Exopolypophatase of Escherichia coli (PPX) is a highly processive enzyme demonstrating the ability to recognize polyphosphates of specific lengths. The mechanisms responsible for the processivity and polymer length recognition of the enzyme were investigated in relation to the manner in which polyphosphate is bound to the enzyme. Multiple polyphosphate binding sites were identified on distant portions of the enzyme and were determined to be responsible for the polymer length recognition of the enzyme. In addition, two independently folded domains were identified. The N-terminal domain contained a quasi-processive polyphosphate active site belonging to the sugar kinase/actin/hsp70 superfAMILY. The C-terminal domain contained a single polyphosphate binding site and was responsible for nearly all of the PPX affinity for polyphosphate. This domain was also found to confer a highly processive mode of action to PPX. Collectively, these results were used to describe the interaction of polyphosphate with PPX.

Polyphosphate up to several thousand phosphate residues in length is known to accumulate in bacteria, fungi, plants, and animals (1). The function of this phosphate biopolymer, although not well understood, is believed to involve energy and phosphate storage, transformation of DNA across cell membranes (2), gene regulation (3), stationary phase survival (4, 5), and response to starvation for amino acids or carbon (6, 7).

Exopolypophatases are found in a variety of organisms and hydrolyze terminal phosphate bonds to yield orthophosphate (P1). Their metabolic role, however, is unclear, as they appear to waste the stored energy of the phosphate bonds. Escherichia coli exopolypophatase, like most exopolypophatases, is highly processive, as it hydrolyzes entire polyphosphate chains greater than 1000 phosphate residues in length to orthophosphate without release of polyphosphate intermediates.

In contrast, several polyphosphate-degrading enzymes produce specific chain length intermediates by progressively removing terminal phosphates from long chain polyphosphates until the specific length intermediate remains and is released. These intermediates range from P40 for guanosine pentaphosphate phosphohydrolase (GppA) (8) to P100, roughly 200 Å in length, for polyphosphate glucokinase of Propionibacterium shermani (9, 10).

We have investigated this release of specific polyphosphate intermediates by PPX under conditions non-optimal for activity. Furthermore, we have identified domains of the enzyme and their functions in polyphosphate binding and processivity in order to describe the interaction of polyphosphate with PPX.

**EXPERIMENTAL PROCEDURES**

**PPX Assays—**Radioactive [32P]polyphosphate was synthesized according to reported procedures (11, 12). This polyphosphate was used to assay PPX activity with minor modifications to reported methods (8, 13). The 15–µl reactions contained 50 mM Tricine-KOH (pH 8.0), 1 mM MgCl2, 175 mM KCl, and 100 mM [32P]polyphosphate chains, assuming a chain length of 750 phosphate residues. Reactions at 37 °C were monitored by sampling 2 µl onto a polyethyleneimine plate, which was then developed in 0.4 M LiCl and 1 M HCOOH. The plates, cut roughly in the middle, were analyzed by liquid scintillation counting.

A nonradioactive assay made use of the same reaction mixture as the radioactive assay except [32P]polyphosphate was replaced by 50 µM PpK (Sigma), and the reaction volume was increased to 50 µl. Reaction progress at 37 °C was monitored by adding 10-µl reaction samples to 750 µl of phosphate assay mix (Sigma).

**Cloning PPX—**Plasmids p4ZKX and p24/18KX were created for overexpressing PPX. Plasmid p4ZKX was constructed in a two-step process. A sequenced 287-base pair, PCR-generated segment of pppk was first inserted into pGEM-4Z (Promega), PstI (5′) to EcoRI (3′), creating p4ZZST. Plasmid pBC9 (13) served as the PCR template with primers 5′-GTG TGT TTC AGC GTT GTA GTC CTT CGG-3′ and 5′-TGT GTG TGA AGC TTC GTG TAT TGG CAT AGG-3′, creating p4ZZST-3, creating p4ZZST. The two-step construction of p24/18KX began with insertion a 240-base pair multiple cloning site linker from pUC18 XbaI to NdeI into the corresponding sites of pET24a (Novagen). The complete pppk and ppx genes from p4ZKX were then inserted PstI (5′) to NdeI (3′), yielding p24/18KX.

