Streptomyces lividans Potassium Channel Contains Poly-(*R*)-3-hydroxybutyrate and Inorganic Polyphosphate†

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ABSTRACT: The *Streptomyces lividans* KcsA potassium channel, a homotetramer of 17.6 kDa subunits, was found to contain two nonproteinaceous polymers, namely, poly-(*R*)-3-hydroxybutyrate (PHB) and inorganic polyphosphate (polyP). PHB and polyP are ubiquitous cellular constituents with a demonstrated capacity for cation selection and transport. PHB was detected in both tetramer and monomer species of KcsA by reaction to anti-PHB IgG on Western blots, and estimated as 28 monomer units of PHB per KcsA tetramer by a chemical assay in which PHB is converted to its unique degradation product, crotonic acid. PolyP was detected in KcsA tetramers, but not in monomers, by metachromatic reaction to *o*-toluidine blue stain on SDS−PAGE gels. A band of free polyP was also visible, suggesting that polyP is released when tetramers dissociate. The exopolyphosphatase of *Saccharomyces cerevisiae* degraded the free polyP, but tetramer-associated polyP was not affected, indicating it was inaccessible to the enzyme. PolyP in KcsA was estimated as 15 monomer units per tetramer by an enzymatic assay in which polyphosphate kinase is used to transfer phosphates from polyP to [^{14}C]ADP, yielding [^{14}C]ATP. The experimentally determined isoelectric point of KcsA tetramer was 6.5−7.5, substantially more acidic than the theoretical pI of 10.3, and consistent with the inclusion of a polyanion. The results suggest that PHB is covalently bound to KcsA subunits while polyP is held within tetramers by ionic forces. It is posited that KcsA protein creates an environment in which PHB/polyP is selective for K^+. The basic amino acids attenuate the negative charge density of polyP, thereby transforming the cation binding preference from multivalent to monovalent, and discrimination between K^+ and Na^+ is accomplished by adjusting the ligand geometry in cation binding cavities formed by PHB and polyP.

Poly-(*R*)-3-hydroxybutyrate (PHB)[1] and inorganic polyphosphate (polyP) are simple homopolymers that are found in a wide variety of organisms ranging from bacteria to mammals (*1*−*14*). The individual and combined molecular properties of PHB and polyP make them well suited to engage in selective transfer of cations across membranes. PHB, a linear polymer of (*R*)-3-hydroxybutyrate, is an amphiphilic polyester that forms ion-conducting complexes with salts (*14*−*19*); polyP, a linear polymer of phosphoryl units joined by high-energy phosphoanhydride bonds, has a high capacity for ion exchange and ability to discriminate among cations by charge (*20*, *21*). Complexes of the two polymers, isolated from bacterial plasma membranes or prepared from the synthetic polymers, form voltage-activated calcium-selective channels in planar lipid bilayers that display many of the characteristics of protein calcium channels (*22*−*25*). PHB is also found in close association with specific proteins in diverse prokaryotes and eukaryotes (*3*−*8*, *26*), and both PHB and polyP are components of the human erythrocyte CaATPase pump (*27*). The ubiquitous presence of these polymers in cells, their inherent aptitude for ion selection and transport, and their association with channel and pump proteins prompted this examination of the potassium channel of the Gram-positive bacterium *Streptomyces lividans* (KcsA), the first ion channel to be analyzed by X-ray crystallography (*28*).

KcsA is a 160 amino acid polypeptide that oligomerizes in a tetramer to form a highly selective potassium channel (*29*−*32*). KcsA has been cloned, overexpressed, and purified to homogeneity, and its stability and electrophysiological behavior have been examined (*29*−*36*). Its small size, high levels of expression, and stability in detergent solutions have made it an excellent candidate for structural and biochemical studies. X-ray analysis of KcsA crystals, with data to 3.2 Å resolution, revealed that residues 23−119 of four identical subunits create an inverted teepee structure with a 12 Å long narrow pore at the outer end (*28*). Each subunit has 2 transmembrane α-helices connected by a pore region of roughly 30 amino acids, a common structural motif in K^+ channels. The outer helix faces the lipid bilayer, and the inner helix faces the central pore. Segments of the amino acid

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[1] Abbreviations: PHB, poly-(*R*)-3-hydroxybutyrate; polyP, inorganic polyphosphate; KcsA, K^+ channel from *Streptomyces lividans*; C_{12}M, *n*-dodecyl-β-D-maltoside; NTA, nitrilotriacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CAPSO, 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid; IEF, isoelectric focusing; p*I*, isoelectric point; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, nitroblue tetrazolium; PEI, polyethylenimine.

