PHB indicate that the length of oligomers with 19 units and 23 units are ca. 57 Å and 69 Å, respectively [96,97]. PHB19/23 did not form channels in bilayers of 16:0 18:1 PC, even at concentrations up to 2.5%. This failure was attributed to a mismatch between oligomer length and bilayer width (48 Å). The oligomers were considered to be too long to remain fully extended in this bilayer and too short to fold properly. To test this hypothesis, the complexes were incorporated into bilayers of 1,2 diecosenoyl phosphatidyl choline (d20:1 PC), and separately into bilayers of 1,2 dierucoyl phosphatidyl choline (d22:1 PC). Since each additional methylene group adds about 1.27 Å to the bilayer [96], the widths of the d20:1 PC and d22:1 PC bilayers were estimated as 53 Å and 58 Å, respectively. At concentrations ≥1% of phospholipids (w/w), PHB19/23 displayed channel activity in both bilayers, but much more frequent channel formation in the wider d22:1 PC bilayer.
It is important to note that the channels formed by short-chain PHB in planar lipid bilayers are essentially non-selective pores. In all studies, the conductance of the channels was highly variable and they exhibited high open probabilities, indicating they were essentially open pores. The channels did not discriminate among cations of the same charge, and they also did not distinguish among cations by charge. The current records of the PHB channels were similar to those observed with ionic and non-ionic detergents, such as Triton X-100, octylglucoside, or sodium dodecyl sulfate, in planar lipid bilayers [98,99].

In summary, the primordial organic biopolymer PHB has important and unique physical properties. Short-chain PHB form non-selective ion channels in planar lipid bilayers, provided that there is a rough correspondence between oligomer length and bilayer width. The data suggest that PHB longer than bilayer width can bend back in hairpin turns to fit within the confines of the hydrophobic region, but each of the folded segments should be of approximately bilayer length. It is postulated that short-chain PHB aggregate in clusters, with molecules arranged parallel to fatty acyl chains. Planar bilayer studies demonstrate the ability of PHB to make bilayers permeable to ions; however, PHB channels cannot select among cations whether by charge, size or coordination geometry.

Moreover, PHB must be incorporated into bilayers at high concentrations, and the resulting channels lack voltage sensitivity. Like PolyP, PHB has some of the essential properties of physiological ion channels but not others. Auspiciously, the two polymers complement each other, and together they possess all the requisite characteristics of well-regulated ion-selective channels.

PHB complexes in prokaryotic membranes

PHB complexes were first discovered in bacteria, by Reusch and Sadoff [64], while observing the thermotropic lipid-phase transitions of the cytoplasmic membranes of the Gram-negative bacterium *Azotobacter vinelandii* during log-phase growth and at stages throughout the development of genetic competence, which develops gradually and peaks at ~ 18 hrs. (Figure 8). The lipid-phase transitions, which reflect changes in phospholipid chain mobility, bilayer thickness, and bilayer viscosity, were observed from 5 °C to 65 °C in whole cell cultures by fluorescence emission spectroscopy using the fluorescent probe, N-phenyl-1-naphthylamine (NPN), which reports lipid phase transitions in reasonable agreement with light scattering and X-ray diffraction [100].

The phospholipid phase transitions, begin below 0 °C and end at ~ 22 °C. As temperatures continue to increase, one observes a sharp increase in fluorescence that begins at ~ 42 °C and peaks at ~ 56 °C. The intensity of this high temperature phase transition correlates positively with the transformation efficiency of the cells and with the concentration of short-chain PHB in the cytoplasmic membranes [101]. The same high temperature phase transition is observed in genetically competent cells of Gram-negative *Escherichia coli* which does not synthesize long-chain PHB and which becomes genetically competent by chemical treatment. The high temperature phase transition is completely absent in log-phase *E. coli* cells (Figure 9A) but very strong in competent cells (Figure 9B), and its intensity correlates positively with the transformation efficiency of the cells and with the concentration of short-chain PHB in the cytoplasmic membranes [101,102].

\[\text{Figure 8: Thermotropic fluorescence spectra of N-phenyl-1-naphthylamine (NPN) in A. vinelandii UW1 cells during log-phase vegetative growth and at stages of increasing genetic transformability. Fluorescence spectra of NPN in A. vinelandii UW1 cells during log phase vegetative growth and at various stages of genetic transformability. Fluorescence intensities are relative. Excitation, 360 nm; emission, 410 nm. Measurements were made at increasing temperature (ca.2 °C/min). From Reusch and Sadoff 1983 [64].}\]
The fluorescence peaks above physiological temperatures suggest that PHB forms an organized complex structure in the bilayer. The intact PHB complexes have no conspicuous effect on the viscosity of the phospholipid bilayer at or below physiological temperatures. However, as the temperature rises above physiological values, there is a pronounced increase in NPN fluorescence indicating a sharp increase in bilayer viscosity, which may be attributed to the release of highly viscous free PHB as the complexes dissociate. This phase transition is irreversible. When temperatures are decreased, the bilayer viscosity remains high indicating that the complexes do not reform (Figure 9C).

The fluorescence curves indicate that log-phase cells contain few if any PHB membrane complexes, but the complexes increase in number rapidly as the cells become genetically competent. When the competent cells are returned to log-phase growth, the high-temperature fluorescence peak rapidly diminishes or disappears, signifying that the complexes have been withdrawn from the bilayer. Inhibition of protein synthesis in *E. coli* had no influence on the NPN fluorescence spectrum or the degree of genetic competence, but inhibition of PHB synthesis prevented both the appearance of the NPN fluorescence peak and the development of genetic competence [101].

Freeze-fracture electron microscopy reveals that the development of genetic competence, whether in *A. vinelandii* by physiological means or in *E. coli* by chemical treatment, coincides with the appearance of semi-regular protein-free plaques containing shallow particles in the cytoplasmic but not the outer membranes [102] (Figure 10). The plaques in both organisms grow in size and frequency as the concentration of PHB in the membranes increases.
In summary, all methods of inducing genetic competence in bacteria examined thus far result in the formation of PHB complexes in the cytoplasmic membranes. In all cases, there is a direct relationship between the concentration of the PHB complexes in the membranes and the genetic competence of the organism. When cells are returned to log-phase growth, the PHB complexes are rapidly withdrawn from the membranes.