**Overexpression and Purification of PPX—** Selective plating (14) and induction studies were used to test plasmid stability in E. coli BLR(DE3) (E. coli BLR(DE3) (pET-DE3::pPpK mB) mB.dcm Δrlc-848, Δι(1805::Tn10(DE3)) (Novagen)). Plasmid p24/18KX showed much greater stability than p4ZKX.

**E. coli BLR(DE3) p24/18KX** was cultured in 3 liters of Luria broth (LB) medium with kanamycin (16 µg/ml) and tetracycline (10 µg/ml). Cells were induced by adding isopropyl-1-thio-D-galactopyranoside to a final concentration of 0.4 mM when the optical density of the culture was at a wavelength of 600 nm (A600) reached 0.5. Two hours later at an A600 of 1.4, cells were harvested by centrifugation. The pellet was resuspended in 85 ml of 50 mM Tris-Cl (pH 7.5), 10% sucrose, frozen in liquid nitrogen, and stored at −87 °C. For lysis, 25 ml of 50 mM Tris-Cl (pH 7.5), 10% sucrose at 50 °C was added to the cells. When thawed, 12 ml of 10× lysis buffer was added. This 10× lysis buffer contained 50 mM Tris-Cl (pH 7.5), 10% sucrose, 100 mM (NH4)2SO4, 50 mM diithiothreitol, and 50 mM EDTA. The mixture was incubated on ice for 1 h, shifted to 37 °C for 6 min, cooled on ice for 10 min, sonicated (2 × 30 s, 80% power), and centrifuged at 48,400 × g for 2 h. All remaining procedures occurred at 4 °C. The undialyzed, combined supernatant was loaded (100 ml at 7 ml/min) on a 600-ml fast flow Q-Sepharose column (Am-
ersham Pharmacia Biotech) pre-equilibrated with 1 liter of buffer A (10% glycerol, 50 mM Tris-Cl (pH 7.5), 2 mM dithiothreitol) with 8 mM (NH$_4$)$_2$SO$_4$. The column was washed with 300 ml of buffer B and then with a 2-liter gradient from 5–100 mM (NH$_4$)$_2$SO$_4$ in buffer A. PPX was then eluted with a 2-liter gradient from 50–400 mM (NH$_4$)$_2$SO$_4$ in buffer A. Fractions (50 ml) were collected and assayed. Fractions with high specific activity were combined and dialyzed against 10 volumes of buffer A with 5 mM (NH$_4$)$_2$SO$_4$. The dialyzed mixture was centrifuged at 48,400 × g for 20 min. A 1-liter fast flow SP-Sepharose column (Amersham Pharmacia Biotech) equilibrated with 1 liter of 5 mM (NH$_4$)$_2$SO$_4$ in buffer A was then loaded with the supernatant. The column was washed with 1.8 liters of 5 mM (NH$_4$)$_2$SO$_4$ in buffer A. PPX was then eluted with a 2-liter gradient from 5–100 mM (NH$_4$)$_2$SO$_4$ in buffer A. Fractions with the highest specific activity were combined and dialyzed in buffer B (10% glycerol, 50 mM K$_2$HPO$_4$/KH$_2$PO$_4$ (pH 7.5), and 2 mM dithiothreitol). A 500-ml ceramic hydroxyapatite column (Bio-Rad) was equilibrated with 850 ml of buffer B and then loaded (at roughly 10 bed volumes/h) on a 2.5-ml Ni$^{2+}$-charged column (Novagen). The column was washed with 25 ml of binding buffer and 15 ml of wash buffer (60 mM imidazole, 350 mM KCl, and 20 mM Tris-Cl (pH 7.9)). The column was then eluted with 15 ml of elution buffer (1 mM imidazole, 350 mM KCl, and 20 mM Tris-Cl (pH 7.9)). The purified protein was then eluted from 50 ml of Tris-Cl (pH 7.5) with 175 mM KCl and concentrated.

The N-terminal domain was produced as a homogenous inclusion body that required solubilization but did not require further purification. Lysed cells were centrifuged and washed with 6 x 1 ml in binding buffer. The N-terminal domain was then solubilized in binding buffer with 8 x 1 ml. The urea was dialyzed away in four stages with a 50 ml Tris-Cl (pH 7.5) and 175 mM KCl buffer containing 2, 500, 100, and no urea. This process was repeated for a total of three cycles.