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sequence of KcsA show similarity to the sequences of eukaryotic K^+ channels (*28*, *29*). The correspondence is particularly strong for the residues of the highly conserved signature sequence in the pore region and is also significant for the residues of the inner helix. The existence of these sequences, critical to the ion conduction and gating processes, indicates that KcsA has essentially the same pore structure and mechanism of potassium selectivity as eukaryotic potassium channels (*37*).

There is no clear evidence of PHB and/or polyP in the electron density maps published by Doyle et al. (*28*) for the truncated KcsA channel; nonetheless, we find here that both polymers are components of the integral channel. The results of this study suggest that the *S. lividans* K^+ channel is a supramolecular structure in which protein, PHB, and polyP combine to carry out the intricate mechanisms of voltage sensing, K^+ selection, and transport.

EXPERIMENTAL PROCEDURES

S. lividans KcsA Potassium Channel (KcsA). Purified KcsA was generously provided by C. Miller (Brandeis University). The protein was prepared from a KcsA clone, modified by additions of a hexahistidine tag immediately following the initial methionine and two amino acids, A161 and S162, followed by a strep tag at the C-terminal end. For details of modification, expression, and purification procedures, see Heginbotham et al. (1997, 1998) (*30*, *33*). Briefly, the gene was transferred into pASK90, and the resulting construct was transformed into *E. coli* strain JM-83. Cells were grown in "Terrific broth", 50 μg/mL ampicillin, and expression was induced by anhydrotetracycline (200 μg/mL). Cells were disrupted by sonication, and pelleted membranes were suspended in 100 mM NaP_i, 5 mM NaCl, pH 7.0, and extracted with 10 mM *n*-dodecyl-β-D-maltoside ($C_{12}M$). After removal of membranes by centrifugation, the extract was incubated with Ni-NTA agarose in the presence of 20−40 mM imidazole, and the protein was eluted with 1 mM $C_{12}M$, 400 mM imidazole, pH 7.0. The protein sample used in this study, diluted into loading buffer (unheated) and subjected to SDS−PAGE gel and Coomassie-staining as described below, displayed a major band at ∼65 kDa, consistent with KcsA tetramer, and a minor band at ∼19 kDa, consistent with KcsA subunit (*30*, *31*).

SDS−PAGE and Western Blotting. KcsA (4 μg in 1 mM $C_{12}M$, 400 mM imidazole, pH 7.0) was diluted into Laemmli loading buffer containing 2% β-mercaptoethanol. The sample was heated in a 70 °C bath for 1 min and resolved on a 12% polyacrylamide gel into tetramer and monomer species using SDS−Tris−glycine buffer. Half the gel was stained with Coomassie Brilliant Blue R250; the other half was transferred to a supported nitrocellulose membrane in CAPS buffer, pH 11.3, using a Mini Trans-Blot electrophoretic cell. The membrane was blocked with 2% gelatin (electrophoresis grade; Bio-Rad) in Tris-buffered saline, pH 7.5, 0.1% Tween-20. Primary incubation was with polyclonal anti-PHB IgG in blocking buffer. The PHB antibody was produced in rabbits to synthetic 8mer of PHB conjugated to gelatin (Metabolix Inc.), and purified by protein A chromatography. Second antibody was goat anti-rabbit alkaline phosphatase conjugate in the same buffer. Color development was performed with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) (Bio-Rad).

Assay for PHB. The procedure was essentially as reported by Huang and Reusch (*26*). Briefly, KcsA (50 μg) was precipitated with 5 mL of cold 95% ethanol, and dried by lyophilization. Concentrated sulfuric acid was added (0.6 mL), and the sample was heated at 120 °C for 40 min. The resulting crotonic acid was extracted, and chromatographed on an Aminex HPX-87H ion exclusion organic acid analysis column (Bio-Rad; 4.1 × 250 mm) using 0.014 N H_2SO_4 as eluent. The crotonic acid peak was identified by its elution time, UV absorption curve, and mass spectrum, and quantitated by comparison of the peak area with those of crotonic acid standards. PHB standards (from *Alcaligenes* spp.), subjected to the same procedure, produced ∼50% of the theoretical yield of crotonic acid.

