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Novel components and enzymatic activities of the human erythrocyte plasma membrane calcium pump

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Abstract The plasma membrane Ca^{2+} pump is essential for the maintenance of cystolic calcium ion concentration levels in eukaryotes. Here we show that the Ca^{2+}-ATPase, purified from human erythrocytes, contains two homopolymers, poly(3-hydroxybutyrate) (PHB) and inorganic polyphosphate (polyP), which form voltage-activated calcium channels in the plasma membranes of *Escherichia coli* and other bacteria. Furthermore, we demonstrate that the plasma membrane Ca^{2+}-ATPase may function as a polyphosphate kinase, i.e. it exhibits ATP-polyphosphate transferase and polyphosphate-ADP transferase activities. These findings suggest a novel supramolecular structure for the functional Ca^{2+}-ATPase, and a new mechanism of uphill Ca^{2+} extrusion coupled to ATP hydrolysis.

Key words: Poly (3-hydroxybutyrate); Polyphosphate; Polyphosphate kinase; Ca^{2+}-ATPase

1. Introduction

Poly(3-hydroxybutyrate) (PHB), a linear polymer of R-3-hydroxybutyrate, and inorganic polyphosphate (polyP), a linear polymer of orthophosphate (Pi) residues linked by phosphoanhydride bonds, are ubiquitous constituents of biological cells [1–5]. PHB is a solvating amphiphilic polymer with a demonstrable capacity to carry ions through hydrophobic media [6,7]; polyP is a phosphorylating agent with a strong ion exchange capacity and a preference for divalent over monovalent cations [8]. The two polymers associate to form voltage-activated calcium ion channels in the plasma membranes of *Escherichia coli* and other bacteria [7]. The structure of the complexes is unknown, but presumably Ca(polyP) forms a salt bridge, solvated by PHB, across the membrane [7,9]. It has been postulated that the PHB/polyP complexes may also function as Ca^{2+} pumps [10]. The polyP chain may be elongated at the cytoplasmic face of the membrane by transfer of the γ-phosphate of ATP [11] or other high energy phosphates [12], and hydrolyzed at the periplasmic face to orthophosphate via polyphosphatases [12,13]. The ultimate effect of these reactions would be to draw Ca^{2+} from the cytoplasm and extrude it into the periplasm.

Inasmuch as both PHB and polyP have been conserved in eukaryotic cells [3–5], the question arose as to whether PHB/polyP membrane complexes have also been conserved in eukaryotic calcium transport systems. In this regard, we examined the Ca^{2+}-ATPase of the human erythrocyte membrane, which is the sole transporter of Ca^{2+} in the cell and was the first plasma membrane enzyme demonstrated to work as a Ca^{2+} pump [14,15]. Subsequently, it has been determined that the Ca^{2+}-pumping ATPase is ubiquitous in plasma membranes of a variety of cell types in eukaryotic organisms [16,17].

2. Materials and methods

2.1. Purification of Ca^{2+}-ATPase

Red-cell ghost membrane proteins were solubilized in the presence of the non-ionic detergent *n*-dodecyl octaethylene glycol monoether ($C_{12}E_8$), and the Ca^{2+}-ATPase was purified by calmodulin affinity chromatography as previously described [18]. The enzyme was stored at −80°C in elution buffer containing 10 mM Tris-maleate, pH 7.4, 130 mM KCl, 0.5 mM $MgCl_2$, 5 mM EGTA, 20% glycerol, 750 μM $C_{12}E_8$, 2 mM dithiothreitol.

2.2. Protein assay

The concentration of enzyme protein was determined by the Bio-Rad Protein Micro-assay, based on the Bradford dye-binding procedure [19]. Bovine serum albumin was used as a standard.

2.3. Determination of PHB

Samples (0.15 mg purified Ca^{2+}-ATPase; 2.6 mg erythrocyte ghosts) were precipitated with 5% trichloroacetic acid (TCA), washed with the same medium 2×, and then washed with acetone. Concentrated sulfuric acid was added (0.5 ml), and the samples were heated at 120°C for 40 min. Samples were diluted with saturated sodium sulfate solution (1.5 ml), and extracted with methylene chloride (4×). Crotonic acid was converted to the sodium salt by addition of 0.1 ml 2 N NaOH, and methylene chloride was evaporated with a stream of dry nitrogen. The samples were acidified with 3 N H_2SO_4, and chromatographed on an HPLC Aminex HPX-87H organic acids column (Bio-Rad) with 0.014 N H_2SO_4 as eluent. Detection was at 212 nm. The crotonic acid peak was identified by elution time, UV and mass spectra, and quantitated by comparison of the peak area with that of crotonic acid standards. It should be noted that none of the amino acids or their homopolymers form detectable amounts of crotonic acid under the assay conditions [20]. Values (average of two determinations) were corrected for incomplete conversion to crotonic acid and crotonic acid degradation [20]. The values are based on standards of granule PHB because there are as yet no suitable standards for protein-associated PHB [20]. Since the conversion of PHB associated with protein to crotonic acid is much less efficient, the actual amounts in protein are significantly higher than given by this measurement.