**Size Exclusion Chromatography—**Size exclusion chromatography was performed using a HitPreg 26/60 Sephacryl 5-200 HR column (Amersham Pharmacia Biotech) with buffer containing 50 mM (NH$_4$)$_2$SO$_4$ and 50 mM Tris-Cl (pH 7.5).

**Cloning of Nested Deletions—**PCR was used to generate a series of C-terminal deletions of PPX. Consecutive deletions differed by roughly 20 amino acids, with the exact position of each deletion chosen to minimize primer secondary structures at PCR-annealing temperatures. The genes were cloned into pET24a, Ndel to EcoRI, without a fusion peptide, creating plasmids p24XE1–12, with 16 amino acids removed from the enzyme expressed by p24XE1 and 279 amino acids removed from the enzyme expressed by p24XE12. The primer for the 5′-end of supernatant and an Ndel site was the same for all plasmids: 5′-AGT ACC TAT GCC AAT ACA CGA TAT AAA ACC TAA ACC CCC CCC TCC TCG TGG. The 3′-end of pET24a added a stop codon and an EcoRI site (17). Specific results are shown only for deletion 5 (104 amino acids deleted), which used 5′-CACA GAG ATT CAC ACC AGT GTC GCC ATC ATC AGC-3′ as a primer for the 3′-end of PCR.

Activity of Nested Deletions—**E. coli BLR(DE3)-containing plasmids p24XE1–6 were cultured in 25 ml of LB medium. At an A$_{600}$ of 0.5, cells were induced with isopropyl-1-thio-β-D-galactopyranoside to 0.25 mM. After 2 h, cells were centrifuged and resuspended in 0.5 ml of 50 mM Tris-Cl (pH 7.5) with 175 mM KCl. Resuspended cells were mixed with 5 μl of a 10 mg/ml lysozyme solution and incubated 30 min on ice. Cells were then sonicated 2× 20 s at 70% power. The lysate was centrifuged at 17,500 × g to yield pellet and supernatant fractions. The activity of the supernatant was measured using the radioactive PP$_4$ assay.

Equilibrium Polyphosphate Binding Assay—**As inactive PPX precipitated with polyphosphate, equilibrium binding was measured as polyphosphate retained by immobilized enzyme. PPX was inactivated by removal of divalent cations and was immobilized using N-hydroxy succinimide (NHS)-activated acrylic beads (Affipler, Bio-Rad). Immobilized N- and C-terminal domains of PPX were prepared from the purified domains. Reaction volumes of 10 μl contained 4 μl of resuspended, immobilized PPX and 6 μl of equilibration buffer containing 10 mM HEPES-KOH (pH 7.5), 175 mM KCl, 0.5 mM EDTA, and polyphosphate. The polyphosphate level in this 6 μl of buffer was such that the equilibrium binding reaction had the desired final polyphosphate concentration assuming 8 μl of liquid reaction volume and 2 μl of solid, immobilized enzyme. After a short equilibration period of approximately 10 s, the reaction was mixed with 100 μl of equilibration buffer without polyphosphate and transferred to an ST 69 cellulose acetate membrane (Schleicher & Schuell) on a vacuum filtration apparatus. The gel was then rinsed with 10 ml of equilibration buffer without polyphosphate, and bound polyphosphate was measured by liquid scintillation counting of the membrane.

**RESULTS**

Generation of Polyphosphate Intermediates—**At 175 mM KCl (data not shown), optimal in terms of specific activity, and at least up to 300 mM KCl, orthophosphate was the main product. A P$_2$ band representing roughly one P$_2$ released per long chain.
was also observed, as was a faint P3 band (Fig. 1).

Between 600 mM and 1.2 M KCl, P50 and P14 bands were generated. Smearing from the P14 band to the P3 band was present at 600 mM KCl; however, interestingly, chain lengths between P50 and P14 were not observed. Above 600 mM KCl, polyphosphate smearing from the wells to the P50 band was very evident and a P2 band was not present for 1.2 M KCl.