Extraction of PolyP. The method used was essentially as described by Crooke et al. and Kumble and Kornberg (*38*, *39*). KcsA was incubated with proteinase K (60 μg/mL) for 2 h at 37 °C, and extracted with phenol/chloroform (1:1, equilibrated with Tris-HCl, pH 7.5). The mixture was separated into phases by centrifugation at 14000*g* for 5 min. The phenol phase was back-extracted 2× with 50 mM Tris-HCl, pH 7.5, 10 mM EDTA. The aqueous phases were pooled and extracted 3× with water-saturated chloroform. PolyP was precipitated with ice-cold *sec*-butanol, collected by centrifugation at 14000*g* for 30 min, and resuspended in 1 mM $C_{12}M$, 400 mM imidazole, pH 7.0. Loss of polyP during this procedure was estimated as ∼10% by addition of ^{32}P-polyP as a tracer (*38*, *39*).

Assay for PolyP. The method used was essentially that of Crooke et al. (*38*) as previously described (*40*). Briefly, polyP purified from KcsA was used as a substrate for conversion of [^{14}C]ADP to [^{14}C]ATP catalyzed by *E. coli* polyphosphate kinase (gift from A. Kornberg, Stanford University). The reaction mixture (10 μL) contained 50 mM KHepes, pH 7.2, 40 mM $(NH_4)_2SO_4$, 4 mM $MgCl_2$, 0.2 mM [^{14}C]ADP, 2000 units of polyphosphate kinase, and polyP sample. Samples were as follows: native KcsA; polyP extracted from KcsA; and synthetic polyP (type 45). After incubation at 37 °C for 45 min, the reaction was terminated by cooling in an ice bath and addition of ADP and ATP to 5 mM. Aliquots were spotted on PEI−cellulose thin-layer plates, and polyP, ATP, and ADP were resolved by development in 1.0 M LiCl, 1.0 M HCOOH. Spots corresponding to ADP and ATP were visualized with a UV lamp, by autoradiography, cut out, and quantitated by liquid scintillation.

Determination of Isoelectric Point (pI). The pI of KcsA was determined by analytical and slab gel isoelectric focusing. Analytical focusing was carried out on 0.4 mm gels of 5% acrylamide/bisacrylamide (33.7:1), 10−30 mM ($C_{12}M$), 5% 3−10 ampholytes on gel support film using a Bio-Rad model 111 Mini IEF cell. IEF standards were from Bio-Rad. Gels were stained with Coomassie Brilliant Blue R-250, crocein scarlet (Bio-Rad IEF stain), and destained with 40% methanol, 10% acetic acid. Voltages were stepped from 100 to 450 V for a total of 90 min. Slab gels were prepared from 5% acrylamide/bisacrylamide (29:1), 5% glycerol, 10−30 mM $C_{12}M$, 2.5% carrier ampholytes (3−10; 40%; Bio-Rad) in a Mini-protean II gel apparatus. Anode buffer was phosphoric acid, pH 2.2; cathode buffer was arginine, lysine, pH 12. Voltages were stepped from 100 to 500 V for total focusing times of 2.5−6 h. Standards, staining, and destaining were as above.

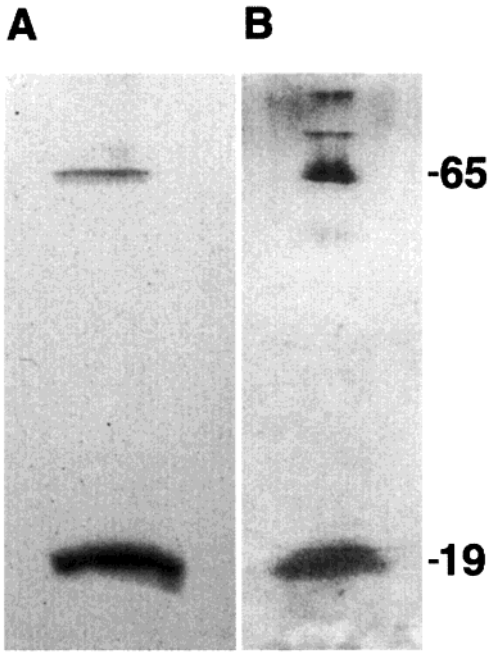

FIGURE 1: Presence of PHB in KcsA tetramers and monomers. The figure shows (A) a 12% SDS−PAGE gel of partially dissociated KcsA visualized with Coomassie Brilliant Blue stain and (B) a Western blot of a similar gel probed with anti-PHB IgG. Second antibody was conjugated to alkaline phosphatase. Samples were heated for 1 min at 70 °C before loading.