Composition of PHB membrane complexes

The PHB complexes were isolated from the membranes of competent *E. coli* cells and analyzed. The molecular size of the PHB, estimated by viscosity measurements [103], size-exclusion high-pressure liquid chromatography [52], and electrospray MS (~12 kDa) [57] was ~140 residues (~12 kDa) (Figure 11). The PHB complexant was identified as PolyP by chemical assay [104], and by enzymatic assays using polyphosphate kinase and exopolyphosphatases [105-107], and its chain length was estimated by acrylamide gel electrophoresis [108] and electrospray MS [57] as ~65 residues (~5 kDa) (Figure 11). The neutralizing cation for PolyP was determined to be Ca\(^{2+}\) by graphite furnace atomic absorption spectrometry [103]. The molecular weight of the complexes was estimated as ~17 kDa by size-exclusion chromatography [78] and electrospray MS [57] (Figure 11), indicating that the complexes are formed from one strand of CaPolyP and one strand of PHB. The extracted complexes, when incorporated into liposomes exhibit a fluorescence peak with NPN in the same temperature range as the complexes in whole cells [103].

![Electrospray mass spectrum (positive mode) of PolyP/PHB complexes](image)

The sum of the data indicate that the complex is composed of the polymers PHB and PolyP in a ratio of ~2:1, and that the complexed cation is Ca\(^{2+}\). The composition of the complex was confirmed by reconstituting it from PHB, extracted from competent *E. coli*, and CaPolyP formed from commercial sodium polyphosphate (average chain length 65) [77]. The reconstituted complex, incorporated into liposomes, displayed the characteristic thermotropic fluorescence peak at 56 °C with NPN.

Molecular structure of PolyP/PHB complexes

The molecular structure of PolyP/PHB complexes remains uncertain; however, some assumptions can be made concerning the general organization of the complexes from the physical properties and sizes of the polymers, and the low dielectric environment they inhabit. It is clear that the highly polar polyanionic PolyP must be shielded within the hydrophobic region of the lipid bilayer by the amphiphilic PHB.

Reusch and Sadoff proposed a structure for the complex in which PHB encircles CaPolyP. A computer model (Charmm) of this putative structure with 14 PHB residues and 7 PolyP residues per turn reveals a complex with a diameter of 24 Å, helical rise of 4.5 Å and average length of 45 Å [103]. The outer lipophilic shell of the complex (Figure 12A) is seen to have alternating linear arrays of methyl and methylene groups that span the hydrophobic region of the bilayer. A horizontal cross section (Figure 12B) shows the PolyP helix within the PHB cylinder, with the calcium ions surrounded by oxygen atoms from the two polymers. The calcium ions link the two polymers by forming strong ionic bonds to PolyP and weak ion-dipole bonds to the ester carbonyl oxygens of PHB.
Each Ca\(^{2+}\) is held in an ionophoretic cage, solvated by eight oxygen atoms in distorted cubic geometry - four phosphoryl oxygens from two adjoining units of PolyP and four ester carbonyl oxygens (two from contiguous PHB units on one turn and two from contiguous PHB units on the turn directly above or below) (Figure 12C). This arrangement has the added effect of crosslinking the turns of the PHB helix (Figure 13). The result is a symmetrical polymer electrolyte structure that forms a salt bridge between the cytoplasm and the periplasm.

Figure 12: PHB exolipophilic-endopolarophilic helical structure. Model showing PHB helix – 14 subunits per turn - in the preferred antiperiplanar arrangement with linear arrays of methyl groups facing outward and linear arrays ester groups lining the inside of the cavity. O-red; C-blue; H; grey

B. Computer model (Charmm) of the CaPolyP:PHB structure. Horizontal cross section showing the PolyP helix within the PHB helix. Each Ca\(^{2+}\) is surrounded by the oxygen moieties of PolyP and PHB. The seven Ca\(^{2+}\) displayed are from two turns of the PolyP helix (3 1/2 Ca\(^{2+}\) per turn). Ca\(^{2+}\) - green; P – yellow; O- red; C-blue; H; grey

C. Binding geometry for individual calcium ions. A. Model (Hyper-Chem). Each Ca\(^{2+}\) is neutralized by the two monovalent negative charges of two adjacent phosphate residues and solvated by eight coordinating oxygens -four oxygens from the two phosphate residues and four ester carbonyl oxygens contributed by two adjacent PHB residues in one turn of the helix and two adjacent PHB residues from the turn directly above or below. Ca\(^{2+}\) - green; P – yellow; O- red; C-blue; H; grey. From Reusch et al. 1995 [77].

Figure 13: Noncovalent bonds between Ca\(^{2+}\) and PHB. View down the PHB cylinder of Fig. 12A showing noncovalent bonds between Ca\(^{2+}\) (closed circles) and carbonyl ester oxygens (open circles) of PHB in a pattern that links each turn of the PHB helix alternatively to the proximal turns above and below. The binding sites form a ladder leading Ca\(^{2+}\) through the channel in small evenly-spaced steps. From Reusch and Sadoff 1988 [103]

Other helical arrangements should be considered. PHB is an elastomer capable of segmental motions at biological temperatures, and the PolyP polyanion is known for its ability to adopt a wide variety of conformations [5]. There may not be a single uniform structure for the complexes in the membrane, but the family of configurations is limited by the need to have conformity between the length of the PHB molecule and the width of the hydrophobic portion of the bilayer.

Seebach and Fritz suggest an alternative structure for the complex in which the PHB chains are folded in a 6Å pitch, 21-helical conformation, thus forming square-shaped sheets of ca. 50 x 50 x 5 Å within the lipid bilayer [58]. The PHB chains would be free to move within the bilayer and thus could organize themselves to form cylinders of various diameters, e.g. eight antiparallel chains in an ellipsoid arrangement would form a 50Å cylinder of 20Å diameter that can accommodate and solvate a helix of CaPolyP (Figure 14).
The former model assumes a coiled conformation for PHB such as it displays in solution [73], while the latter model suggests that PHB maintains the lamellar form of its solid state [68,69]. The result of both arrangements is a pliant structure of two discrete polymers bridged together by lanes of calcium cations. Normal molecular motions of the polymers such as twisting or stretching movements, or sliding or rotation of PolyP within the homogeneous environment provided by PHB, may modify channel geometry.