**Limited Proteolysis Domains and Their Functions—** Limited proteolysis with either trypsin or *S. aureus* V8 protease (Glu-C) produced two discrete fragments (Fig. 2). The 24-kDa Glu-C fragment was submitted for N-terminal sequencing (Biomolecular Resource Center, University of California, San Francisco). This sequencing revealed two nearly identical populations representing cleavage at glutamate 304 and glutamate 306. Extended digestion with Glu-C resulted in a single highly protease-resistant 24-kDa fragment.

**PPX Domains—** Genes for the N- and C-terminal domains were generated using PCR. When overexpressed, the C-terminal fragment was a soluble protein, whereas the N-terminal domain was an inclusion body. Purified C-terminal domain, N-terminal inclusion body, and solubilized, refolded N-terminal domain all lacked polyphosphatase activity using the standard PPX assay. However, polyphosphatase activity could be recovered when the purified N- and C-terminal domains were combined.

Size exclusion chromatography confirmed PPX to be a 116-kDa dimer as previously reported (13); however, purified C-terminal domain (24 kDa) was found to be monomeric. Purified N-terminal domain (34 kDa) was 60% monomeric and 40% dimeric.

**PPX Nested Deletions—** Nested C-terminal deletions of PPX were created using PCR. These deletions were spaced roughly 20 amino acids apart, with the largest deletion removing slightly more than the entire C-terminal domain. These proteins were overexpressed in *E. coli* BLR(DE3), and their activities were measured under conditions optimal for native PPX activity (175 mM KCl, 50 mM Tris-HCl (pH 7.5), and 1 mM MgCl2). Polyacrylamide gels of reaction samples showed a similar behavior among all deletions (data not shown). This behavior is clearly observed using deletion 5 (104 amino acids deleted), with smearing directly from the wells nearly to the P14 region, where a homogenous intermediate band accumulated (Fig. 3). Although a P2 band is also present, a P50 band is not observed despite smearing through the P50 region. This behavior was first detectable with deletion 3, and by deletion 5, it was very pronounced. Even with deletion 10 (237 of 513 amino acids removed), 28 amino acids shorter than the N-terminal domain, the same deletion 5 banding pattern (P14 and P2 bands) could still be observed. Activities of all deletions were not quantified; however, polyphosphatase activity fell with each deletion and became difficult to detect beyond deletion 10.

**Equilibrium Polyphosphate Binding—** Equilibrium binding data for immobilized, native PPX are shown in both a semi-log plot and a Scatchard plot (Fig. 4). Due to the poor fit of the standard equilibrium dissociation constant curve at low and high substrate concentrations and the nonlinearity of the Scatchard plot, effective dissociation constants were determined independently at low and high substrate concentrations. These were calculated by nonlinear fit of the data to standard single site dissociation constant equation (Kaleidagraph, Synergy Software, Reading, PA). The data were divided to maximize the

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**FIG. 1.** Polyacrylamide gel electrophoresis of PPX reaction products. Reactions were performed at the indicated KCl concentrations. Samples were taken when approximately 20% (1), 40% (2), and 60% (3) of the polyphosphate was hydrolyzed to orthophosphate. The lengths of reaction products indicated were determined using the size ladders in the center of the gel. Size ladders were resolved to individual bands by expanding the central region and adjusting the contrast of the PhosphorImager.

**FIG. 2.** Generation of N- and C-terminal domains of PPX by limited proteolysis. Upper panel, samples were analyzed on a 10–20% gradient SDS-polyacrylamide electrophoresis gel. Three identical lanes of PPX proteolysed by *S. aureus* V8 protease (Glu-C) at 6 μg/lane are shown in the middle of the gel. Unproteolysed PPX at 0.6, 2, and 6 μg/lane are shown on the right. Lower panel, N- and C-terminal domains of PPX with the position of Glu-C cleavage, Glu-304, indicated. Consensus sequences to the sugar kinase/actin/hsp70 superfamily are found within the N-terminal domain up to Ala-284. The position of the C terminus of deletion 5 (Fig. 3) is also shown.
correspondence of the Scatchard plot data to best-fit lines for high and low substrate concentrations. For immobilized PPX, the data were divided at 800 nM polyphosphate (as chains), whereas for the C-terminal domain, data were divided at 400 nM polyphosphate. The effective dissociation constants for polyphosphate binding by immobilized PPX and purified N- or C-terminal domain are given in Table II. In all cases curve-fitting gave $R^2$ values greater than 0.9, and the standard deviation in equilibrium binding data points was less than 20%.