RESULTS

PHB in KcsA. Purified KcsA was partially dissociated by brief heating (1 min at 70 °C) in SDS buffer and then separated by SDS−PAGE into tetramer and monomer species. Transfer of the tetramer to a nitrocellulose membrane in Tris−glycine buffer, pH 8.3, was nearly complete; however, only traces of the monomer transferred at this pH or in CAPSO buffer, pH 10.0. Successful transfer of monomer (and tetramer) was achieved in CAPS buffer, pH 11.3. Both forms reacted positively to anti-PHB IgG on a Western blot (Figure 1). PHB is uncharged and does not itself migrate on electrophoretic gels; hence, its comigration with protein indicates it is tightly bound. Although PHB is soluble in chloroform, repeated extraction with warm chloroform did not separate it from the protein, suggesting that the bonding is covalent.

The presence of PHB in purified KcsA was confirmed, and the amount was estimated by a chemical assay in which PHB is converted to crotonic acid via β-elimination by heating in concentrated sulfuric acid. The crotonic acid is then extracted, separated by HPLC chromatography, and quantitated by comparison of the peak area with those of standards (see Experimental Procedures). Under the conditions of the assay, no amino acids or homopolymers of amino acids produce significant amounts of crotonic acid (*26*). The method was originally developed by Karr et al. (*41*) for measuring high molecular weight granule PHB in bacteria. Huang and Reusch (*26*) found that PHB conjugated to protein required higher temperatures (120 vs 90 °C) and longer incubation times (40 vs 20 min) to achieve maximum yields of crotonic acid. Under these more severe conditions, there are considerable losses due to thermolytic degradation of PHB and crotonic acid (*42*). The loss was estimated experimentally for granule PHB as ∼50% (*25*), but is unknown for protein-conjugated PHB. Since there are presently no suitable standards for protein-conjugated PHB, values are given relative to granule PHB. In the case of KcsA, PHB was estimated at 34 μg/mg of protein, indicating ∼28 monomer units of PHB per tetramer, presumably 7 units per monomer. While this assay furnishes proof of the presence of PHB, the value it provides should be considered as minimal and may not accurately reflect the in vivo concentration.

PolyP in KcsA. KcsA was heated for 1 min at 70 °C and separated into tetramer and monomer species by SDS−PAGE, and the gel was stained with the cationic dye *o*-toluidine blue. With this dye, polyPs with five or more residues exhibit metachromasy and shift the absorption maximum of the dye toward shorter wavelengths, i.e., from a maximum at 507 nm (blue) to 530 nm (violet) (*43*). The tetramer band showed the characteristic purple−red color of polyP, whereas the monomer band did not. There was an additional faint band near the dye front that stained purple−red with *o*-toluidine blue but did not stain with Coomassie Brilliant Blue 250, suggesting the absence of protein. This band, which migrated to the same position as synthetic polyPs in similar SDS−PAGE gels, was considered to be free polyP, released during dissociation of tetramers. Unheated KcsA subjected to electrophoresis in the same gel was predominantly tetramer (>85%); staining with *o*-toluidine blue revealed polyP in the tetramer, but free polyP was not detected.

The identity of polyP was tested by observing the effects of treatment with exopolyphosphatase from *S. cerevisiae*. This enzyme removes orthophosphate units processively from the end of polyP chains in the range of 3−1000 units (*44*). Exopolyphosphatase (550 units) and 2 mM $MgCl_2$ were added to the sample after cooling, and the mixture was incubated for 1 h at room temperature. After SDS−PAGE and staining with *o*-toluidine blue, the tetramer still showed the characteristic purple−red color of polyP, but the band attributed to free polyP had disappeared. This supports the inference that the nonproteinaceous band is free polyP and also indicates that polyP in the tetramers is not accessible to exopolyphosphatase. Unfortunately, small amounts of low molecular weight polyP tend to dissolve during destaining to a degree that makes the reactions difficult to document.