2.4. Determination of polyP

The method used was essentially as described by Crooke et al. [21]. The Ca^{2-}-ATPase protein (0.2 mg) was precipitated with 5% cold trichloroacetic acid, washed with acetone (3×), and extracted (2×) with 10 mM EDTA, pH 7.5. The extract was washed with phenol:-chloroform (1:1), and then chloroform (3×). The lyophilized extract was added to 50 mM HEPES, KOH, pH 7.2, 40 mM $(NH_4)_2SO_4$, 4 mM $MgCl_2$, 20 μM [U-^{14}C]ADP (0.2 Ci/mmol), 2000 units PPK, and the mixture was incubated for 45 min at 37°C. The reaction was terminated by cooling in an ice bath. ADP and ATP were added (5 mM each) to act as carriers. Aliquots were chromatographed on polyethyleneimine-cellulose thin layer plates with 1.0 M LiCl, 1.0 M HCOOH as developer. The radioactivity that migrated with ADP, ATP, and that which remained at the origin (visualized with UV

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irradiation) were determined by liquid scintillation counting. Standard $polyP_{45}$, treated in the same manner, was nearly completely (90%) converted to ATP under these conditions. Total [^{32}P]Pi was estimated, from liquid scintillation counts, as 1.4 nmol (average of two determinations). Value given is the average of two determinations.

2.5. Formation of polyP from ATP

The method used was essentially that of Ahn and Kornberg [22]. The reaction mixture (100 μl) contained 0.5 mM [γ-^{32}P]ATP (3000 cpm/nmol), 100 mM KCl, 4 mM $MgCl_2$, 0.1 mM $CaCl_2$ in 50 mM HEPES-KOH (pH 7.4) and Ca^{2+}-ATPase (2 μg). Acetyl phosphate (2 mM) and acetate kinase (20 μg/ml) were also added to regenerate ATP. After incubation at 37°C for 20 min, the reaction was stopped by cooling and by the addition of an equal volume of 7% $HClO_4$. Bovine serum albumin (0.1 mg) was added as carrier. Controls were the same except they contained denatured Ca^{2+}-ATPase. Precipitable material was collected on 0.45 μm Millipore HA filters, and washed with 3 ml of 1 M HCl, 0.1 mM sodium pyrophosphate (4×), and 95% ethanol (2×).

2.6. Chain elongation of polyP by ATP

The reaction mixture (50 μl) contained $polyP_5$ (10 μg), 1 μg Ca^{2+}-ATPase, [γ-^{32}P]ATP (30 Ci/mmol), 50 mM HEPES, KOH, pH 7.4, 130 mM KCl, 8 mM $MgCl_2$, 0.15 mM $C_{12}E_8$, 20 μM 1-palmitoyl, 2-oleoyl, phosphatidylcholine, 1 mM EGTA, 1 mM $CaCl_2$. Controls were either without $CaCl_2$ and with added EGTA (10 mM) or without Ca^{2+}-ATPase. After incubation at 37°C for 40 min, a portion of the samples was added to loading buffer (10 μl) and subjected to electrophoresis on 20% acrylamide, 7 M urea minigels (1:10 acrylamide:bisacrylamide) with Tris/borate/EDTA (90/90/2.7 mM) pH 8.3 buffer. Standards were $(polyP)_5$, [^{32}P]Pi, xylene cyanol, and brom phenol blue. The position of the two dye markers was previously calibrated against short-chain polyPs that were prepared and measured as described by Clark and Wood [23]. Visualization was by exposure to Hyperfilm-MP (Amersham) for 12 h.