**DISCUSSION**

**Binding Site-Active Site Distances**—The results shown in Fig. 1 clearly indicate that at KCl concentrations greater than 600 mM, PPX produces discrete P50, P14, and P2 intermediates. The appearance of smearing from the wells at the higher salt concentrations, representing a progressive shortening of all chains in the reaction, is indicative of a mode of action that is not highly processive. Such a mode of action can be nonprocessive with a single hydrolysis step per chain binding event or quasi-processive, defined here as having on average fewer than 200 hydrolysis steps per chain binding event. The presence of a substantial P50 band and the absence of slightly longer chains at 600 mM KCl suggest a quasi-processive mode of action. The mode of action becomes increasingly nonprocessive at higher KCl concentrations. These results support bond hydrolysis and processivity being not strictly coupled processes.

The range of discrete intermediates produced by exopolyphosphatases (and polyphosphate phosphotransferases) including P2, P3 (minor), P14, and P50 with PPX, P4 and P40 with guanosine pentaphosphate phosphohydrolase (GppA) (8), and finally, P100 with polyphosphate glucokinase (9, 10) suggests the intermediates form due to an enzyme-dependent phenomenon and not due to an independent physical property of polyphosphate. This is also supported by the fact that polyphosphate does not have a rigid secondary structure but is a highly flexible, freely rotating chain as reflected by its short $8\text{-Å}$ persistence length (18, 19).

The enzyme dependence of chain lengths recognized by these enzymes suggests there are at least three discrete sites on PPX that interact with polyphosphate. These sites are located roughly 50, 15, and 3 phosphate residues from the active site, with the P50 site and the active site potentially greater than 100 Å apart. These interaction sites could directly bind polyphosphate, could affect a polyphosphate binding site elsewhere on the enzyme, or could affect the kinetics at the active site.

These possibilities were addressed through additional experiments. Under quasi-processive conditions, C-terminal deletions 2 through 10 did not produce a P50 band (Fig. 3), implying the P50 interaction site was no longer functional. Furthermore, deletion of portions of the C terminus caused the enzyme to become quasi-processive at low salt concentrations, suggesting weakened binding of polyphosphate. In fact, PPX and purified C-terminal domain were found to have nearly equal affinity for polyphosphate, confirming the existence of a high affinity polyphosphate binding site on the C-terminal domain. That the functionality of the P50 site is lost, accompanied by weakened binding for C-terminal deletions 2 through 10 and that the C-terminal domain has a high affinity polyphosphate binding site suggest the P50 site may directly bind polyphosphate.

The P14 interaction site also appears to directly bind...
Polyphosphate Binding of PPX

above 600 mM, KCl native PPX produces smearing between the P14 and P3 bands (Fig. 1). This is consistent with the P14 site directly binding polyphosphate with chains shorter than P14 released with higher frequency and bound with lower frequency than longer chains.

The distinct sizes of the P50 and P14 intermediates imply the corresponding binding sites are discrete regions on the enzyme. However, this does not rule out additional positive polyphosphate-PPX interactions at other positions or the existence of a large binding pocket running 50 phosphates from the active site.

The P3 interaction site was not characterized further to determine if it directly binds polyphosphate. The results of Fig. 1, however, do confirm that the P2 product is generated from sequential hydrolysis of phosphates from longer polyphosphate until only the final P2 chain remains. This is observed in 1.2 M KCl as the interaction of polyphosphates shorter than P14 appears to be weakened to the extent that the chains effectively do not bind. Hydrolysis under these conditions terminates at P14, and the P3 intermediate is not observed.

It is interesting that at 600 mM KCl smearing between the P50 and P14 bands is not observed, indicating that chains longer and shorter than P50 are processively hydrolyzed, whereas P50 chains are released. What is it that causes PPX to have low affinity for P50 but seemingly higher affinity for longer and shorter chains, and is this result inconsistent with the P50 site functioning to directly bind polyphosphate? We believe the P50 site binds polyphosphate with much higher affinity than the P3 or P14 sites. Based on this, we speculate that a P50 chain bound to the P50, P14, and P3 sites may on average release from and rebind to the P14 and P3 sites many times before releasing from the P50 site. When release from the P50 site occurs, the P50 chain may be bound to the P14 and P3 sites, in which case the chain can advance into the active site and can be processively hydrolyzed to P14 or shorter. Alternatively, the P50 chain may not be bound to the P14 and P3 sites when release from the P50 site occurs. We speculate further, that in this case the P50 chain is freed from the enzyme.

