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Gating Kinetics of *E. coli* Poly-3-Hydroxybutyrate/Polyphosphate Channels in Planar Bilayer Membranes

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Abstract. Nonproteinaceous calcium channel complexes from *Escherichia coli*, composed of poly-(R)-3-hydroxybutyrate (PHB) and inorganic polyphosphate (polyP), exhibit two distinct gating modes (modes 1 and 2) in planar lipid bilayers. Here we report the kinetic characterization of the channel in mode 2, a mode characterized by two well-defined conductance levels, a fully open state (87 ± 3 pS), and a major subconductance state (56 ± 2 pS). Other subconductance states and full closures are rare (<0.5% of total time). Several kinetic properties of the channel showed asymmetric voltage-dependence indicating an asymmetry in the channel structure. Accordingly, single channels responded to potential change in one of two mirror-image patterns, postulated to arise from opposite orientations of the asymmetrical channel complex in the bilayer. The fraction of time spent in each conductance level was strongly voltage-sensitive. For channels reported in this study, presumably all oriented in the same direction, residence time in the fully open state increased as clamping potentials became more positive whereas residence time in the major subconductance state increased at more negative potentials. Analysis of open time distributions revealed existence of two kinetically distinct states for each level. The shorter time constants for both conductance states exhibited weak voltage-sensitivity; however, the longer time constants were strongly voltage-sensitive. A kinetic scheme, consistent with the complex voltage dependence of the channel, is proposed.

Key words: Poly-3-hydroxybutyrate — Polyphosphate — Calcium channel — Channel gating — Subconductance — Single channel recording

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Introduction

There is increasing evidence that calcium signaling is implicated in a variety of bacterial functions including chemotaxis, cell division, and signal transduction (Lynn & Rosen, 1987; Tisa, Olivera & Adler, 1993; Tisa & Adler, 1994; Norris et al., 1996). Although calcium channel activity has been identified in cell extracts (Rosen & McClees, 1974; Matsushita, Hirata & Kusaka, 1989), specific channel proteins have not yet been described. Albeit, nonproteinaceous complexes, composed of poly-(R)-3-hydroxybutyrate (PHB) and inorganic polyphosphate (polyP), were discovered by Reusch et al. (1983, 1986, 1987, 1988) in the cytoplasmic membranes of *Escherichia coli* and other bacteria. The PHB/CapolyP complexes form calcium-selective channels in planar bilayers that display many of the characteristics of protein calcium channels, i.e., permeability to Ca^{2+}, Sr^{2+}, and Ba^{2+}, selectivity for divalent cations over monovalent cations, and blockade by La^{3+} in a concentration-dependent manner (Reusch et al., 1995; Das et al., 1997). Recently, it was demonstrated that single channel characteristics of synthetic PHB/CapolyP complexes in planar bilayers were indistinguishable from those of *E. coli* complexes (Das et al., 1997). This provided conclusive proof that the observed channel activity was affected solely by the cooperative behavior of PHB and polyP, two simple homopolymers that are ubiquitous constituents of both prokaryotic and eukaryotic cells (Reusch, 1989; Reusch & Gruhn, 1997).

Both PHB and polyP have molecular characteristics consistent with a role in ion conduction (Reusch, 1992). PHB has salt-solvating properties that arise from the frequent and regular recurrence of electron-donating ester carbonyl oxygens along its flexible backbone (Armand, 1987; Gray, 1992; Reusch, 1992). Accordingly, PHB transports cations across methylene chloride layers in U-tubes (Bürger & Seebach, 1993), forms ion-con-

ducting complexes with lithium perchlorate (Reusch & Reusch, 1993), and nonselective ion channels in planar lipid bilayers (Seebach et al., 1996*a*). PolyP has a high density of monovalent negative charges that create a large capacity for ion exchange and a greater affinity for multivalent over monovalent cations (Majling & Hanic, 1980; Corbridge, 1985). The inherent preference of polyP for multivalent cations is attributable to the substantially higher bonding energies and the low energy barrier to rotations about the P-O-P backbone (Corbridge, 1985).

Polymers capable of solvating salts do so by forming multiple coordinate bonds between electron donating groups on the polymer backbone and salt cations (Armand, 1987; Gray, 1992). PHB solvates CapolyP by encircling it and replacing water of hydration about calcium ions by coordinate bonds to its ester carbonyl oxygens. The result is a flexible structure of two discrete polymers bridged together by parallel lanes of calcium ions (Reusch & Sadoff, 1988; Reusch et al., 1995; Seebach et al., 1994, 1996*b*). Such multi-lane, multi-binding site channels may be expected to have complex gating kinetics. In this paper, we analyze the single channel gating kinetics of *E. coli* PHB/CapolyP complexes in mode 2, more complex of two gating modes observed in planar lipid bilayers.

Materials and Methods

Solutions and Media

SOB medium (Hanahan, 1983): 2% Bacto-tryptone (Difco, Detroit, MI), 0.5% yeast extract (Difco), 10 mM NaCl, 2.5 mM KCl. Transformation buffer (in mM): 100 KCl, 45 $MnCl_2$, 10 $CaCl_2$, 10 4-morpholineethanesulfonic acid (MES) (Sigma, St. Louis, MO) neutralized to pH 6.3 with KOH. Solution for bilayer measurements (in mM): 200 $CaCl_2$, 5 $MgCl_2$, 10 Tris Hepes, pH 7.4.

Preparation of competent cells of *E. coli* DH5α

E. coli DH5α cells were made genetically competent by a variation of the method of Hanahan (1983) as previously described (Reusch et al., 1986). This procedure has been shown to effect a 50- to 100-fold increase in the concentration of complexes in the plasma membranes (Reusch & Sadoff, 1983; Reusch et al., 1986). Briefly, cells were cultured in SOB medium to an absorbance at 550 nm of ca 0.4. The cells were pelleted at low centrifugal speed (800 × g) at 4°C, and gently resuspended in ⅓ volume of transformation buffer for 30 min at 4°C. The cells were then collected under the same conditions as above.

Extraction of PHB-Calcium Polyphosphate Complexes

The pellet of *E. coli* competent cells was washed with methanol (2×), methanol:acetone 1:1 (2×), and acetone (2×), and the dry residue was extracted overnight at 4°C with chloroform (10^9 cells/ml $CHCl_3$). Since the complexes are sensitive to temperature and moisture, all solvents were ice-cold and dried with Molecular Sieves — 3Å for alcohol, acetone, and 4Å for chloroform (Aldrich, Milwaukee, WI).