Since Ca²⁺ was the intracellular messenger and modulator of the most primitive biological systems, the first channel to appear was likely a Ca²⁺ channel [109]. In order to serve as an intracellular messenger, the intracellular concentration of an ion must be very low and precisely controlled, so that small transmembrane fluxes of the ion would significantly change its intracellular concentration to modulate cellular mechanisms. In addition, it must bind to its substrate selectively. Ca²⁺, with its low intracellular concentration (10⁻¹⁰⁻¹⁰₀ nM), high coordination number and irregular coordination geometry meets these requirements. The association of PolyP with PHB in the bilayers of early cells provided a means to specifically sequester Ca²⁺. It also established a system for the rapid import of Ca²⁺, controlled by the membrane electrical potential, thereby setting the stage for cell signaling.

**PolyP/PHB complexes as calcium channels**

Since Ca²⁺ was the intracellular messenger and modulator of the most primitive biological systems, the first channel to appear was likely a Ca²⁺ channel [109]. In order to serve as an intracellular messenger, the intracellular concentration of an ion must be very low and precisely controlled, so that small transmembrane fluxes of the ion would significantly change its intracellular concentration to modulate cellular mechanisms. In addition, it must bind to its substrate selectively. Ca²⁺, with its low intracellular concentration (10⁻¹⁰⁻¹⁰₀ nM), high coordination number and irregular coordination geometry meets these requirements. The association of PolyP with PHB in the bilayers of early cells provided a means to specifically sequester Ca²⁺. It also established a system for the rapid import of Ca²⁺, controlled by the membrane electrical potential, thereby setting the stage for cell signaling.

The composition and physical characteristics of the PolyP/PHB complexes suggest they can function as calcium channels. In support of this hypothesis, membrane vesicles, prepared from the envelopes of genetically competent *E. coli* cells displayed Ca²⁺-channel activity in planar lipid bilayers composed of synthetic 16:0,18:0 phosphatidylcholine [77]. When the envelopes were separated into cytoplasmic and outer membrane fractions, Ca²⁺-channel activity was observed with the cytoplasmic membrane fraction but not with the outer membrane fraction. This is consistent with the presence of PHB in cytoplasmic but not outer membrane fractions of competent cells [64], and with freeze-fracture studies that reveal the presence of plaques with shallow particles in cytoplasmic but not outer membranes of competent cells [102]. The complexes were extracted from *E. coli* competent cells and incorporated into planar lipid bilayers as above, wherein they again displayed Ca²⁺-channel activity [77].

To establish the composition of the calcium channels still further, PolyP/PHB complexes were reconstituted from PHB recovered from *E. coli* and CaPolyP prepared from commercial sodium polyphosphate and calcium chloride by [77]. This blend was premixed with phospholipids and used to form a planar bilayer. Next, the complexes were formed *in situ*. PHB was added to the phospholipids before painting the bilayer; CaPolyP was then added to the aqueous bathing solutions, and a potential was applied to induce the formation of the complexes within the bilayer.
Finally, to rule out contamination by polypeptide molecules, the complex was reconstructed from synthetic PHB, commercial PolyP and calcium chloride [110]. Lengweiler et al used an exponential fragment-coupling strategy to synthesize monodisperse polymers of R-3HB composed of 128 units [111]. The MW of this synthetic polymer is ~11.4kD, which is close to that of the \textit{E. coli} PHB (~12 kD). CaPolyP was prepared from sodium polyphosphate glass (average residue number 65) and calcium chloride. The current records of channels formed in planar bilayers by the synthetic complexes of PHB\(_{128}\)CaPolyP\(_{65}\) were indistinguishable from those formed by the CaPolyP /PHB complexes extracted from \textit{E. coli} (Figure 15).

![Figure 15: Representative single-channel current fluctuations of synthetic and \textit{E. coli} PHB/polyP complexes. Left. Synthetic PolyP/PHB\(_{128}\) complexes; Right. PolyP/PHB/ complexes extracted from competent cells of \textit{E. coli} DH5a. The PolyP/PHB complexes were each incorporated into planar lipid bilayers composed of 16:0,18:1, phosphatidylcholine and cholesterol (5:1; w/w) between aqueous bathing solutions of 200 mM CaCl\(_2\), 5 mM MgCl\(_2\), 10 mM Tris Hepes, pH 7.4 at 22 °C. The bars at the side of each profile indicate the fully closed state of the channel. Clamping potentials with respect to ground are indicated at the left side. From Das et al 1997 [110].](image)

Single-channel Ca\(^{2+}\) currents were obtained with each of these preparations. In all cases, the concentration of PHB in the bilayer was restricted to one-hundredth or less of the amount required to form channels composed solely of PHB. All natural and all synthetic complexes displayed the signal characteristics of protein Ca\(^{2+}\) channels: voltage-activation, permeance to both monovalent and divalent cations but strong preference for divalent cations, strong preference for Ca\(^{2+}\) over Mg\(^{2+}\), and block by transition metal cation such as La\(^{3+}\) and Al\(^{3+}\). The conductance of synthetic and \textit{E. coli} channels were also equivalent, 101 ± 6 and 104 ± 12pS, respectively [77,110].

These signal characteristics of protein Ca\(^{2+}\) channels are consistent with the physical properties of PolyP and PHB. In the lipid bilayer, the PolyP molecule is enclosed within the PHB sheath, so that only its terminal residues are exposed to an aqueous environment. At physiological pH, the terminal phosphate units bear a divalent negative charge, thus binding of divalent Ca\(^{2+}\) and Mg\(^{2+}\) is strongly preferred over binding of monovalent Na\(^{+}\) and K\(^{+}\). Due to a lower hydration energy, Ca\(^{2+}\) is slightly favored over Mg\(^{2+}\) when binding to the terminal phosphate unit, but coordination geometry becomes the determining factor as the cations move into the PolyP/PHB channel. The very strong preference of PolyP/PHB complexes for Ca\(^{2+}\) over Mg\(^{2+}\) indicates that the cation binding sites within the complex are ionophoretic [112,113]. Only multidentate ligands are capable of selecting between cations of the same charge with high efficiency [114,115], and this indicates that the ester carbonyl oxygens of PHB participate in the selection process. The irregular arrangement and number of oxygen ligands provided by PolyP and PHB strongly favor Ca\(^{2+}\) over Mg\(^{2+}\). Ca\(^{2+}\) has a preference for eight ligands, a high tolerance for variability in its bond lengths and angles, a lower hydration energy and more rapid hydration kinetics [5,112,113,116]; Mg\(^{2+}\) is disfavored by a strong preference for six ligands in a regular octahedral arrangement, a high hydration energy, and slow rate of ligand exchange [116]. Transition metal cations, such as La\(^{3+}\), bind very tightly to PolyP at the interface, but the electrochemical gradient is insufficient to move them into and through the channel, and consequently they block the ion flow.

