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Purification and Characterization of Polyphosphate Kinase from *Neisseria meningitidis*

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The important human pathogens *Neisseria meningitidis* and *Neisseria gonorrhoeae* accumulate phosphate in the form of polyphosphate (A. Noegel and E. C. Gotschlich, J. Exp. Med. 157:2049–2060, 1983), and the localization of more than half of this long-chain polymer on the exterior of the cells suggests a function as a protective, capsule-like coating. To enable further genetic investigation of the role of polyphosphate in *Neisseria* spp., the enzyme polyphosphate kinase (PPK), which catalyzes the synthesis of polyphosphate from ATP, was purified from *N. meningitidis* BNCV. The activity is dependent on Mg^{2+} and phosphate or polyphosphate and is inhibited by ADP. The K_m for ATP is 1.5 mM, and the turnover number is 47 phosphate residues per polypeptide per s. Analysis of PPK labelled with [γ-^{32}P]ATP indicates that the enzyme is phosphorylated during the reaction, probably at an arginine residue. N-terminal and two internal amino acid sequences were derived from the purified protein and will allow the design of synthetic oligonucleotides for cloning and genetic manipulation of the *ppk* gene.

Since the discovery of polyphosphates in a yeast (23, 24), their occurrence and function in cells have been the topic of much research. Inorganic polyphosphate (linear polymeric phosphate, linked by acid anhydride bonds) has been detected in species from all major groups of organisms (20, 33), and various enzymes which are involved in the synthesis and breakdown of polyphosphate and link its metabolism to other biochemical pathways have been discovered. It has been suggested that polyphosphate in microorganisms might serve as an energy or as a phosphate store (11, 18–20), as a carrier of activated phosphate groups, substituting for ATP in some reactions (29, 35), as a buffer for the maintenance of constant intracellular phosphate concentration (11, 17, 20), or, in association with poly-β-hydroxybutyrate and Ca^{2+}, as a hydrophilic membrane pore (30).

In several *Neisseria* species, high-molecular-weight polyphosphate (around 200 residues in length) was found to constitute more than 10% of the total cellular phosphate (27). About half of the polyphosphate was loosely attached to the surface of the neisseriae, in contrast to the other species investigated. This suggested that it may play a part in interactions with the environment of the bacteria, perhaps providing an antiphagocytic "capsule," chelating ions necessary for complement fixation, or complexing such essential nutrients as iron.

Accumulation of high-molecular-weight polyphosphate is known to occur in microorganisms by the enzyme polyphosphate kinase (PPK) (polyphosphate:ADP phosphotransferase):

$$\text{ATP} + \text{polyP}_{(n)} \rightleftharpoons \text{ADP} + \text{polyP}_{(n+1)}$$

where polyP is polyphosphate and n is the degree of polymerization. The activity was first demonstrated in yeast cell extracts by Yoshida and Yamataka (38), and the enzyme has subsequently been purified to a greater or lesser extent from a number of microorganisms, namely; *Escherichia coli* (1, 18, 22), *Corynebacterium xerosis* (25), *Salmonella minnesota* (26), *Saccharomyces cerevisiae* (8), *Propionibacterium shermanii* (31, 32), and *Sulfolobus acidocaldarius* (33).

PPK was purified from *Neisseria meningitidis* with a view to investigation of its properties and comparison with enzymes from other organisms. Sequence analysis of the protein would allow cloning of the gene encoding PPK and the study of the effects of overexpression or inactivation of the gene on the physiology and virulence of the bacteria.

MATERIALS AND METHODS

SDS-PAGE and staining of gels. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the discontinuous buffer system of Laemmli (21) with gels (80 by 90 by 0.75 mm) containing 11% acrylamide-bisacrylamide (37.5:1). Protein markers for determination of apparent molecular weight were purchased from Sigma Chemical Co.

Staining with Coomassie blue was performed as described by Fairbanks et al. (7).

Silver staining was carried out essentially as described by Hitchcock and Brown (13).

Assay for production of polyphosphate. Two methods were used to assay PPK activity.

(i) Assay by incorporation of [^{32}P]phosphate. Volumes (5 to 20 μl) of the samples to be tested were added to tubes containing 200 μl of ATP reagent. ATP reagent consisted of 40 mM glycylglycine-KCl, 10 mM potassium phosphate (pH 7.0), 10 mM $MgCl_2$, 15% (vol/vol) glycerol, and 1 mM ATP ([γ-^{32}P]ATP was added to give a specific activity of 1 μCi μmol^{-1}). Since the enzyme was strongly inhibited by ADP, 6 mM phosphoenolpyruvate and 10 U of pyruvate kinase (enzyme type II from rabbit muscle; Sigma) ml^{-1} were added to the assay mixture as an ATP-regenerating system. After incubation at room temperature for 30 min, the reaction was stopped and the product was precipitated by the addition of 60 μl of 0.5 M Tris–0.25 M EDTA (pH 8.0) containing 2.5% cetyl-trimethylammonium bromide and 0.25 M NaCl (CTAB reagent).