Determination of PHB

PHB was moded by a modification of the method of Karr, Waters & Emerich (1983) as previously described (Reusch et al., 1995). Briefly, PHB was converted to crotonic acid by heating the dry sample in concentrated sulfuric acid at 90°C for 30 min. The crotonic acid was extracted, separated by HPLC chromatography (Jasco Chromatography System, Easton, MD) on a Bio-Rad Organic Acids Column HPX87H (Hercules, CA), and quantitated by comparison of peak area, measured on a Shimadzu C-R3A Chromatopac Integrator (Columbia, MD), with that of crotonic acid standards.

Purification of PHB/polyP Complexes by Size-Exclusion Chromatography

The chloroform extract was filtered, using an 0.2 μm PTFE syringe filter (Whatman, Hillsboro, OR), and the filtrate was chromatographed at 4°C on an HPLC nonaqueous size-exclusion column (Shodex K803; 8 mm × 300 mm) using chloroform as an eluent at a flow rate of 0.5 ml/min. Molecular weight standards were synthetic PHBs (courtesy of D. Seebach, ETH, Zürich) and polyisoprenes (Polysciences, Warrington, PA). Eluent fractions (250 μl each) were tested for channel activity as described below.

Reconstitution of PHB/polyP Channels in Lipid Bilayer Membranes

Lipid bilayer membranes were formed across an aperture of ~200 μm diameter in a Delrin® cup (Warner Instruments, Hamden, CT) with a lipid mixture of synthetic 1-palmitoyl, 2-oleoyl, phosphatidylcholine (40 mg/ml) (Avanti Polar Lipids, Birmingham, AL) and cholesterol (8 mg/ml) in decane. The bilayer was formed between symmetric bathing solutions of 200 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM Tris-Hepes, pH 7.4. All salts used in the bathing solutions were ultrapure (>99%) (Aldrich, Milwaukee, WI). Plasma membrane vesicles, prepared as previously described (Reusch et al., 1986), were added to the aqueous solution and allowed to incorporate into the bilayer spontaneously. PHB/CapolyP complexes were incorporated into the bilayer by direct addition to the lipids before forming the bilayer; 1–2 μl of a chloroform solution of the complexes was added to 250 μl decane solution of the above lipids (ratio of PHB to phospholipid was <1:1000), and after removal of chloroform by evaporation with a stream of dry nitrogen gas, the solution was used to form a bilayer. The primary preparations were generally very active. Typically, two or more dilutions of the initial preparation with the above lipid solution were required to obtain a bilayer with less than 3 channels (*see* Fig. 1*B*). Single channel currents were recorded with an Axopatch 200A integrating patch clamp amplifier (Axon Instruments, Foster City, CA). The *cis* solution (voltage command side) was connected to the CV 201A head-stage input and the *trans* solution was held at virtual ground via a pair of matched Ag-AgCl electrodes. Currents through the voltage-clamped bilayers (background conductance <6 pS) were low pass filtered at 10 kHz (−3dB cutoff, Bessel type response) and recorded on videocassettes after digitization through an analog-to-digital converter (VR 10B, Instrutech, USA). Using standard voltage conventions, positive clamping potentials are quoted as potentials with respect to the ground (*trans* chamber) and positive currents are shown as "upward" traces.

Single-Channel Recording and Data Analyses

At the dilution used for recordings, only one channel was observed in the course of the experiment (2–3 hr), even during long periods of recording and at clamping potentials where the open probability of the channel was very high (>0.7). Recording noise was ~0.7–1.2 pA peak to peak at 1.5 kHz (−3 dB, low pass 8-pole Bessel filter). Data were analyzed after filtration through an 8 pole Bessel filter (902LPF, Frequency Devices, Haverhill, MA) at 1–2 kHz as mentioned in the figure legends using pClamp software (version 6.0.4, Axon Instruments); additional standard nonlinear fitting routines were applied where necessary. Sampling was done using a TL-1 interface (Axon Instruments, CA) at 5–20 kHz. Channel amplitudes and the proportion of time spent in individual conductance states were measured by fitting multiple Gaussian distributions to all point amplitude histograms. The proportion of time spent in individual conductance states was plotted as a fraction of histogram area against clamping potential. For analysis of open time distributions, where the nature and extent of low pass filtering were critical, an iterative approach was adopted to determine the minimum cutoff frequency. Briefly, the filter's −3 dB point for each recording was set such that bilayer noise (in the absence of a channel) just failed to lead to the detection of false "openings" to the lowest amplitude conductance level (determined after a preliminary amplitude analysis of a corresponding recording containing a channel; e.g., *see* Clark, Murray & Ashley, 1997). Under ideal conditions, the shortest directly measureable duration should theoretically have had a rise time of $0.3321/f_c$, where f_c is the −3 dB point in kHz (Colquhoun & Sigworth, 1983). That is, use of a cutoff frequency of 2 kHz should allow the duration of events as short as 0.166 msec to be measured. Thus with a corner frequency of 2 kHz, corresponding to a rise time of 0.17 msec, the true width of events lasting longer than ~0.2 msec could be reliably detected. The filter cutoff frequencies and the limits of our analysis are indicated in the Results and the figure legends. For analysis of open time distribution, data were low pass filtered at 2 kHz using an 8-pole Bessel filter and acquired at 20 kHz. We have chosen to display results of kinetic analysis as apparent rates leaving open the possibility of applying different correction procedures for missed events caused by frequency response limitations (e.g., Neher, 1983; Colquhoun & Sigworth, 1983). We have interpreted the results in a conservative manner and none of our conclusions is critically dependent on the frequency response.

The open time distribution data were fit using simplex least square and Levenberg-Marquardt methods, both of which yielded comparable values for the kinetic constants. The optimal number of components required to fit each distribution was assessed by gradually increasing the number of components from 1 to 6 and assessing the resultant fit by using f-statistics through a comparison program built into the pClamp software.

Results

Activity of *E. coli* PHB/CapolyP Channel Preparations

We first compared the calcium channel activity, under identical experimental conditions, of PHB/CapolyP complexes in plasma membrane vesicles with that of PHB/CapolyP complexes extracted from cells into chloroform. In some cases, the extracted complexes were further purified by nonaqueous size-exclusion chromatography (*see* Materials and Methods). As shown in Fig. 1*A*, the channel conductances observed in plasma membrane vesicles, crude extracts and HPLC-purified extracts are indistinguishable, and the channel gating characteristics are very similar. In all three preparations, the channels demonstrated selectivity for Ca^{2+} and block by La^{3+} as previously reported (Reusch et al., 1995).