Accordingly, the PolyP/PHB complexes act as dynamic molecular sieves, selecting Ca\(^{2+}\) from other physiological cations, first by charge and hydration energy and then by coordination geometry and rapid rate of ligand exchange. The Ca\(^{2+}\) are tightly held to PolyP by strong ionic bonds, thus the transport of Ca\(^{2+}\) from one phosphate anion to the next along the PolyP chain requires the application of a sizeable potential force. The electrical potential across lipid bilayers is itself insufficient so that additional energy must be provided by the chemical potential. Ca\(^{2+}\), although present in very low extracellular concentrations (~2.5 mM), achieves a
large chemical potential by maintaining internal Ca\(^{2+}\) concentrations at extremely low levels (~0.0001 mM), providing a > 20,000 fold chemical inward gradient [112,113]. The 'gate' is established by the equilibrium between the electrochemical potential and the bond energy. When the total electrochemical potential is stronger than the ionic bonds between Ca\(^{2+}\) and the phosphate residues of PolyP, Ca\(^{2+}\) will flow along the PolyP chain into the cells, i.e. the channel is 'open.' When the electrochemical potential is insufficient, the channel is 'closed'.

**Effect of pH on selectivity of PolyP/PHB complexes**

The channels formed by PolyP/PHB complexes are highly selective for Ca\(^{2+}\) at physiological pH. However, it is important to the biogenesis of ion channels that the selectivity of PolyP/PHB channels can be altered by a small decrease in pH. Since the hydrogens of the terminal phosphate subunits are weak-acids (pK\(_2\) ~ 6.8-7.2), only a small decrease in local pH can reduce the charge of the terminal phosphate anion to monovalent, and then binding of monovalent cations is preferred [5]. Clearly the terminal phosphate units of PolyP - the first binding sites for cations that are attracted to the channels - play a critical function in cation selection.

Evidence for the sensitivity of PolyP/PHB complexes to pH was provided by the studies of Das and Reusch, who examined the selectivity of PolyP/PHB channels for Ca\(^{2+}\) and Na\(^{+}\) in planar lipid bilayers as a function of solution pH [118]. Since Ca\(^{2+}\) and Na\(^{+}\) are similar in coordination geometry and size (1.06 Å vs 0.98 Å), the two cations are distinguished primarily by charge. The PolyP/PHB channels are strongly Ca\(^{2+}\)-selective at pH 7.4. However, this preference for Ca\(^{2+}\) diminishes within half a pH unit. The complexes become nonselective at pH ~7.0, and weakly Na\(^{+}\)-selective at pH < 7.0. Accordingly, lowering the pH abolishes the preference for Ca\(^{2+}\) over Na\(^{+}\). Whereas the controlling factor for the selection of cations to penetrate into the channel formed between PolyP and PHB is coordination geometry, the controlling factor for the initial selection of cations by the terminal unit of PolyP is local pH.

This remarkable shift in preference from divalent to monovalent cations over a narrow pH range near physiological values provides the basis for a molecular mechanism for regulation of the transport of monovalent cations by PolyP/PHB channels and by PolyP-containing protein channels. The pH around PolyP termini can be altered *in vivo* by a proton flow or by strategic arrangement of basic amino acid residues of the polypeptides around the channel entry (see KcsA).

**PolyP/PHB complexes as calcium pumps**

The capacity of PolyP/PHB to function as a Ca\(^{2+}\) pump derives from the nearly equal phosphorylating potentials of PolyP and ATP [5].

\[
\text{ATP} + \text{PolyP}_n \leftrightarrow \text{ADP} + \text{PolyP}_{n+1}
\]

The structural features of PolyP/PHB channels, coupled with the ability of PolyP to 'grow' by accepting phosphoryl groups from ATP and other phosphate anhydrides [4-6,14,29], form the basis for a hypothetical mechanism for export of Ca\(^{2+}\) from cells. The CaPolyP helix traversing the PHB pore creates a bridge that connects the pools of Ca\(^{2+}\) in the cytoplasm with the Ca\(^{2+}\) in the external medium. Although there is a strong inward Ca\(^{2+}\) gradient across the membrane, the exceptionally strong ionic bonds between Ca\(^{2+}\) and the PolyP anions prevent the inward flow of Ca\(^{2+}\) under equilibrium conditions. At the same time, Ca\(^{2+}\) is held to PHB by its relatively weak ion-dipole bonds to carbonyl oxygens. This organization implies that the CaPolyP channel can be secreted through the PHB sheath and out of the cell by extending the PolyP chain at the cytoplasmic side of the membrane.

In bacteria, the enzyme PolyP kinase transfers phosphates from ATP to the end of a PolyP chain [118]. As the appended phosphate units move into and through the PHB channel, Ca\(^{2+}\) is sequestered from the cytoplasm. The Ca\(^{2+}\)-PolyP helix winds like a screw in the "threads" of the PHB helix. Alternatively, since the Ca\(^{2+}\) ions form linear vertical arrays inside the complex, an entire turn of the Ca\(^{2+}\)-PolyP helix may form and then rise within the PHB channel. At the periplasmic face, PolyP is discharged into the periplasm, or hydrolyzed or degraded by exopolyphosphatases [105-107,118-120]. The ultimate effect of these reactions is to expend ATP energy to draw Ca\(^{2+}\) from the cytoplasm and extrude it into the periplasm. This is, in effect, a non-proteinaceous CaATPase pump.

**PolyP/PHB complexes as DNA channels.**

The strong positive correlations in diverse bacteria – Gram-negative *A. vinelandii*, *H. influenza*, *E. coli*, and Gram-positive *B. subtilis* - between concentrations of PolyP/PHB complexes in the cytoplasmic membranes and genetic transformatibility and the rapid withdrawal of PolyP/PHB complexes from the bilayer during DNA uptake, led Reusch and Sadoff to propose that the complexes function as sites for entry of ssDNA into bacterial cells [103].