The presence of polyP in tetrameric KcsA was confirmed and the amount estimated by an enzymatic assay in which *E. coli* polyphosphate kinase is used to transfer terminal phosphates from polyP to [^{14}C]ADP, yielding [^{14}C]ATP (*45*). This enzyme is highly specific for polyP; with ADP in excess, PPK converts nearly 90% of polyP to ATP. PolyP isolated from KcsA (see Experimental Procedures) and native KcsA were each incubated in a reaction mixture containing 0.2 mM [^{14}C]ADP, 2000 units of polyphosphate kinase (PPK) in 50 mM KHepes, pH 7.2, 40 mM $(NH_4)_2SO_4$, 4 mM $MgCl_2$ for 45 min at 37 °C (*25*) (Figure 2, lanes 3, 4). The products were separated by thin-layer chromatography on PEI−cellulose plates. ATP and ADP were visualized with UV irradiation and autoradiography, and quantitated by liquid scintillation counting. Controls were synthetic polyP (type 45) in the same reaction mixture (Figure 2, lane 1), and polyP isolated from KcsA with polyphosphate kinase omitted (Figure 2, lane 2). Allowing for 10% loss during purification (*37*, *38*), the amount of polyP isolated from KcsA was

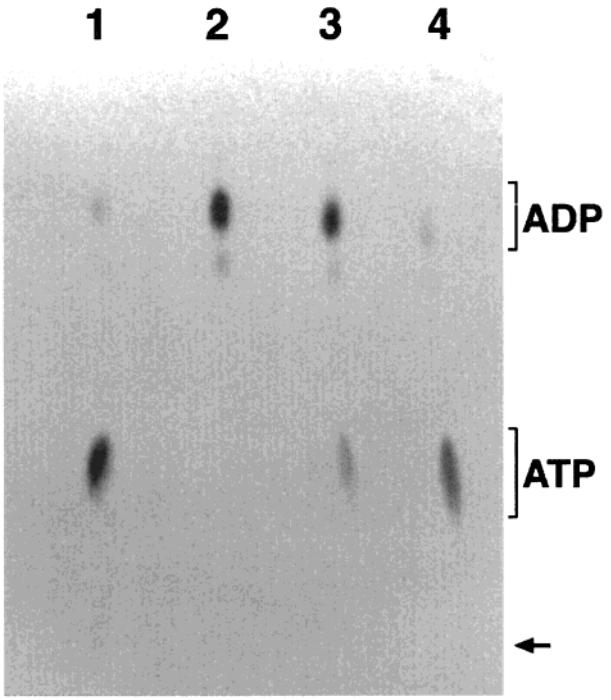

FIGURE 2: Autoradiogram showing formation of [^{14}C]ATP from [^{14}C]ADP and polyP in KcsA. Samples were incubated with 50 mM KHepes, pH 7.2, 40 mM $(NH_4)_2SO_4$, 4 mM $MgCl_2$, 0.5 mM [^{14}C]ADP, and 2000 units of polyphosphate kinase at 37 °C for 45 min. ADP and ATP (5 mM each) were added as carriers, and ADP and ATP were resolved by thin-layer chromatography on PEI−cellulose and visualized by UV and autoradiography. Brackets indicate regions of the chromatogram occupied by ADP and ATP standards after development with 1 M LiCl, 1 M HCOOH. Lane 1, 0.1 μg of polyP (type 45); lane 2, as in lane 4 but without polyphosphate kinase; lane 3, native KcsA; lane 4, polyP isolated from KcsA as described under Experimental Procedures. The arrow indicates the origin.

Table 1: Determination of PolyP in KcsA

reaction mixture[a]	polyP in native KcsA (μg of polyP/mg of KcsA)	polyP isolated from KcsA[b] (μg of polyP/mg of KcsA)
no additions	3	17
+5 μg of $polyP_{45}$/mg of KcsA	6.5	22
+exopolyphosphatase[c]	0	0