The channel preparations were generally very active, and repeated dilutions of the primary preparation (Materials and Methods) with lipid/decane solutions were required to obtain bilayers with single channels. Fig. 1*B* shows a representative 75 sec trace of a primary preparation containing at least 7 channels (top panel). The lowest closed level observed was 24 pA below the baseline, indicating the presence of several more channels (6 to 8) for which full closures were not observed.

Two Modes of Gating

The channel displayed two principal modes of gating at both positive and negative clamping potentials. In mode 1, the channel exhibited long openings of the order of several seconds with infrequent and brief closures to the fully closed state (Fig. 2*A*). In mode 2, the channel activity was characterized by long bursts with fast flickering transitions between the fully open state and a distinct subconductance state, interrupted occasionally by closures to the fully closed state (Figs. 2*B* and 4*A*). In both modes, the channel occasionally entered a long closed state of several seconds duration (*data not shown*). Switching between modes 1 and 2 was observed but such occurrences were infrequent (Fig. 2*C*).

Major Subconductance State of Mode 2

All points amplitude histograms were analyzed at various clamping potentials. The histograms for current records at +120 and −120 mV are shown in Fig. 3*A*. The amplitude histograms at all potentials could be best fit (simplex least square fitting) by two Gaussian distributions (the components are shown as points and the resultant fits as solid lines in Fig. 3*A*), e.g., mean amplitudes at +120 mV were 9.7 pA and 6.3 pA. For both conductance states, the current-voltage relationships, constructed by analysis of all point histograms at indicated potentials, are linear over the range of potentials tested, i.e., +120 to −120 mV (Fig. 3*B*). Conductances of the fully open and subconductance states were respectively 87 ± 3 pS and 56 ± 2 pS.

Voltage-dependent Responses of PHB/polyP Single Channels

Several kinetic properties of PHB/polyP channels showed asymmetric voltage dependence. A given single

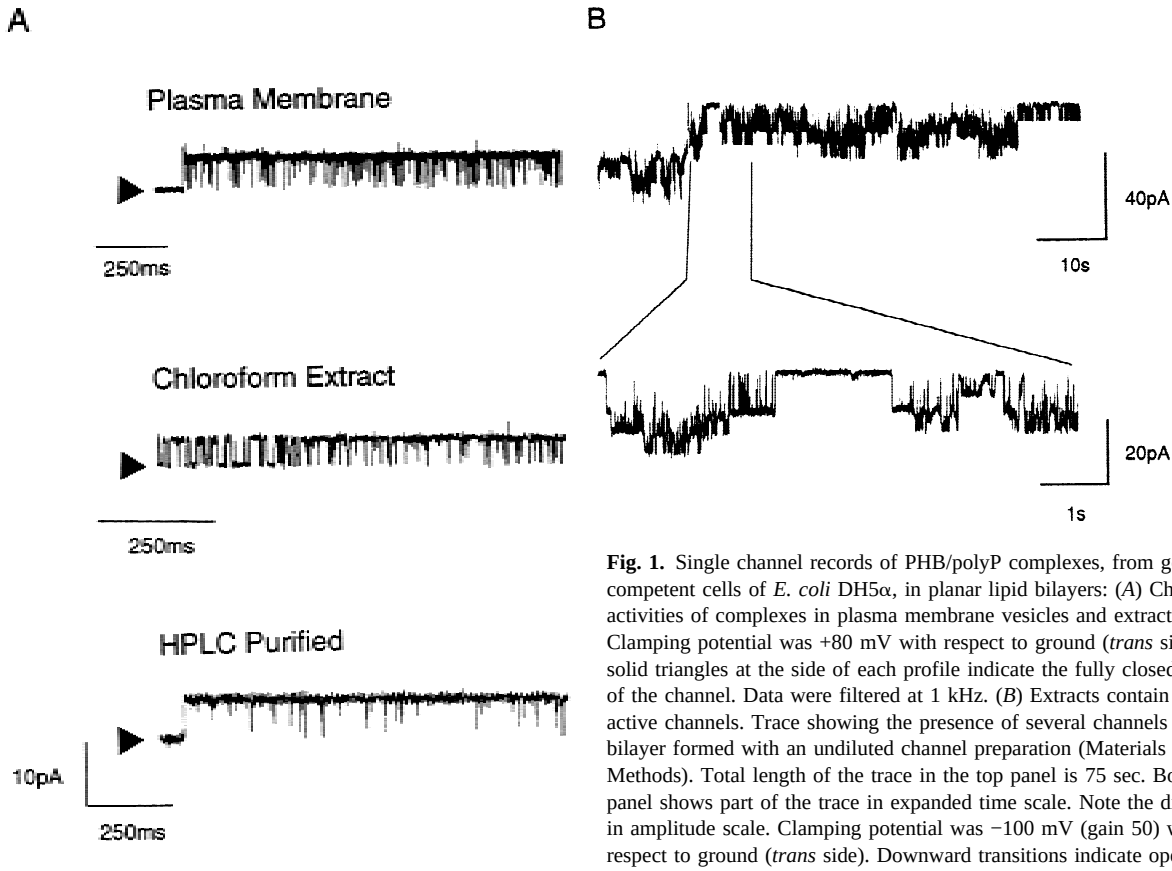

Fig. 1. Single channel records of PHB/polyP complexes, from genetically competent cells of *E. coli* DH5α, in planar lipid bilayers: (*A*) Channel activities of complexes in plasma membrane vesicles and extracts. Clamping potential was +80 mV with respect to ground (*trans* side). The solid triangles at the side of each profile indicate the fully closed state of the channel. Data were filtered at 1 kHz. (*B*) Extracts contain many active channels. Trace showing the presence of several channels in a bilayer formed with an undiluted channel preparation (Materials and Methods). Total length of the trace in the top panel is 75 sec. Bottom panel shows part of the trace in expanded time scale. Note the difference in amplitude scale. Clamping potential was −100 mV (gain 50) with respect to ground (*trans* side). Downward transitions indicate opening of a channel. Data were filtered at 500 Hz. Channels were incorporated into planar lipid bilayers, composed of synthetic 1-palmitoyl, 2-oleoyl, phosphatidyl-choline and cholesterol (5:1 w/w), between symmetric bathing solutions of 200 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM Tris-Hepes, pH 7.4 at 22°C as described in Materials and Methods.

channel displayed one of two voltage-dependent responses that were mirror images of each other. This indicates an asymmetry in the channel structure and suggests the complexes can assume two opposite orientations in the bilayer. For clarity, we report below the voltage-dependent responses of single channels that display one of these patterns, presumably channels in the same orientation. Channels with the putative reverse orientation show equal but opposite voltage-dependent responses.