The varied protocols for development of genetic competence differ for each organism, but the result is always a conspicuous increase in the concentration of PolyP/PHB complexes in the cytoplasmic membranes [64,103]. When the formation of the complexes is prevented by any means, transformation is inhibited [103,121]. An excess of Ca\(^{2+}\) is provided, and this has the effect of cross-bridging the terminal phosphate anion of PolyP to the terminal phosphate anion of a DNA strand. When the cells are returned to log-phase growth, the concentration of membrane PolyP/PHB complexes quickly diminishes. As the PolyP is retrieved from the PHB channel by cytoplasmic enzymes, the bound ssDNA molecule may be drawn into and through the PHB channel and into the
cytoplasm. The inner diameter of the proposed pore is 16.4 Å [77] – larger than the ~15 Å diameter of the alpha hemolysin pore which accommodates ssDNA [122] - and PHB is more flexible at physiological temperatures than the alpha hemolysin polypeptides. In some cases, as in E. coli, a "heat pulse" is applied [123]. This would have the effect of further increasing PHB flexibility, thus facilitating the necessary conformational adjustments. The equivalence and symmetry of the PHB ligands would ease the transfer of ssDNA through the channel. In support of this hypothesis, donor 32P-labelled ssDNA was found complexed to PHB when DNA uptake in E. coli RR1 was interrupted [51].

Protein-conjugated PHB/PolyP complexes in prokaryotes

As the evolution of ion channels progressed, no other cellular molecules appeared that could equal or surpass the capacity of PolyP to attract cations, select among cations by charge, and conduct cations in an orderly fashion in response to an electrochemical potential. However, the roles of PHB in ion channels were gradually assumed and improved upon by polypeptides. PHB can form pathways across the bilayer but they cannot control access to the PolyP conductor at either side of the bilayer and cannot influence the local pH around the end units of PolyP. As a result, PolyP/PHB channels will preferentially transport Ca2+, either into the cell in response to the electrochemical potential or out of the cell by the addition of phosphate residues to the PolyP chain at the intracellular side by ATP or other nucleoside triphosphates.

Polypeptides can form more diverse structures than PHB. In addition to providing cross-bilayer pathways for PolyP, they can also control access to the PolyP conductor at one or both sides of the bilayer, and/or influence local pH at the channel entry and thereby adjust the charge of the PolyP end unit. Accordingly, polypeptides can make the PolyP conductor available to monovalent cations. Although they are less flexible than PHB at physiological temperatures, this disadvantage can be offset by the attachment of short chains of PHB at critical sites to improve complexation interactions with the cations. Since the flexibility of PHB is highly influenced by temperature, channels formed by PHB-modified polypeptides will function optimally at physiological temperatures. The postulate is that as ion channels evolved, PolyP continued to serve as the conductive cores of ion channels formed by PHB-modified polypeptides.

PolyP/PHB in a potassium channel

Potassium channels are ubiquitous integral membrane structures. They are found in all kingdoms of life and are involved in a wide range of physiological processes. In a landmark paper in 1995, Schrempf et al identified, cloned, and expressed the gene for KcsA, a potassium channel from the prokaryote, Streptomyces lividans [124].

Structure of S. lividans KcsA

S. lividans KcsA is a 160-amino acid polypeptide that oligomerizes to form a tetrameric potassium channel with 2 domains: a transmembrane domain of 120 residues and a cytoplasmic domain of 40 residues. There have been numerous physical studies to determine the three-dimensional structure of the four polypeptides. X-ray crystallography shows that the four identical subunits form an inverted teepee surrounding a large central cavity leading to a narrow pore at the extracellular end [125-128]. Site-directed spin labeling and EPR spectroscopy and NMR studies indicate that the N terminus of KcsA forms a short amphipathic α-helix, whereas the C-terminal domains form a helical bundle that extends ~50 Å into the cytoplasm [129-133] (Figure 16A).

In this structure, the presence of the PolyP anion within the channel has not been recognized or acknowledged. Since KPolyP is water-soluble, it would be easily lost during the purification of the tetramer and certainly lost when the tetramer is dissociated into monomers. The existence of PolyP within the KcsA tetramer was first signaled by the metachromatic reaction of the tetramer, but not the monomer, to o-toluidine blue stain on SDS-PAGE gels [134]. S. cerevisiae exopolypophosphatase degraded the PolyP after its release from the tetramers, but had no influence on PolyP within intact tetramers, indicating that PolyP in the KcsA tetramer was inaccessible to the enzyme. Strong confirmatory evidence for the presence of PolyP in the tetramer is provided by the large difference between the theoretical isoelectric point - 10.3 - and experimental isoelectric point - 6.5-7.5 - of the KcsA tetramer (Figure 17) [134].

The length of PolyP in KcsA was estimated as ~15 monomer units per tetramer by an enzymatic assay with polyphosphate kinase (PPK) in which the PolyP is used to convert [14C]ADP to [14C]ATP [106]. This estimate is likely to be low due to hydrolysis of PolyP during isolation procedures. Hegermann et al. visualized PolyP in S. lividans cells, using energy-filtered electron microscopy (EFTEM) and lead sulfide precipitation. They observed structured PolyP precipitates at the inner face of the cytoplasmic membrane in wild-type cells, but not in KcsA-minus S. lividans mutant cells [135].

It is hypothesized that the narrow PolyP chain (~ 4 Å across) is held within the C-terminal helical bundles of the four polypeptides in which it extends from the channel entrance to the cytoplasmic side of the ‘selectivity pore’ (Figure 16B). The only connections between PolyP and the KcsA polypeptides would be made via K+, which forms strong ionic bonds to PolyP but only weak coordinate bonds to ester carbonyl oxygens of amino acid residues of the polypeptides.
Figure 16: Model of KcsA. A. Ribbon representation of the KcsA tetramer. Four identical subunits create an inverted teepee. The inner helix of each subunit faces the central pore while the outer helix faces the phospholipid membrane. There is a relatively wide water-filled cavity (about 10 angstroms wide) near the middle of the membrane that narrows into the selectivity filter at the extracellular end (from Doyle et al. [125]. B. Putative supramolecular structure of KcsA. The PolyP chain is held within the four polypeptides and extends from the cytoplasmic end to the ‘selectivity pore’. Each polypeptide is modified at serines S102 and S129 by short-chain PHB [61].

Figure 17: Analytical Isoelectric focusing of KcsA. Focusing was carried out on 0.4 mm gels of 5% acrylamide:bis (33.7:1), 20 mM n-dodecyl-β-D-maltoside, 5% pH 3-10 ampholytes formed on gel support film. The gel was stained with Coomassie Brilliant Blue R-250, crocein scarlet and destained with 40% methanol, 10% acetic acid.