2.7. Formation of ATP from polyP

[^{32}P]polyP was prepared by incubating 500 μCi [γ-^{32}P]ATP (30 Ci/mmol) in 50 mM HEPES-KOH, pH 7.2, 40 mM ammonium sulfate, 4 mM $MgCl_2$, 0.1 mM $(polyP)_5$, acetyl phosphate (2 mM), acetate kinase (20 μg/ml) and PPK (10^4 units) at 37°C for 2 h. The reaction was stopped by addition of EDTA (50 mM) and the [^{32}P]polyP was purified as described by Akiyama et al. [13]. The resulting [^{32}P]polyP had an average chain length >300 residues based on polyacrylamide gel analysis [23].

[^{32}P]polyP was incubated with Ca^{2+}-ATPase (2 μg), 1 mM ADP, 100 mM KCl, 4 mM $MgCl_2$, 0.1 mM $CaCl_2$ in 10 mM HEPES.KOH (pH 7.4) for 30 min at 37°C [21]. Control was without Ca^{2+}-ATPase. ATP and ADP (15 nmol each) were added to the samples as carriers, and ADP, ATP and polyP were resolved by thin layer chromatography on a polyethyleneimine-cellulose plate using 1 M LiCl and 1 M HCOOH. After autoradiography, ADP and ATP and polyP at the origin were visualized by UV, and quantitated by cutting out the appropriate areas and measuring the radioactivity by liquid scintillation counting.

2.8. Phosphorylation of Ca^{2+}-ATPase by [^{32}P]polyP

[^{32}P]polyP was added to Ca^{2+}-ATPase (2 μg) in a solution of 10 mM HEPES-KOH, pH 7.2, containing 100 mM KCl, 1.0 mM $MgCl_2$, 750 μM $C_{12}E_8$, 50 μM $CaCl_2$ (total volume 25 μl) and the mixture was incubated for 2 min at room temperature. Polyphosphatase (PPX) (40 units) was added to degrade remaining polyP, and a portion of the mixture was separated by polyacrylamide (10%) gel electrophoresis, and visualized by exposure to Hyperfilm-MP for 36 h.

3. Results and discussion

The Ca^{2+}-ATPase constitutes only 0.1% or less of the total erythrocyte membrane protein; nonetheless, it can be purified to homogeneity by calmodulin affinity chromatography [18,24]. We first probed the purified Ca^{2+}-ATPase on Western blots with anti-PHB IgG [20]. The protein, composed of a single polypeptide of molecular mass 135 kDa, gave a strong

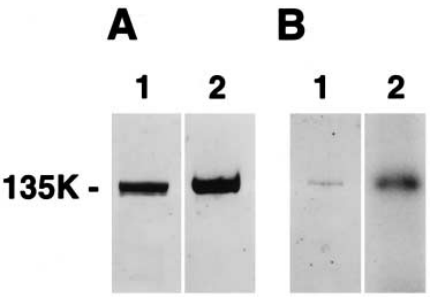

Fig. 1. (A) Reaction of Ca^{2+}-ATPase protein to anti-PHB IgG. Purified Ca^{2+}-ATPase protein (8.5 μg per lane) was separated by electrophoresis on a 10% polyacrylamide gel. Lanes: 1, Coomassie blue stain of Ca^{2+}-ATPase protein; 2, Western blot of Ca^{2+}-ATPase protein probed with rabbit anti-PHB IgG[21]. Second antibody was anti-rabbit IgG conjugated to alkaline phosphatase. Color development was with the alkaline phosphatase substrate kit (Bio-Rad). (B) Phosphorylation of the Ca^{2+}-ATPase by [^{32}P]polyP. Purified Ca^{2+}-ATPase protein (2 μg) was phosphorylated at room temperature by [^{32}P]polyP as described in the Methods (see Section 2), and separated by electrophoresis on a 10% polyacrylamide gel. Lanes: 1, Coomassie blue stain of phosphorylated Ca^{2+}-ATPase; 2, Autoradiogram of phosphorylated Ca^{2+}-ATPase.

positive reaction to the antibody (Fig. 1A). The co-migration of PHB with the Ca^{2+}-ATPase indicates the lipidic polymer is tightly complexed or covalently bound to the protein. PHB is highly insoluble in water and uncharged, so that uncomplexed PHB does not migrate on electrophoretic gels. Furthermore, although PHB is soluble in chloroform, it was not possible to remove PHB from the Ca^{2+}-ATPase by repeated extraction with this solvent.