Voltage-dependence of All Points Amplitude Histograms and Residence Time

Current traces of the channel showed preference for the fully open state at positive potentials and for the major subconductance state at negative potentials (Fig. 4*A*). The relative proportions of fit components, corresponding to two conductance states in the amplitude histograms, were voltage dependent (Fig. 4*B*). As clamping potentials became more positive, the area corresponding to the fully open state increased and that for the substate decreased (Fig. 4*B*). At −40 mV, the channel showed equal residence in the two states. It may be noted that neither state was empty over time (>150 sec) at any potential. Corresponding voltage dependence was shown by the residence times in each conductance state (Fig. 4*C*). The fraction of time spent by the channel in the fully open state increased linearly with increase in clamping potential, whereas the reverse was true for the major subconductance state. It is also interesting to note that the time spent by the channel in the fully closed state did not change appreciably with potential and remained constant at about 0.5% of total time.

Voltage-dependence of Open Probability

The probability of openings to the fully open state from the major subconductance state (P_{os}), shown in Fig. 4*D*, were calculated by considering the subconductance level as the base level. The monotonic dependence on clamping potential reiterates the same trends observed in Fig. 4*B* and *C*. The linear voltage dependence indicates an increasing probability of detecting the channel in the

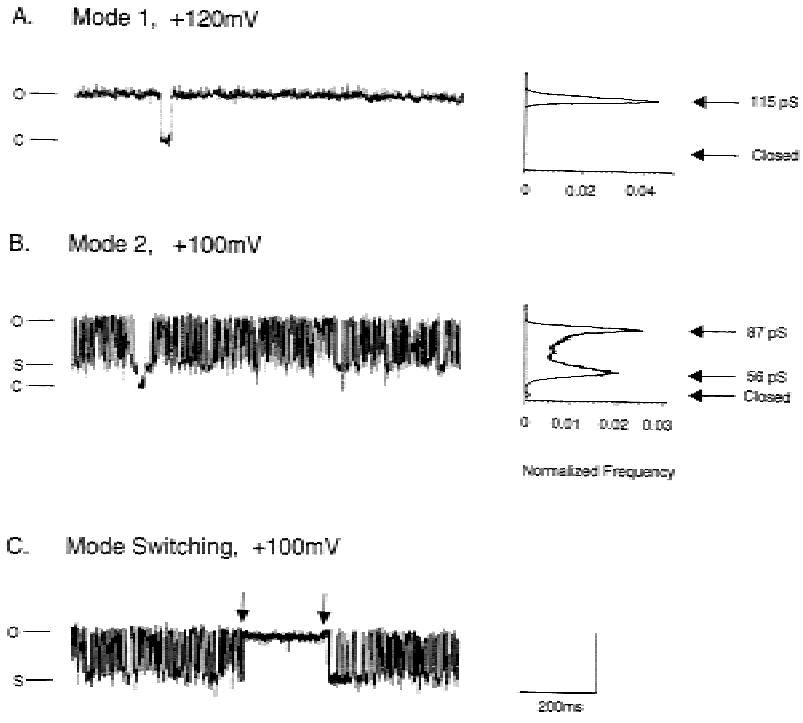

Fig. 2. Gating of the PHB/polyP channel in two modes: (*A*) Mode 1. (*B*) Mode 2. (*C*) Transition between modes. Recordings show ion channel activity of the extracted complexes in two kinetically distinct modes of gating: (*A*) mode 1 and (*B*) mode 2. Positions of fully closed state, fully open state and the major subconductance state of mode 2 are indicated as C, O and S, respectively, on the left side of the tracings. Note that the vertical bar indicating current scale is 15 pA for *A* and 10 pA for *B* and *C*. All point amplitude histograms for selected traces in each mode are shown at the side of respective traces with corresponding mean conductances. In *C*, transitions from mode 2 to mode 1 and back are indicated by arrows. Clamping potential with respect to ground (*trans*) was −120 mV for *A* and +100 mV for *B* and *C*. Data shown were filtered at 1.5 kHz for *A* and *B* and at 1 kHz for *C*.

Complexes were extracted from competent *E. coli* Dh5α and incorporated into planar lipid bilayers composed of synthetic 1-palmitoyl, 2-oleoyl, phosphatidyl-choline and cholesterol (5:1 w/w) between symmetric bathing solutions of 200 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM Tris-Hepes, pH 7.4 at 22°C as described in Materials and Methods.

fully open state at more positive clamping potentials. Thus the channel (in this orientation) is more likely to be in the fully open state at higher positive potentials and in the major subconductance state at higher negative potentials.

Analysis of Gating Kinetics

Due to the very low frequency of occurrence of the fully closed state, only transitions between the major subconductance state and the fully open state were examined. As above, the major subconductance level was considered the base level. Thus, the dwell states 0 and 1 correspond to the major subconductance state and the fully open state, respectively. Figure 5 shows the open time distributions at +120 and −120 mV for the fully open (*A* and *B*) and the major subconductance states (*C* and *D*). At all potentials, the data for both the fully open and major subconductance states could be best fit with two exponentials, indicating the presence of two kinetically distinct states at each level. The data reported here were obtained by the simplex least square fitting method; Levenberg-Marquardt fitting routines yielded nearly identical values for the kinetic time constants (τ). For the fully open state, τ values were 0.5 and 3.0 msec at +120 mV, and 0.4 and 0.6 msec at −120 mV; for the major subconductance state, τ values were 0.3 and 1.0 msec at +120 and 0.4 and 2.2 msec at −120 mV. It should be noted that with the corner frequency of 2 kHz used for data reported here, corresponding to a rise time of 0.17 msec, the true width of events lasting longer than ~0.2 msec could be reliably detected.

Analysis of the data for various potentials indicates that the shorter time constants, for both the fully open and the subconductance states, display only weak sensitivity to voltage change and exhibit a symmetrical response to increasing positive and negative potentials (Fig. 6*A*). On the other hand, the longer time constants for both states show significant and asymmetric voltage dependence (Fig. 6*B*). The longer time constant for the major subconductance state decreases as the clamping potential approaches zero from negative potentials, and then shows a marginal increase at positive clamping potentials. The longer time constant for the fully open state increases steeply with increasing positive clamping potentials and is insensitive to voltage at negative clamping potentials.