Lane 1, pI standards; lane 2, unheated KcsA - the intact KcsA tetramer complex has a near neutral pI; lane 3, KcsA heated for 5 min. at 90 °C – the uncomplexed KcsA protein moves off the bottom of the gel indicating it has a pI > 10. From Reusch 1999 [134].

The early structural studies of KcsA polypeptides also failed to discern the modification of the polypeptides by short-chain PHB. This simple polyester could easily be overlooked in X-ray studies of KcsA since it would resemble lipid or detergent molecules. PHB was identified in both monomers and tetramers of KcsA by Western blot assays using anti-PHB IgG [134]. PHB could not be extracted from the protein with chloroform, indicating it was conjugated to amino acid residues of the polypeptides. The amount was estimated by chemical assay as ~28 monomer units of PHB per KcsA tetramer or 7 units per monomer. This value may not accurately reflect the in vivo concentration due to hydrolysis of the ester groups of PHB during purification procedures.

Negoda et al. identified the PHB conjugation sites in KcsA as serines 102 and 129 [61]. The loss of conjugated PHB, achieved by mutating the serines to glycines, had no discernible effect on tetramer formation or tetramer stability; however, the mutations negatively influenced the incorporation and/or retention of PolyP by the tetramer. Planar lipid bilayer studies of the PHB mutants indicate that the absence of conjugated PHB has adverse effects on channel formation and channel function [61]. Channel conductance was greatly reduced and/or highly irregular. The placement of the flexible PHB ligands at strategic sites along the polypeptide housing may help to stabilize and orient the KPolyp molecule by providing additional complexation sites at these positions (Figure 16B). One may conclude that PHB-modification of KcsA plays a critical role in maintaining the PolyP molecule in an optimal transbilayer position for efficient K⁺ transport.
Mechanism of K⁺ selection and export by *S. lividans* KcsA

The *S. lividans* KcsA channel provides us with an excellent model for tracing the evolution of PolyP/PHB calcium channels into potassium channels in biological cells. The three-dimensional structure of the KcsA channel demonstrates how polypeptides can harness the conductive potential of PolyP for the controlled export of K⁺ from the cytoplasm under physiological conditions. In biological cells, the K⁺ gradient (outward) is in the opposite direction from the Ca²⁺ gradient (inward). In order to overcome the strong preference of PolyP for Ca²⁺, a K⁺ channel would have to 1) prevent Ca²⁺ from accessing the PolyP conductor at the extracellular side, and 2) alter the local pH around the terminal phosphate anion to prevent access of Ca²⁺ or Mg²⁺ to the PolyP chain at the intracellular side.

Early studies of KcsA, which were based on the three-dimensional structure of the polypeptides, proposed that hydrated cations enter at the intracellular side of the KcsA channel and form a single file leading to the selectivity pore at the extracellular end [137] (Figure 16A). Selectivity for K⁺ and against Na⁺ and other ions was posited to occur at the entryway to the selectivity pore. Ions are dehydrated as they enter the selectivity pore, and differences in size and coordination geometry favor the passage of K⁺ [136-139]. The means by which rejected ions return to the intracellular solution is unclear. It has been suggested that Na⁺ can ‘punch through’ the selectivity filter if a sufficient cytoplasmic positive voltage is applied [142]. In this mechanism, Na⁺ escapes from its blocking site in the wide hydrated vestibule at the intracellular side of the selectivity pore and moves through the selectivity filter. However, in this case, selectivity has been circumvented.

In the KcsA model suggested by Reusch, the four identical PHB-modified polypeptides form a symmetric complex around a central core molecule of PolyP [134]. The narrow pore at the extracellular end, called the "selectivity pore", is instead viewed as a ‘barrier pore’. It is posited to function as a one-way valve that permits the exit of dehydrated K⁺ while denying entry, and thus access to the PolyP conductor, at the extracellular side to Ca²⁺, which has a strong inward gradient. The ‘barrier pore’ fulfills the first requirement for a K⁺ channel – preventing Ca²⁺ from binding to the PolyP conductor.

At the intracellular side, cations are attracted to the PolyP anion. Selectivity occurs at the entrance. Divalent cations are deterred from binding by the collar of eight terminal arginines, which surround the PolyP end unit and reduce its effective negative charge to monovalent. K⁺ is the major intracellular monovalent cation and it is further preferred over Na⁺ by its lower heat of hydration and by its coordination geometry [140]. Some of the water of hydration of K⁺ is removed on binding to the PolyP end unit, and carbonyl oxygens of amino acid residues replace the remaining water of hydration as K⁺ enter the channel. The dehydrated K⁺ ions are held by strong ionic bonds to the PolyP backbone, which extends from the cytoplasmic face to the narrow pore at the extracellular side (‘barrier pore’). The outward K⁺ chemical potential (K⁺ gradient) of ~30:1 is counter-balanced by the strength of the K⁺PolyP- ionic bonds and by the inward membrane electrical potential of ~60 mV. ‘Gating’ depends on the equilibrium between these forces. When the electrochemical potential is equal to or less than the binding energy between K⁺ and PolyP-, the channel is ‘closed’. A decrease in the negative electrical potential or increase in the positive chemical potential ‘opens’ the channel and K⁺ ions move out through the narrow pore until equilibrium is reestablished.

Physiological studies of *S. lividans* KcsA

In the first physiological studies of KcsA, Schrempf et al. [124] reported single-channel activity in liposome-protoplast vesicles at pH 7.2. However despite the fact that physiological pH in *S. lividans* is fairly constant at neutral values, KcsA soon after became widely regarded as a pH-dependent, proton-activated K⁺ channel [136,137]. One factor that led to regarding KcsA as a pH-dependent channel was the failure to reconstitute the KcsA channel to its native conformation. Since the modification of the polypeptides by PHB was not known, the importance of temperature on the conformation of the KcsA polypeptides was not recognized. The cytoplasmic C-terminal segments become disorder during purification procedures, thus exposing the PolyP end unit to medium pH. In this case, an acidic medium pH is needed to reduce the charge on the end unit of PolyP to monovalent in order to favor binding of K⁺ to PolyP. However, incubation of the channel at temperatures > 25 °C allows the PHB-modified C-terminal segments to relax into their native position in which the eight terminal arginine residues of the four polypeptides surround the PolyP end unit. In this arrangement, the charge on the PolyP end unit is reduced to monovalent, and an acidic pH is unnecessary. Zakharian and Reusch showed that when purified KcsA is maintained at temperatures < 25 °C, it displays sensitivity to pH; i.e. it shows a preference for divalent cations at pH 7.2 and monovalent cations at pH 6.8 [144]. However, when incubated at temperatures >25 °C, KcsA undergoes a transition to its mature conformation in which it exhibits high selectivity for K⁺ regardless of solution pH.