[a] The reaction mixture (10 μL) contained 50 mM KHepes, pH 7.2, 40 mM $(NH_4)_2SO_4$, 4 mM $MgCl_2$, 0.2 mM [^{14}C]ADP, 2000 units of polyphosphate kinase, and polyP sample. [^{14}C]ADP and [^{14}C]ATP were resolved on PEI−cellulose thin-layer plates with 1.0 M LiCl, 1.0 M HCOOH. Quantitation was by scintillation counting (see Experimental Procedures). [b] Isolation procedure is given under Experimental Procedures. Value is corrected for loss of polyP during isolation procedure, estimated as 10% (*26*, *27*). [c] Sample was incubated with 550 units of *S. cerevisiae* exopolyphosphatase in 400 mM imidazole, pH 7.0, 2 mM $MgCl_2$, 1 mM $C_{12}M$, for 1 h at room temperature before assay.

estimated as ~17 μg/mg of KcsA or ~15 polyP monomer units per tetramer (Table 1). Loss of polyP during purification of KcsA could not be estimated.

PolyP in native KcsA produced 18% as much ATP (~3 μg/mg of protein) as the polyP isolated from the K^+ channel. About 30% of this difference could be attributed to the inhibition of polyphosphate kinase by KcsA protein; this was assessed by comparing the increase in ATP formation when polyP type 45 was added to the two samples (Table 1). When native KcsA was treated with exopolyphosphatase as above before assay, no ATP was formed (Table 1). The results can be interpreted to mean that polyP in the tetramer is not accessible to polyphosphate kinase and that ATP is formed only by polyP released during dissociation of the oligomer. In this regard, the amount of ATP observed in native KcsA was consistent with the degree of dissociation, evaluated by observing the tetramer:monomer ratio on SDS−PAGE gels stained with Coomassie Brilliant Blue using an Ambis optical imaging system.

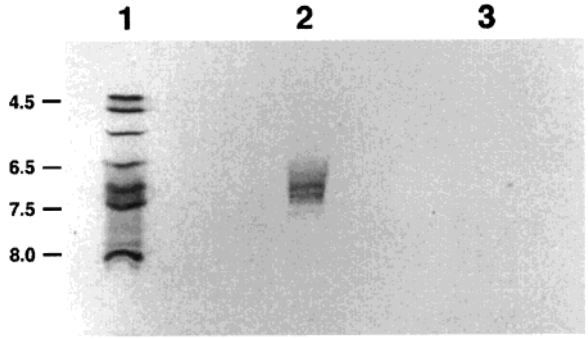

FIGURE 3: Analytical isoelectric focusing of KcsA. Focusing was carried out on 0.4 mm gels of 5% acrylamide/bisacrylamide (33.7:1), 20 mM ($C_{12}M$), 5% pH 3−10 ampholytes formed on gel support film. The gel was stained with Coomassie Brilliant Blue R-250, crocein scarlet, and destained with 40% methanol, 10% acetic acid. Lane 1, p*I* standards; lane 2, unheated KcsA; lane 3, KcsA heated for 5 min at 90 °C.

Isoelectric Points of KcsA Tetramer and Monomer. The theoretical isoelectric point (pI) of KcsA subunits was calculated as 10.30 using the ExPASy Compute pI/Mw tool (http://www.expasy.ch/tools/pi_tool.html) (*46*, *47*). This very basic pI reflects the excess of positively charged residues (19+, 13−), mainly arginines, in the polypeptide. KcsA used in this study had been modified by addition of a hexahistidine tag at the N-terminal end and a strep tag at the C-terminal end (*30*). These modifications produced subunits with a slightly higher theoretical pI of 10.53.

The pI of tetrameric KcsA was determined experimentally by analytical acrylamide gel focusing. In this method, an inverted horizontal gel (composed of 5% acrylamide, 20 mM dodecyl maltoside, 5% pH 3−10 carrier ampholytes) rested directly on graphite electrodes. Samples were applied near the center of the gel, thus avoiding pH extremes The oligomer moved from the position of application (pH 7.6) toward the anode and focused in a broad band, composed of a series of closely spaced bands, extending from pH 6.5 to pH 7.5 (Figure 3). Longer focusing times did not reduce the heterogeneity. This result indicates that the oligomers are a family of structures of diverse net surface charge, with the majority having approximately equal numbers of negatively and positively charged residues on the protein surface.