Proposed Kinetic Scheme

A kinetic scheme, consistent with the experimental observations, is shown in Fig. 7. States associated with the fully open level are represented as O_1 and O_2, and those with the major subconductance level as S_1 and S_2, with subscripts 1 and 2 referring to shorter and longer time constants, respectively. States with longer time constants (O_2 and S_2), shown inside the dashed box, display higher sensitivity to clamping potential than states with shorter time constants (O_1 and S_1). At high negative potentials, S_2 dominates and nearly all transitions to the

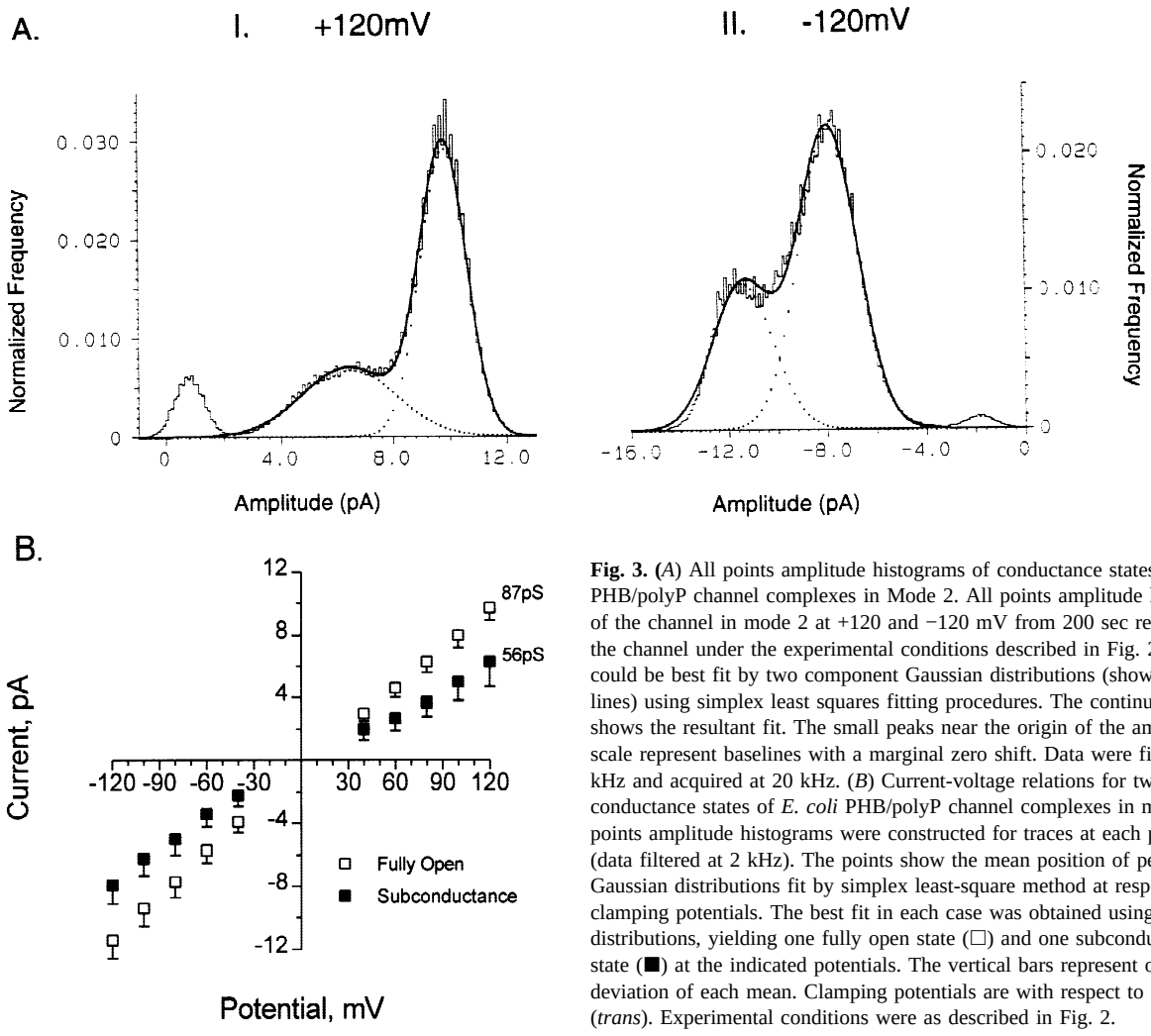

Fig. 3. (*A*) All points amplitude histograms of conductance states of *E. coli* PHB/polyP channel complexes in Mode 2. All points amplitude histograms of the channel in mode 2 at +120 and −120 mV from 200 sec recordings of the channel under the experimental conditions described in Fig. 2. The data could be best fit by two component Gaussian distributions (shown as dotted lines) using simplex least squares fitting procedures. The continuous line shows the resultant fit. The small peaks near the origin of the amplitude scale represent baselines with a marginal zero shift. Data were filtered at 2 kHz and acquired at 20 kHz. (*B*) Current-voltage relations for two conductance states of *E. coli* PHB/polyP channel complexes in mode 2. All points amplitude histograms were constructed for traces at each potential (data filtered at 2 kHz). The points show the mean position of peaks of Gaussian distributions fit by simplex least-square method at respective clamping potentials. The best fit in each case was obtained using two distributions, yielding one fully open state (□) and one subconductance state (■) at the indicated potentials. The vertical bars represent one standard deviation of each mean. Clamping potentials are with respect to ground (*trans*). Experimental conditions were as described in Fig. 2.

fully open state consist of fast openings to O_1. Under these conditions, the kinetic scheme reduces to a simpler scheme i.e., $S_2 \leftrightarrow O_1 \leftrightarrow S_1$. At high positive potentials, most transitions to the fully open state lead to longer openings, indicating dominance of O_2, and the scheme takes the form of a linear Markov chain i.e., $O_2 \leftrightarrow S_1 \leftrightarrow O_1$. Thus at negative potentials, O_2 becomes progressively less accessible from other states, and at increasingly positive potentials, S_2 becomes less accessible from other states. This effect is readily observable from the traces shown in Fig. 4*A*. At intermediate potentials, transitions from S_1 to O_1 and O_2, from S_2 to O_1 and O_2 and *vice versa* are observed. Transitions between O_1 and O_2 or between S_1 and S_2 may occur but are experimentally unobservable.