The importance of the terminal arginine residues to KcsA selectivity was demonstrated in studies by Negoda et al., which showed that when only one of the two C-terminal arginines of each polypeptide is replaced by a neutral residue, the channels remain highly selective for K⁺ over Mg²⁺, independent of medium pH; however, when both C-terminal arginines are replaced with neutral residues, the channels are selective for Mg²⁺ when pH is >7 and for K⁺ when pH is <7 [145]. Further support for this model of KcsA function is provided by studies of Negoda et al., in which one or both of the C-terminal arginines were replaced with residues of progressively lower basicity -lysine, histidine, valine, asparagine [146]. Planar bilayers studies of these mutants show that as the basicity of the C-terminal residues decreases, Mg²⁺ block increases, and Mg²⁺ becomes permeant when medium pH is greater than the pI of the C-terminal residues. The results indicate that interactions between PolyP and the C-terminal arginines of the polypeptides are decisive elements in selectivity for K⁺ by KcsA.
A second factor that led to KcsA being regarded as a proton-activated channel was the failure of the channel to activate in planar lipid bilayers with solely an electrical potential [137]. *S. lividans* is a soil bacterium and, since concentrations of K⁺ are very low in soils, KcsA evolved to function with a large outward chemical potential. Accordingly, KcsA cannot be activated in planar bilayers by the electrical potential alone, not because it is pH-gated *in vivo* but because an electrical potential large enough to break the strong ionic bonds between K⁺ and PolyP would rupture the planar bilayer *in vitro*. It is relevant that the first physiological studies of KcsA by Schrempf et al. [124] were performed with a K⁺ gradient of 5:1 at pH 7.2. Zakharian and Reusch [56] confirmed that the KcsA channel opens readily at physiological pH in the planar bilayer with an outward K⁺ gradient > 2.5:1.

In conclusion, the evidence suggests that *S. lividans* KcsA is a supramolecular complex of four identical polypeptides, each modified by PHB at serines 102 and 129, which surround a PolyP molecule that is postulated to extend from the narrow pore at the extracellular end (‘selectivity pore’ or ‘barrier pore’) to the intracellular mouth of the channel. The PolyP molecule is surrounded at the intracellular entry by a collar of eight arginine residues that repel divalent cations and reduce the effective charge of the PolyP end unit to monovalent, and thereby favors the binding of K⁺ at the channel entry. Due to the strength of the ionic bonds between K⁺ and PolyP, both an electrical and a chemical potential are required to ‘open’ the KcsA channel in planar lipid bilayer studies.

**PolyP/PHB complexes in eukaryotic membranes**

The ubiquity of PolyP and PHB led to an investigation of the occurrence of PolyP/PHB complexes in eukaryotes. Reusch analyzed a variety of plant and animal tissues and all were found to contain PHB associated with PolyP [50-52]. The eukaryotic PolyP had chain lengths of 120-200 subunits, and was associated with PolyP of somewhat greater length (150-220 units) than the bacterial counterpart (60 units). Suzuki et al. isolated PolyP/PHB complexes from sugar beet (*Beta vulgaris L.*) with a slightly shorter chain length of 106 residues and with a narrower molecular weight distribution [147]. Their analysis of the end groups by 31P NMR, 1H NMR and GC-MS, indicated that the carboxyl end groups were unmodified, while ~ 70 mol-% of the hydroxyl end were modified by short-chain alkanolic and alkanedioic acids.

**PolyP/PHB complexes in mitochondria**

There have been a number of studies that confirm the presence of PolyP/PHB complexes in mitochondria. PolyP/PHB complexes were isolated from the mitochondria, microsomes and plasma membranes of bovine liver by Reusch [51]. Seebach et al. confirmed the presence of PolyP in beef heart mitochondria by ³¹P NMR [52]. Pavlov et al. isolated PolyP/PHB complexes from rat liver mitochondria and found that the complexes form large conductance, weakly selective channels with multiple conductance states in planar lipid bilayers [148]. The channel activity closely resembled the performance of the Ca²⁺-induced mitochondria permeability transition pore channel (mPTP), a nonselective pore complex in the inner mitochondrial membrane. Lichko et al. identified PolyP in mitochondria of *Saccharomyces cerevisiae*, [149] and Kulakovskaya et al. isolated PolyP of ~15 residues from the membranes and inter-membrane space of the mitochondria [150].

Abramov et al. discerned a role for PolyP in mitochondrial function [151]. When levels of PolyP in mammalian mitochondria were reduced by expression of polyphosphatase, there was an increase in the capacity of mitochondria to accumulate Ca²⁺. This led to the suggestion that PolyP plays a role in the activation of the mPTP. Pavlov et al. found that PolyP levels in mitochondria were dynamic and responsive to various inhibitors and activators of mitochondrial respiration [152]. Seidlmayer et al. isolated short-chain PolyP of ~ 25 units from rabbit heart mitochondria and demonstrated that mitochondrial PolyP contributes to the opening of the mPTP in cardiac mitochondria, thus supporting the view that PolyP plays a key role in activation of the mPTP [153]. Smithen et al. isolated PolyP composed of up to 120 residues from mammalian mitochondria and suggested that PHB may also play a significant role in mitochondrial uptake of Ca²⁺ [154]. The mitochondrial PHB was altered enzymatically by targeted expression of the bacterial PHB depolymerase, PhaZ7, in mammalian cultured cells. The reduction in PHB resulted in decreased mitochondrial membrane potential in HepG2 cells, but not in U87 and HeLa cells, suggesting tissue specificity. Elustando et al. used a fluorescein derivative of PHB (fluo-PHB) to demonstrate that PHB preferentially accumulates inside the mitochondria of HeLa cells [155]. They confirmed a role for PHB in facilitating ion transport across the membranes, including transport of Ca²⁺, and further showed that PHB induces mitochondrial membrane depolarization. This membrane depolarization was significantly delayed by Cyclosporin A, an inhibitor of the mPTP.