To confirm the identity of PHB, erythrocyte ghosts and purified Ca^{2+}-ATPase were each analyzed using a chemical assay in which PHB is converted by β elimination to its unique degradation product, crotonic acid (Fig. 2). According to this assay, the erythrocyte ghosts contained 0.06 μg PHB/mg protein and the pure Ca^{2+}-ATPase protein 1.3 μg PHB/mg protein, indicating a greater than twenty fold increase in concentration of PHB in the Ca^{2+}-ATPase as compared to the ghosts. It should be emphasized that the PHB determinations furnish proof of the identity of PHB and provide us with relative concentrations in the two samples, but they do not accurately reflect the in vivo concentrations. Not only is the amount of the polymer underestimated by the unavailability of proper standards for the assay (see Section 2), but PHB, as a polyester, is considerably degraded before measurement by enzymatic and chemical hydrolysis.

We next tested the Ca^{2+}-ATPase for the presence of polyP by staining the protein on SDS-PAGE gels with the cationic dye, *o*-toluidine blue. PolyP of comparatively high molecular weight (>5 residues) exhibits metachromasy, and causes a shift in the absorption maximum of the *o*-toluidine blue towards shorter wavelengths, i.e. from a maximum at 607 nm (blue) to 530 nm (violet) [25]. The 135 kDa Ca^{2+}-ATPase band reacted with the dye to give the characteristic violet color of polyP. The identity of polyP was then confirmed by an enzymatic assay in which polyP is used to convert ^{14}C-ADP to ^{14}C-ATP in the presence *E. coli* polyphosphate kinase (PPK) [21]. The amount of polyP in this sample was estimated as 0.5 μg/mg protein. However, as in the case of PHB above, this value does not accurately reflect its in vivo concentration. PolyP is subject not only to chemical and en-

zymatic hydrolysis during purification and storage, but the highly insoluble calcium salt believed to exist in the Ca^{2+}-ATPase may be partially converted to the water-soluble potassium salt in the aqueous buffer system. The data prove the presence of polyP in the Ca^{2+}-ATPase, but provide only a minimal value for its concentration.

Previous studies have demonstrated that purified Ca^{2+}-ATPase can be phosphorylated by [γ-^{32}P]ATP [26], generating an acyl phosphoenzyme which can transfer the phosphoryl group back to ADP and regenerate ATP [18,27]:

$$Ca^{2+}\text{-ATPase} + [\gamma\text{-}^{32}P]ATP \leftrightarrow Ca^{2+}\text{-ATPase}\cdot[^{32}P]Pi + ADP \quad (1)$$

It is of interest that the polyphosphate kinase (PPK) of *E. coli* behaves similarly; this enzyme is also phosphorylated by ATP, generating an N-linked phosphoenzyme intermediate, which can then transfer the phosphoryl group back to ADP [22]:

$$PPK + [\gamma\text{-}^{32}P]ATP \leftrightarrow PPK\cdot[^{32}P]Pi + ADP \quad (2)$$

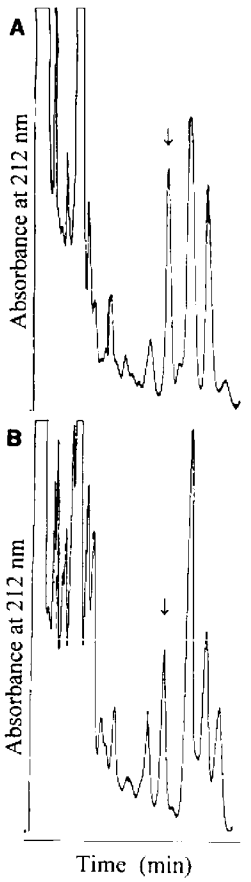

Fig. 2. HPLC elution profile showing the presence of crotonic acid (arrow) in concentrated sulfuric acid digests of human erythrocyte ghosts and purified Ca^{2+}-ATPase (see the Methods in Section 2). Chromatography was done on an HPLC Aminex HPX-87H organic acids column (Bio-Rad) with 0.014 N H_2SO_4 as eluent. Crotonic acid was identified by its elution time, UV absorption curve, and co-elution with standard crotonic acid, and its identity was confirmed by mass spectroscopy. Quantitation was by comparison of the peak area with that of crotonic acid standards. (A) 2.6 mg human erythrocyte ghosts. (B) 0.15 mg purified Ca^{2+}-ATPase.