Discussion

It has now been established by total synthesis (Das et al., 1997) that PHB/CapolyP polymer electrolyte complexes form calcium-selective channels in planar bilayer membranes with properties resembling those of proteinaceous calcium channels (Reusch et al., 1995). The complexes select for divalent over monovalent cations, are permeable to Ca^{2+}, Ba^{2+}, and Sr^{2+}, and show concentration-dependent block by La^{3+}. Here we report that the kinetic properties of PHB/CapolyP channels display a complexity similar to that observed in proteinaceous channels (Fig. 1).

Although this report focuses on the channel activity in planar bilayers of PHB/CapolyP complexes extracted from *E. coli* DH5α cells, the gating behavior in planar bilayers of PHB/CapolyP complexes in *E. coli* plasma membrane vesicles exhibit the same modes and subconductance states (Fig. 1; *unpublished data*).

The channel displays two major modes of gating. In mode 1, the channel openings are long (of the order of several seconds) with infrequent brief closures. After a period of such activity, the channel may close for longer intervals of the order of several seconds (*data not*

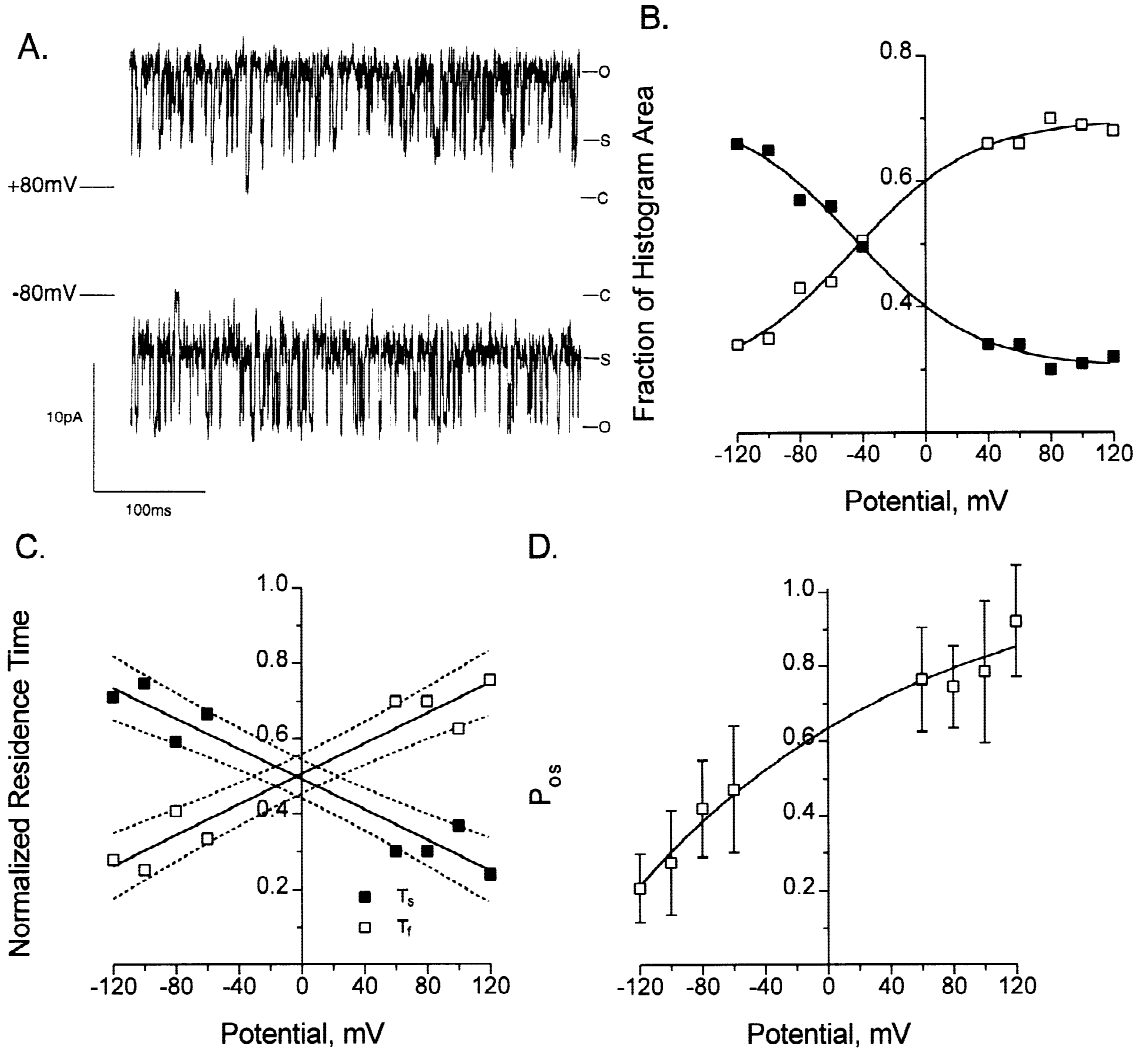

Fig. 4. Voltage dependence of PHB/polyP channel complexes in mode 2. Experimental conditions and analysis were as described in Fig. 2. In all cases, analysis was done on traces exceeding 150 sec; the fully closed state made up less than 0.5% of this time. The filtered at 2 kHz and acquired at 20 kHz. (*A*) Preference for specific conductance states. Representative traces indicating the preference for the fully open state (O) at positive potential (+80 mV) and for the major subconductance state (S) at negative potential (−80 mV). *C* indicates position of the fully closed state. (*B*) Normalized areas corresponding to fully open and major subconductance states in the all points amplitude histograms. For each potential, the data show the proportion of components corresponding to the fully open (□) and subconductance (■) states for best fit of the all points amplitude histogram. Data were normalized with respect to the total area under the curves, fit by Gaussian distributions at each potential (*C*) Normalized residence times of the *E. coli* PHB/polyP channel complexes in the two major conductance states. Data show the average residence time of the channel in the fully open state (T_f, □ and the subconductance state (T_s, ■), at indicated clamping potentials. Results of fitting by linear regression are shown by solid lines. Dotted lines indicate 95% level of confidence. (*D*) Open probability of the fully open state of mode 2. The data show the probability of opening of the channel, P_{OS} to the fully open state from the subconductance state at indicated clamping potentials. Fit by nonlinear regression (Boltzman) is shown by the solid line.

shown). In mode 2, the channel opens to a distinct subconductance state from which fast flickering openings to the fully open state and closures take place (Fig. 2*B*). The channel records show occasional full closures from this intermediate conductive state. The two modes of gating are kinetically connected as switching between modes is observed (*see* Fig. 2*C*). The switching can take place in either direction — from mode 1 to mode 2 or the reverse. The factors that determine preference for a specific mode or affect switching between modes are presently unknown. Other subconductance states are also observed but with much lower frequency.