**PolyP/PHB in the Ca²⁺-ATPase of human erythrocytes**

The first protein ion pump found to contain both PolyP and PHB was the Ca²⁺-ATPase of the human erythrocyte plasma membrane [156], which was also the first enzyme demonstrated to work as a Ca²⁺ pump [157]. The presence of PolyP in the Ca²⁺-ATPase was revealed by metachromatic reaction of o-toluidine stain and by enzymatic assay using PolyP kinase [158]. The presence of PHB was disclosed by Western blot and chemical assay. The PHB could not be removed by extraction with chloroform, suggesting that its association with the ATPase polypeptides is covalent.
In order for PolyP to ‘pump’ Ca\(^{2+}\) out of the cell, the PolyP chain must be extended at the cytoplasmic side. As discussed above, polyphosphate kinases transfer high-energy phosphates from ATP to PolyP in bacteria, \([12-14,30]\). Polyphosphate kinases have not been detected in eukaryotes; however, Niggli et al. \([159]\) showed that the human erythrocyte Ca\(^{2+}\)-ATPase could emulate some of the activities established for polyphosphate kinase (PPK) by Ahn and Kornberg \([105]\). Both enzymes are phosphorylated by ATP, and both produce a phosphoenzyme that can transfer the phosphoryl group back to ADP and regenerate ATP \([160]\). A histidine residue is phosphorylated in PPK, and an aspartate residue in the Ca\(^{2+}\)-ATPase.

\[
{[^{32}P]}ATP + PPK \rightleftharpoons ADP + {[^{32}P]}PPK
\]

\[
{[^{32}P]}ATP + Ca^{2+}\text{ATPase} \rightleftharpoons ADP + {[^{32}P]}Ca^{2+}\text{ATPase}
\]

ATP and PolyP have similar phosphorylating potentials. The ability of PolyP to phosphorylate the CaATPase was demonstrated by autoradiography.

\[
{[^{32}P]}\text{PolyP}_n + Ca^{2+}\text{ATPase} \rightleftharpoons {[^{32}P]}\text{PolyP}_{n-1} + {[^{32}P]}Ca^{2+}\text{ATPase}
\]

The capacity of the Ca\(^{2+}\)-ATPase to transfer the phosphate back to ADP or to PolyP was also demonstrated.

\[
{[^{32}P]}Ca^{2+}\text{ATPase} + ADP \rightleftharpoons Ca^{2+}\text{ATPase} + ATP
\]

\[
{[^{32}P]}Ca^{2+}\text{ATPase} + \text{PolyP}_n \rightleftharpoons Ca^{2+}\text{ATPase} + {[^{32}P]}\text{PolyP}_{n+1}
\]

The sum of these reactions indicate that the human erythrocyte CaATPase has all of the enzymatic activities associated with bacterial polyphosphate kinases, i.e. it exhibits ATP-PolyP transferase and PolyP-ADP transferase activities. The Ca\(^{2+}\)-ATPase may be considered a supramolecular enzyme composed of PHB-modified polypeptides surrounding a PolyP molecule that transports Ca\(^{2+}\) out of the cytoplasm by catalyzing the transfer of phosphoryl groups from ATP to PolyP.

**PolyP/PHB complexes in TRP channels**

Transient receptor potential channels (TRP channels) are ion channels in the plasma membranes of mammals. They are composed of six membrane-spanning helices with intracellular N- and C-termini. The channels are expressed throughout the body wherein they are non-selectively permeable to several cations, including Na\(^{+}\), Ca\(^{2+}\) and Mg\(^{2+}\). They are activated and regulated by a variety of stimuli such as pain, heat, cold, taste, cooling agents, pungent chemicals, pressure, and vision.

Kim and Cavanaugh \([161]\) found that TRPA1, a member of the mammalian TRP channel superfamily, was readily activated by pungent chemicals in whole cells but failed to do so in excised membrane patches. They reasoned that an essential, water-soluble cytosolic factor was missing from the membrane patches. They identified PolyP as the soluble factor and found that PolyP of 4 to 65 residues were most effective in restoring activity.

Zakharian et al., using whole-cell patch-clamp and fluorescent calcium measurements, found that the transient receptor potential melastatin 8 (TRPM8) channel forms a stable complex with PolyP \([162]\). TRPM8 is expressed in sensory neurons wherein it is responsible for sensing environmental cues. The protein exhibits steep temperature-dependence and is a major sensor of environmental cold temperatures. Channel openings are accompanied by large changes in entropy and enthalpy, indicating considerable conformational changes of the protein. They further demonstrated that the presence of PolyP is essential for normal channel activity, i.e. they found that enzymatic degradation of PolyP by exopolyphosphatase (scPPX1) inhibited the channel activity.

Biochemical analysis of the TRPM8 polypeptides by Zakharian et al. also disclosed the modification of the polypeptides by PHB \([163]\). Cao et al. identified a number of PHB-modified peptides in the N-terminus of the TRPM8 protein and in its extracellular S3–S4 linker by mass spectrometry \([164]\) (Figure 18). The sensitivity of the PHB backbone to temperature may play an important role in the temperature-dependence of the channel. Cold temperatures would induce substantial changes in the numerous noncovalent interactions of PHB with the protein, resulting in significant changes in the conformation of the TRPM8 protein. Accordingly, Cao et al. found that removal of PHB by enzymatic hydrolysis or by site-directed mutagenesis of serine residues that serve as covalent anchors for PHB result in significant inhibition of TRPM8 channel activity \([164]\). One may conclude that both PolyP and PHB have fundamental roles in the structure and function of the TRPM8 channel.

**Conclusions and comments**

Early in the evolution of biological cells, two simple polymers –PolyP and PHB - were created in the environment from inorganic phosphate, methane and carbon monoxide. Each polymer possesses singular physical properties, which complement each other so that cooperatively, PolyP/PHB complexes contain all of the physical features essential to the formation of selective ion channels that establish ion and voltage gradients across lipid bilayers which provide energy for cellular reactions. The channels also enable intracellular messaging and facilitate the exchange of genetic information between cells.
PolyP/PHB channel complexes are the progenitors of supramolecular proteinaceous ion channels and ion pumps in which the conductive properties of PolyP are exploited and adapted by PHB-modified polypeptides, whose diversity and three-dimensional structures enable the regulated transmembrane transport of calcium and other physiological cations. Progress in the biophysics of ion transport requires the elucidation of the fundamental roles of PolyP and PHB in the selection and transport of ions into and out of biological cells.

References