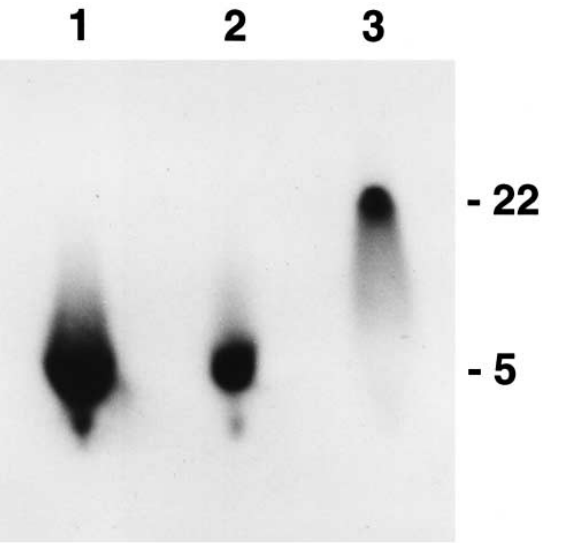

Fig. 3. Elongation of polyP$_5$ by [γ-^{32}P]ATP. PolyP$_5$ (10 μg) was incubated with 2 μg Ca^{2+}-ATPase, [γ-^{32}P]ATP (30 Ci/mmol), 50 mM HEPES-KOH, pH 7.4, 130 mM KCl, 8 mM $MgCl_2$, 0.15 mM $C_{12}E_8$, 20 μM 1-palmitoyl, 2-oleoyl, phosphatidylcholine, 1 mM EGTA, 1 mM $CaCl_2$. Electrophoresis was performed on 20% acrylamide, 7 M urea minigels. PolyP$_5$ was selected for this assay because increases in chain length are more noticeable on gels for short-chain polyphosphates. Standards were polyP$_5$, [^{32}P]Pi, xylene cyanol and brom phenol blue. The position of the dye markers was calibrated against short-chain polyPs that had been prepared and measured as described by Clark and Wood [23]. Visualization was by autoradiography. Lanes: 1, incubation mixture contained all of the above except Ca^{2+}-ATPase; 2, incubation mixture contained all of the above except $CaCl_2$ was omitted and 10 mM EGTA was added; 3, incubation mixture contained all of the above.

However, PPK can also be phosphorylated by polyP, presumably generating the same phosphorylated intermediate, which can then transfer the phosphoryl group to the end of a polyP$_n$ chain (Eq. 3). As a consequence of these reversible reactions, ATP can elongate a polyP chain and/or polyP can be used to form ATP from ADP:

$$PPK + [^{32}P](polyP)_n \leftrightarrow PPK\cdot[^{32}P]Pi + [^{32}P](polyP)_{n-1} \quad (3)$$

Inasmuch as acyl phosphates have greater phosphorylation potentials than N-linked phosphates, we investigated the ability of the Ca^{2+}-ATPase to catalyze the transfer of the γ-phosphate of ATP to exogenous and endogenous polyP. The former was demonstrated by observing the increase in chain length of a short-chain polyP ((polyP)$_5$) on acrylamide gels after incubation with [γ-^{32}P]ATP and Ca^{2+}-ATPase in the presence of Ca^{2+} (Fig. 3). The ability of the Ca^{2+}-ATPase, itself, to produce acid-precipitable polyP from ATP was confirmed by the method used by Ahn and Kornberg [22] to establish this activity for PPK. Incubation of the ATPase (15 pmol) with 0.5 mM ATP for 20 min at 37°C in the presence of an ATP regenerating system produced an average of 14 nmol of acid-precipitable polyP (two samples). The observed reaction for both of these studies (Eq. 4) is the sum of the forward reaction in Eq. 1 and the reaction to transfer of the phosphoryl group from Ca^{2+}-ATPase·[^{32}P]Pi to polyP. It is clear that the Ca^{2+}-ATPase not only contains polyP, but also can transfer phosphoryl residues from the γ phosphate of