The molecular structure of PHB/CapolyP channel complexes remains uncertain; however, it is evident from the physical properties of these two polymers that the polyanionic polyP should be shielded from the hydrophobic bilayer by the amphiphilic PHB. The models proposed for the channel complexes by Reusch et al.

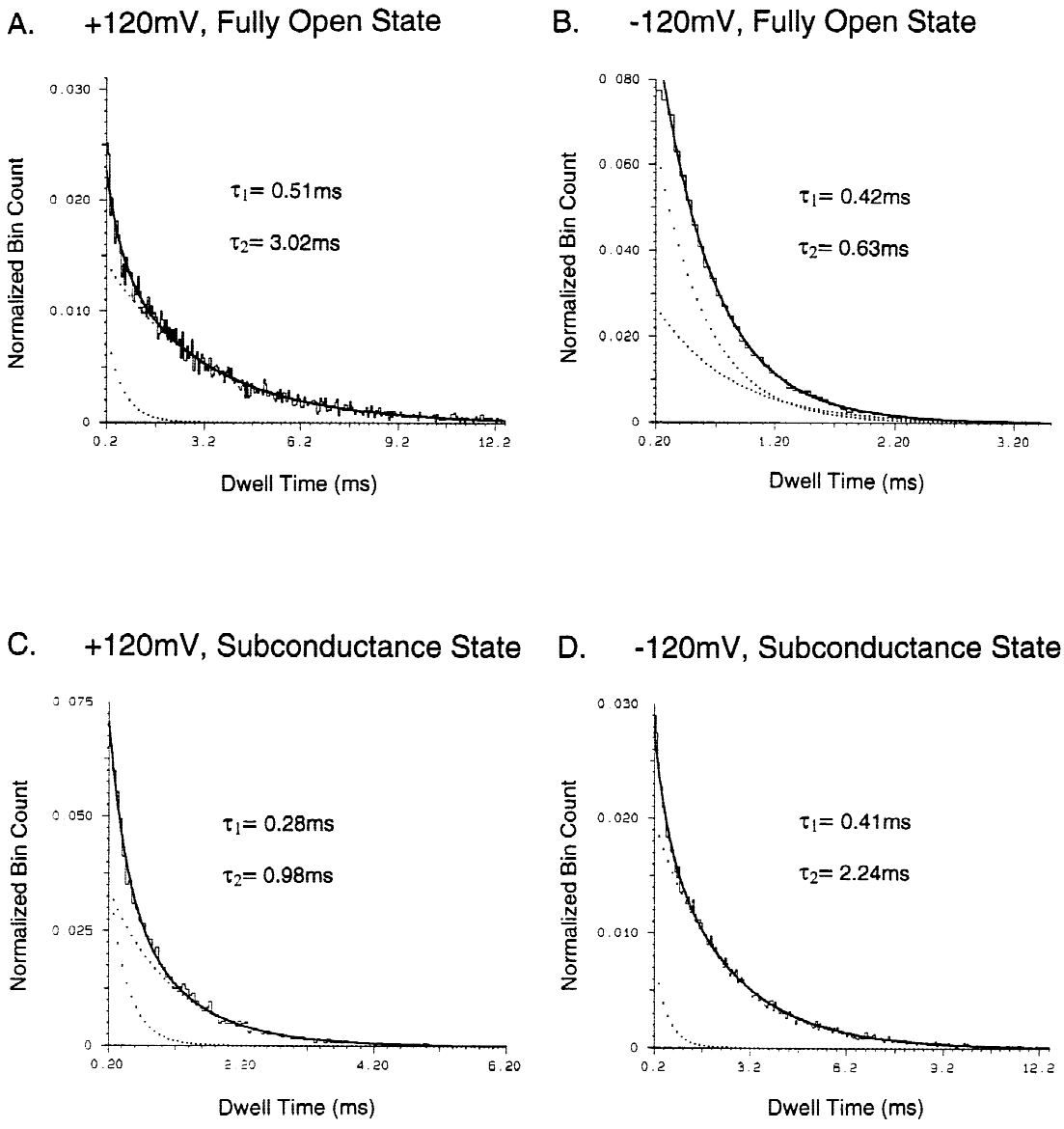

Fig. 5. Open time distributions of the channel at +120 mV (*A* and *C*) and −120 mV (*B* and *D*). For both the fully open (*A* and *B*) and the major subconductance (*C* and *D*) states, data were best fit by two exponential terms. Curves are shown in conventional binning format. Dotted lines indicate individual components and the solid line indicates the resultant fit (*see* Materials and Methods). Bin width was 0.05 msec. Experimental conditions were as described in Fig. 2. In all cases, the total time of recordings analyzed exceeded 150 sec. The time spent by the channel in the fully closed state was less than 0.5%. The filtered at 2 kHz and acquired at 20 kHz. Levenberg-Marquardt and simplex least square fitting methods yielded nearly identical values for the kinetic constants.

(1988, 1995) and Seebach et al. (1994, 1996*b*) have this general structure. The former model proposes that PHB has a coiled conformation such as it displays in solution (Marchessault et al., 1970; Akita et al., 1976) while the latter suggests that PHB maintains the folded helix form of its solid state (Seebach et al., 1996*b*). A consequence of both arrangements is the formation of multiple parallel lanes between the two polymers, with multiple cation-binding sites lining each lane (Reusch et al., 1995; Das et al., 1997). 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 alter channel geometry and effect changes in current amplitude and gating. One would expect that some channel conformations are more stable than others and consequently more probable, e.g., the fully open state of mode 1. The major subconductance state of mode 2 may represent another particularly stable conformation of PHB/polyP in this bilayer. Accordingly, in this multiple-lane channel the rare subconduc-