Limited Proteolysis Domains—S. aureus V8 protease (Glu-C) is capable of hydrolyzing PPX at 32 positions. With limited proteolysis, Glu-C hydrolyzes PPX predominantly at either glutamate 304 or 306, generating relatively protease-resistant N- and C-terminal domains. Interestingly, consensus sequences to the sugar kinase/actin/hsp70 superfamily are found in the N-terminal 284 amino acids of PPX (20, 21). Furthermore, the high stability of the 24-kDa C-terminal domain in the absence of the remainder of the protein suggests the folded structures of these domains are independent.

Enzymes of this superfamily have two similarly folded domains, domains I and II, on each side of a large cleft. Each domain consists of two similar subdomains, a and b, with subdomains Ia and Iia nearer the bottom of the cleft (20). Many members of this superfamily have large insertions between conserved regions that give rise to large and variable subdomains Ib and Iib of the top of the cleft. Insertions between these regions in PPX are among the smallest in the superfamily and presumably give rise to an N-terminal domain with a relatively shallow conserved active site cleft (subdomains Ia and Iia) and negligible Ib and Iib subdomains. PPX is also unique among members of the superfamily in that it has a large 229-amino acid C-terminal addition, which gives rise to the independently folded C-terminal domain.

However, despite the apparent independence in the folded structures of the domains, neither had polyphosphatase activity when expressed separately. This did not appear to be a domain-folding problem, as mixing the separate domains allowed recovery of polyphosphatase activity.

PPX-nested Deletions—Nested C-terminal deletions were able to confirm an N-terminal active site. Additionally, these deletions revealed the C terminus to be involved in the processivity of PPX, either indirectly through interactions with the N terminus or by direct interaction or binding with polyphosphate. The loss of even small portions of the C terminus caused the mode of action at 175 mM KCl to become quasi-processive, causing a variety of chain lengths to accumulate (Fig. 3). At this same salt concentration and up to at least 300 mM KCl, the native enzyme is highly processive, completely hydrolyzing P750 in a single chain binding event. It is interesting that despite weakening the polyphosphate-PPX interaction, these nested deletions do not give rise to a P50 band. Weakened polyphosphate binding and the absence of a P50 band under quasi-processive conditions for deletions 3 through 10 suggest that removal of the C terminus cause the loss of the P50 binding site.

Comparison of Polyphosphate Binding by PPX and by Its N- and C-terminal Domains—The C-terminal domain accounts for nearly all of the affinity between PPX and polyphosphate. Although misfolding of the N-terminal domain might explain weak polyphosphate binding, folding of both purified domains seems to be correct based on recovery of activity for the combined purified domains.

Although PPX is dimeric, the similarity of polyphosphate binding by PPX and its monomeric C-terminal domain supports the polyphosphate-PPX interaction being predominantly with only one monomer subunit of the enzyme. This implies that PPX dimers have two strong C-terminal polyphosphate binding sites.

Conclusion—Collectively, these results allow a model to be proposed for the interaction between PPX and polyphosphate. Within this model, PPX dimers contain 2 functional, N-terminal active sites and 6 polyphosphate binding sites positioned 3, 14, and 50 phosphate residues from each active site. Furthermore, at high concentrations of P50−chains, dimers may bind and hydrolyze two polyphosphate chains simultaneously, with each polyphosphate chain bound to three sites (i.e. a P3, a P14, and a P50).

It is interesting that the P50 site binds polyphosphate tightly such that more than 1000 hydrolysis steps may occur without release of intermediate chains even at monovalent salt concentrations exceeding 300 mM. Due to the probable destabilization of the nested deletion mutants, it is difficult to say if the P3 and P14 sites are independently capable of conferring the highly processive mode of action of PPX. However, given the properties of the P50 binding site, the P3 and P14 sites need not be highly processive. These sites need only be quasi-processive, allowing the final 50 hydrolysis steps to occur without release of an intermediate polyphosphate chain.

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