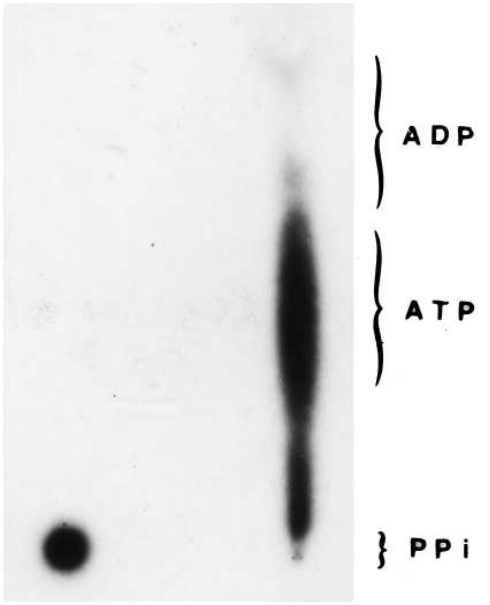

Fig. 4. Formation of [^{32}P]ATP from [^{32}P]polyP and ADP by Ca^{2+}-ATPase. The [^{32}P]polyP, with average chain length >300 residues, was incubated with 1 mM ADP, 100 mM KCl, 4 mM $MgCl_2$, 0.1 mM $CaCl_2$ in 10 mM HEPES-KOH (pH 7.4) and Ca^{2+}-ATPase for 30 min at 37°C [23]. ADP and ADP (15 nmol each) were added as carriers, and ADP, ATP, and polyP were resolved by thin layer chromatography on PEI cellulose, visualized by UV, and autoradiography. Brackets indicate regions of the chromatogram occupied by long chain polyP, ATP, and ADP standards after development. Right lane: reaction in the presence of Ca^{2+}-ATPase. Over 80% of the radioactive phosphate in the product co-migrated with ATP. The remainder, which appears to be short-chain [^{32}P]polyP resulting from incomplete reaction, formed a band between the origin and the ATP band. Left lane: result in the absence of Ca^{2+}-ATPase.

ATP to the polyP chain:

$$[\gamma -^{32}\mathrm{P}]\mathrm{ATP} + (\mathrm{polyP})_n \rightarrow \mathrm{ADP} + [^{32}\mathrm{P}](\mathrm{polyP})_{n+1} \quad (4)$$

Since polyP and ATP have similar phosphorylation potentials [1,2], we investigated the ability of the Ca^{2+}-ATPase to carry out the reverse reaction. The capacity of [^{32}P]polyP to phosphorylate the Ca^{2+}-ATPase is shown in the autoradiogram in Fig. 1B. The identity of the amino acid phosphorylated by polyP is not known; however, the ability of the phosphorylated residue to transfer the phosphoryl group to ADP and generate ATP was demonstrated by incubating the Ca^{2+}-ATPase with [^{32}P]polyP and ADP [23] (Eq. 5). After separation of ADP, ATP, and polyP by thin layer chromatography, nearly 80% of the radioactive phosphate was found to comigrate with ATP (Fig. 4). This result indicates that the Ca^{2+}-ATPase can be phosphorylated by polyP as well as by ATP, yielding the intermediate Ca^{2+}-ATPase·Pi, which then transfers the phosphoryl group to ADP, as in Eq. 1:

$$[^{32}\mathrm{P}](\mathrm{polyP})_n + \mathrm{ADP} \rightarrow [^{32}\mathrm{P}](\mathrm{polyP})_{n-1} + [^{32}\mathrm{P}]\mathrm{ATP} \quad (5)$$

There are a number of ways in which these novel polymers and newly discovered enzymatic activities may expedite the extrusion of Ca^{2+} by the Ca^{2+}-ATPase. We suggest that the Ca^{2+}-ATPase may be considered to be a supramolecular structure consisting of protein, PHB, and polyP. The PHB may be held within the protein by non-covalent associations with amino acid side-chains, but also may be covalently bound to an amino acid at one of its termini. Solvation of polyP by PHB could allow this polyanion to penetrate into the bilayer portion of the Ca^{2+}-ATPase. Since both ends of the polyP molecule can accept or deliver phosphoryl groups, polyP may act as an agent for long-distance phosphoryl transfer. For example, one end of the polyP chain at the cytoplasmic face may accept a phosphoryl group from ATP while the other end delivers a phosphoryl group to an acyl group of an amino acid residue situated within the bilayer region. Coordination of these transfers would maintain a constant chain length. Furthermore, the polyP molecule, itself, may serve as an agent to sequester and transfer Ca^{2+}. The polyP helix may adapt conformations in which adjacent phosphoryl groups form binding sites selective for divalent cations [8]. Both Mg^{2+} and Ca^{2+} are attracted to such sites; however, Ca^{2+} would be preferentially selected as the polyP chain extends into the lower dielectric constant medium of the bilayer region. Factors in this selection of Ca^{2+} include its larger ion diameter, greater coordination number, tolerance for variability in bond lengths and angles, and more rapid hydration kinetics [28].

The presence of these novel polymers in the Ca^{2+}-ATPase pump and their singular properties may open new insights into the molecular mechanism by which Ca^{2+} is exported. Moreover, thermodynamic considerations suggest that polyP synthesis and degradation may occur not only in the plasma membrane Ca^{2+}-ATPase pump, but in other proteins that serve as intermediates in phosphoryl transfer.

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