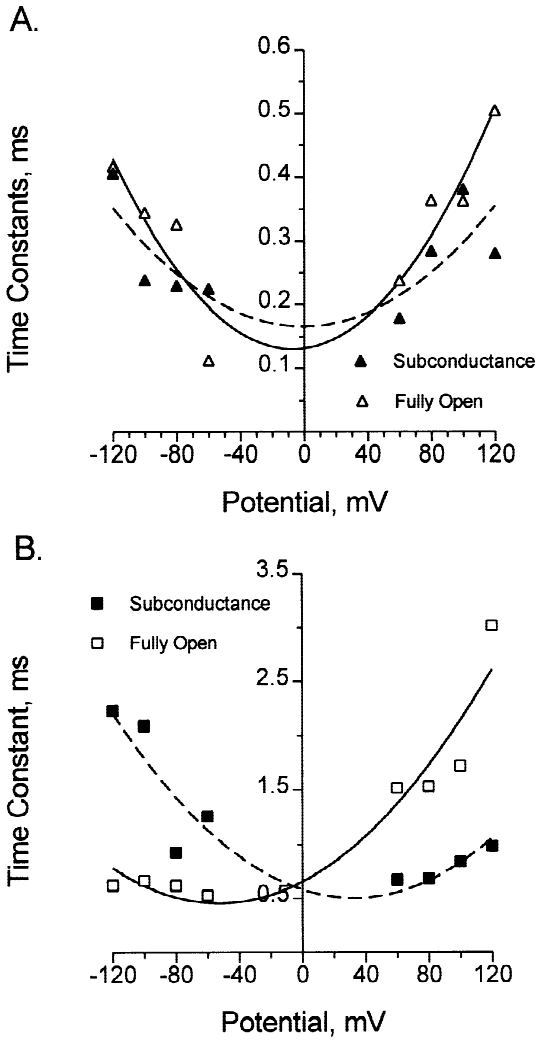

Fig. 6. Voltage dependence of the time constants for fully open and major subconductance states: *A.* Shorter and *B.* Longer time constants. Time constants for exponential components for best fit of open time distributions are plotted against respective clamping potentials (△ and □ for the fully open state; ▲ and ■ for the major subconductance state). Note the difference in the time scale of the axis for time constant between *A* and *B.* Experimental conditions were as described in Fig. 2. For both the fully open and the subconductance states, data were best fit by two exponential components. In all cases, the total time of recordings analyzed exceeded 150 sec. The time spent by the channel in the fully closed state was less than 0.5%. Solid lines indicate fitting of data by polynomials of third order. The data were filtered at 2 kHz using an 8-pole Bessel filter and acquired at 20 kHz. Note that the shorter time constants (triangles) are only weakly sensitive to changes in clamping potential whereas the longer time constants (squares) are strongly voltage sensitive.

tance states may result from less stable conformations or the block of one or more lanes within the conductive core.

In mode 2, several kinetic properties of the channel displayed asymmetric voltage-dependence: the relative contributions of fully open and major subconductance states in the all points amplitude histograms (Fig. 4*B*);

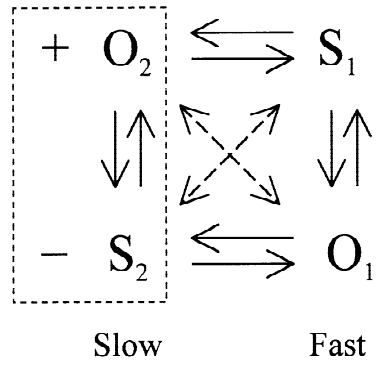

Fig. 7. Kinetic scheme for transitions between fully open and major subconductance states. States associated the fully open level are represented as O_1 and O_2, and those with the major subconductance level as S_1 and S_2, with subscripts 1 and 2 referring to shorter and longer time constants, respectively. The states with longer time constants, inside the dashed box, show marked sensitivity towards the clamping potential as indicated by + and − signs. Transitions between O_1 and O_2 and S_1 and S_2 are experimentally not observable.

the residence time of the channel in each conductance state (Fig. 4*C*); the probability of full openings from the subconductance state (Fig. 4*D*); and the longer time constants for both the states (Fig. 6*B*). We surmise that the pattern of voltage-dependence exhibited by a given single channel is determined by the orientation of the asymmetric PHB/polyP complex in the bilayer. Whereas polyP is completely symmetrical, the end residues of PHB differ. PHB has a carboxyl group at the head and hydroxyl group at the tail, creating a structural asymmetry that permits the complex to assume one of two opposite orientations in the bilayer (Fig. 8). The importance of end residue structure was demonstrated by the strong inhibition of ion transport activity by PHB channels (absent a polyphosphate core), when the hydroxyl or carboxyl end groups were derivatized (Seebach et al., 1996*a*). Synthetic tris(macrocycle) cation channels have also shown remarkable sensitivity to structural changes at the distal ends (Abel et al., 1997). In cells, PHB synthesis takes place at the cytoplasmic side, thus it is likely that all PHB/polyP channels in the plasma membrane are oriented in the same direction and display the same pattern of voltage dependence.

Since the gating profiles of PHB/polyP complexes resemble those of protein channels, we have adopted some of the assumptions used for analyses of protein channel gating, in particular that the observed gating is the net result of random transitions between various kinetic states that form a Markov chain (Colquhoun & Hawkes, 1983; Colquhoun & Sigworth, 1983). The requirement for two exponential functions for best fit of the open time distributions of the fully open state indicates that there are at least two kinetically distinct open states of the channel that may be accessed from the subconductance state. Similarly, the requirement for two

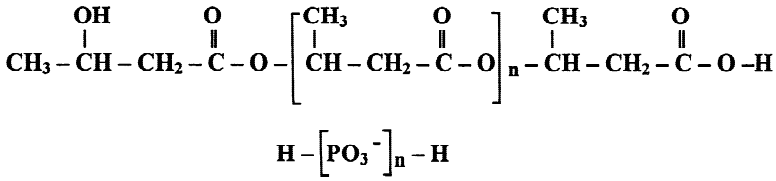

Fig. 8. Structural formulas showing end group asymmetry of PHB and end group symmetry of polyP.

exponential functions for best fit of the time distribution of the major subconductance state indicates the presence of two kinetically distinct states associated with this level. Thus, the gating kinetics of this channel in mode 2 is quite complex. A kinetic scheme consistent with the above experimental observations is shown in Fig. 7. For a thorough understanding of the kinetic scheme, it would be useful to know the kinetic constant(s) for the fully closed state; however, full closures were so rare that membrane instability made this determination impractical (Fig. 3).

To summarize, PHB/polyP channels in planar lipid bilayers displayed complex gating characterized by two distinct modes, namely mode 1 and mode 2. Under the conditions of this study, the major gating mode, mode 2, was characterized by the presence of a major subconductance state from which there were frequent transitions to the fully open state and rare transitions to the fully closed state. Several kinetic properties of the channel exhibited asymmetric voltage-dependence. It is suggested that the complex gating kinetics of the PHB/polyP channels is a direct consequence of the asymmetry of the PHB molecule and the multitude of conductive conformations that may be adopted by a flexible polymer electrolyte complex in response to potential differences.

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