The monomer species proved too insoluble in the nonionic detergent, $C_{12}M$, to enter the gel. The monomer remained on the surface of gels containing $C_{12}M$ at concentrations up to 30 mM (Figure 3). Efforts to measure the p*I* of monomer by slab gel isoelectric focusing (5% acrylamide, 20 mM $C_{12}M$, 2.5% pH 3−10 carrier ampholytes) were also unsuccessful. The monomer remained in a pocket of the gel whether added to the cathode (pH 12) or the anode (pH 2.2) side. However, it should be noted that the calculated pI for the monomer and the experimental pI for the tetramer are

consistent with the pH conditions required to transfer the two species from gels to membranes.

DISCUSSION

These studies establish the presence of two nonproteinaceous polymers, PHB and polyP, in the *S. lividans* potassium channel, KcsA. Western blot analysis of SDS−PAGE gels indicates that PHB is covalently bound to each monomer unit (Figure 1), and chemical assay roughly estimates ∼28 monomer units of PHB per oligomer. The accumulated evidence, obtained from polyphosphate kinase assay (Figure 2), analytical isoelectric focusing (Figure 3), and reaction to *o*-toluidine stain and exopolyphosphatase, indicates that a polyP chain of ∼15 units is held by ionic forces within KcsA oligomers. Given the molecular characteristics of PHB and polyP (*17*−*21*) and their demonstrated roles in ion selection and transport (*22*−*25*), one should not find their presence in an ion channel surprising. PHB, by virtue of its electron-donating oxygens closely spaced along a flexible backbone, is capable of solvating salts of hard cations; its amphiphilic nature allows it to penetrate hydrophobic regions inaccessible to water. PolyP, a polymorphic polyanion with a high density of negative charge, strongly attracts cations and has a well-known ability to discriminate among them by charge (*20*, *21*).

PHB is highly insoluble in water; in all probability, it is located in a hydrophobic region of the protein. K^+ and Na^+ salts of polyPs are water-soluble. It is possible that polyP is ionically bound to positive residues on the outside of the intracellular portion of the tetramer, but ionic bonds are weak in aqueous salt solutions and it seems unlikely that polyP would remain attached to KcsA throughout purification and SDS−PAGE. The affinity of polyP for PHB, demonstrated by the formation of synthetic PHB/polyP channels in lipid bilayers (*24*), suggests that the polyP is complexed to PHB within the channel.

Neither PHB nor polyP is discernible in the electron density maps of KcsA recently published by Doyle et al. (*28*), but there is no contradiction between the modeled crystal structure and the present study. Only 60% of the channel residues, comprising the most hydrophobic region (residues 23−119), were imaged. Much of the intracellular entryway (residues 126−158) had been excised, and most remaining intracellular residues (1−22, 120−125, 159−160) were disordered. The excision of 15% of the protein, the most highly charged region (18 of 23 residues; 9+, 9−), may also have eliminated the polyP and much of the PHB. Even if present, both PHB and polyP are polymorphic and hence disordered and difficult to image.

By all reports, KcsA is remarkably stable. The tetramer resists dissociation in nonionic detergent for over a month at room temperature and for at least an hour in the ionic detergent sodium dodecyl sulfate (*30*, *31*, *33*). It also survives exposure to heat up to ∼60 °C for at least 30 min (*31*). It is interesting to note here that PHB/polyP complexes dissociate at 50−60 °C (*22*). The stability of the tetramer is difficult to reconcile with the high net positive charge of the subunits (19+, 13−). The partial X-ray structure images only 9 (5+, 4−) of the 32 charged residues in each subunit. The acidic residues face inward and the basic residues mainly outward, indicating that this portion of the channel has a positive surface charge. However, the pI of the tetramer indicates that the surface charge of the integral channel is nearly neutral, and this makes it essential to consider the contribution of the 23 charged residues (14+, 9−) that were not imaged. Since these residues are all at the intracellular end, the neutral surface charge implies that a plurality of the excised or disordered basic residues of the intracellular entryway face inward. If these conjectures are correct, it is difficult to understand how K^+ ions are attracted to this entryway.

The perceived electrostatic problems can be resolved by placing polyP within the intracellular entryway of the channel. As a polyanion, polyP can attract and bind together the four basic monomers. At the same time, its presence converts the net charge from positive to negative, and provides an attractive entryway and staircase of negative charges for K^+ transport. By drawing basic residues inward, it leaves the protein surface with a neutral to acidic charge. The ladder of closely spaced bands formed by KcsA oligomers on isoelectric focusing gels (Figure 3), indicating heterogeneity of structure, may be attributed to polyP scission that occurs primarily at end residues (*20*). Although polyP in KcsA apparently was not accessible to enzymes, this does not rule out hydrolysis of terminal phosphates during extended exposure to aqueous solutions. As the chain of phosphates shortens, the net charge of the channel becomes less positive. This interpretation suggests that the in vivo pI is in the more acidic part of the observed range. It is also possible that the heterogeneity in p*I* reflects a diversity of conformations assumed by C-terminal charged amino acids and the polymorphic polyP under the influence of an electric field.

Since polyP has a well-known preference for binding divalent cations, and PHB/polyP are particularly selective for Ca^{2+} (*22*−*24*), one may question its presence in a K^+ channel. This binding preference of polyP is rooted in the greater binding energy of divalent ions, the high charge density of polyP at or near physiological pH, and the exceptional rotational flexibility of the P−O−P bond that enables two neighboring phosphoryl units to approach closely enough to form a divalent binding site (*20*). However, if the charge density is decreased, binding of divalent cations is inhibited, and polyP will select monovalent cations.

These data form the basis of a hypothetical mechanism in which all three polymers cooperate to carry out KcsA functions. It is assumed that each subunit contains a chain of PHB, covalently bound at its carboxy end to an amino acid side chain, and that the polyP is held in the center of the channel by equal ionic attraction to all four subunits. PolyP acts as a voltage sensor and attracts cations to the intracellular entryway. Strategic placement of basic residues of the protein (primarily arginines) attenuates the negative charge density of polyP, transforming its cation selectivity from divalent to monovalent. All three polymers participate in discriminating between K^+ and Na^+. PHB and polyP provide the oxygen ligands that form the binding cavities, but protein architecture defines the distance between ligands of PHB and polyP, and between PHB ligands on neighboring subunits. The methyl groups of PHB are attracted to hydrophobic amino acids along the cavity wall, and the ester carbonyl oxygens face inward to solvate K^+. By attaching a chain of PHB to each subunit, the discriminating ligands are kept too far apart to solvate Na^+. Transport of K^+ then

proceeds by stepwise movement up the polyP ladder. In the narrow pore region, the carbonyl oxygens of the peptide bonds may replace carbonyl oxygens of PHB as solvating agents.

Estimates of molecular size based on van der Waal radii of component atoms indicate that the width of a PHB chain is ~4 Å and that of polyP ~3 Å. The Pauling radius of K^+ is 1.33 Å. This implies that a cavity of ~13.5 Å diameter would accommodate a PHB/polyP/K^+ complex, though considerably less space may be required if the methyl groups of PHB are nestled within hydrophobic pockets along the protein wall. According to the X-ray structure, there is a ~10 Å cavity lined with hydrophobic amino acids in the center of the channel (*28*). Allowing for experimental error, this area could contain a PHB/polyP/K^+ complex with the polyP chain trailing down to an arginine-rich region of the intracellular entryway. It is also possible the polymers are located entirely in the intracellular portion that was not imaged.

It is recognized that this hypothetical mechanism may not satisfy all experimental data acquired for this and other potassium channels; e.g., there is a wealth of mutation and blocking data pointing to the highly conserved narrow pore region as the selectivity filter (e.g., refs *47*−*51*). More experimental work is needed to clarify the locations and roles of PHB and polyP, and it may be necessary to reevaluate earlier experimental results in light of this new information. Mutations may inhibit posttranslational addition of PHB, alter interactions of hydrophobic residues with PHB or interactions of charged residues with polyP, or modify protein architecture. The relative ease with which the polyP, held only by ionic forces, may be lost in electrophysiological systems should also be considered.

In summary, it is posited that the *S. lividans* K^+ channel is a supramolecular structure in which selection and transport of K^+ is effected by cooperative interactions of protein, PHB, and polyP. These studies add to a growing body of evidence attesting to the importance of PHB and polyP in ion transport. The polymers, themselves, associate to form voltage-activated calcium channels in bacterial plasma membranes (*21*−*24*), they are constituents of the human erythrocyte CaATPase plasma membrane pump (*26*), and here we find they are components of the *S. lividans* potassium channel. This represents a very small but quite diverse group of ion transporters. One may conclude that it would be prudent to examine all ion channels and pumps for the presence of these efficacious nonproteinaceous polymers.

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