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After 15 min at room temperature, the reaction mixtures were filtered through a cellulose acetate membrane (0.8-μm pore size) with a Bio-dot filtration apparatus (Bio-Rad), and the precipitated polyphosphate was washed extensively with a 1/5 dilution of the CTAB reagent. The filter was dried and subjected to autoradiography, and then the radioactive spots were excised for measurement of radioactivity in a beta-counter. One unit of activity was defined as that amount which effected the transfer of 1 nmol of phosphate from ATP to polyphosphate in 1 min.

The assay described above was used for localization of activity during the purification of PPK. In investigations of the mechanism, activators, and inhibitors of purified PPK, activity was assayed in the absence of the ATP-regenerating system.

(ii) Assay by measurement of metachromasy. Polyphosphate produced as in the assay described above was confirmed as such by its characteristic reaction with the basic dye toluidine blue-O. Polyphosphate was precipitated with CTAB reagent, pelleted by centrifugation, washed with ethanol containing 50 mM sodium acetate to remove detergent, and redissolved in water. Reaction with toluidine blue (36) caused the absorption maximum of the dye to be shifted from 630 to 530 nm.

Preparation of outer membranes from *N. meningitidis* and detergent extraction of proteins. Membranes were prepared from *N. meningitidis* BNCV (a nonencapsulated variant of M986 [10]). Bacteria were grown for 16 h with shaking at 37°C. The cells from 4 liters of Trypticase soy broth (Difco) were harvested by centrifugation at 10,000 × *g* for 20 min at 4°C. The pellet was resuspended in 100 ml of 0.1 M Tris-HCl (pH 7.5)–5 mM EDTA–20% sucrose containing 40 mg of lysozyme (chicken egg white) and left on ice for 20 min. After the addition of 5 ml of 1 M $MgCl_2$, the mixture was centrifuged at 10,000 × *g* for 30 min at 4°C and the supernatant containing the periplasm was removed. The pellet was resuspended in 100 ml of 10 mM $MgCl_2$ containing 5 mg of DNase I (Sigma; from beef pancreas) and then freeze-thawed four times by using a dry ice-ethanol bath and a 30°C water bath. The suspension was centrifuged at 100,000 × *g* for 1 h at 4°C, and the supernatant containing the cytoplasm was removed.

The membrane pellet was resuspended at 0°C in 100 ml of 20 mM glycylglycine-KCl (pH 7.0) containing 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 5% (wt/vol) Emulphogene BC-720 (isotridecylpolyoxyethylene ether; Rhône-Poulenc, Cranbury, N.J.), and, after extraction for 1 h at 4°C, the suspension was centrifuged at 100,000 × *g* for 1 h. The reddish supernatant was removed, and the pellet was resuspended in $MgCl_2$-Emulphogene buffer and reextracted. Three further extractions were performed at 4°C, each with 100 ml of 20 mM glycylglycine-KCl (pH 7.0) containing 5 mM EDTA, 1 mM DTT, and 5% Emulphogene.

Purification of the PPK from detergent extracts. Material extracted from the bacterial membranes in 20 mM glycylglycine-KCl (pH 7.0)–5 mM EDTA–1 mM DTT–5% Emulphogene was dialyzed against equilibration buffer (25 mM Tris-HCl [pH 7.5], 1 mM EDTA, 1 mM DTT, 0.1% Emulphogene, 15% [vol/vol] glycerol) and then subjected to ion-exchange chromatography on a DEAE-Sepharose (Fastflow; Pharmacia) column. Fractions containing the kinase activity were pooled, mixed with an equal volume of affinity chromatography starting buffer (50 mM glycylglycine-KCl [pH 7.0], 10 mM $MgCl_2$, 1 mM DTT, 0.1% Emulphogene, 15% glycerol), and applied to a column of ADP-agarose (ADP linked by ribose hydroxyls to agarose beads; Sigma catalog no. A5048). The column was washed with 6 bed volumes of starting buffer and then eluted with a step gradient of ATP in starting buffer. The affinity chromatography was performed at 0°C.

Amino acid sequence analysis of the purified kinase. Samples of purified PPK (around 30 μg) eluted from the ADP-agarose column were precipitated by the addition of trichloroacetic acid to 10% (wt/vol) and centrifugation at 16,000 × *g* for 20 min. The precipitate was washed with 0.5 ml of ethanol containing 50 mM sodium acetate and then with 0.5 ml of acetone. The redissolved protein was subjected to SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore). The bands corresponding to the PPK, visualized by staining with Ponceau S, were excised and subjected to N-terminal sequence analysis with an Applied Biosystems model 475A protein sequencer (9).

To obtain sequence information from regions of the protein other than the N terminus, portions of the PPK bound to polyvinylidene difluoride were subjected to proteolytic cleavage (4) by using endoproteinase Lys-C (EC 3.4.99.30; Boehringer-Mannheim, catalog no. 1047 825). The fragments generated were separated by microbore high-performance liquid chromatography, and two of the peptides producing clearly separated peaks were subjected to N-terminal sequence analysis.

Production and analysis of [^{32}P]PPK. Purified PPK (2.4 μg) was reacted for 30 min at 0°C in a mixture (100 μl) containing 40 mM glycylglycine-KCl, 10 mM KP_i (pH 7.0), 8 mM $MgCl_2$, 0.08 mM ATP, 0.8 mM DTT, 12% glycerol, 0.08% Emulphogene, and 1 mCi of [^{32}P]ATP. The reaction mixture was added, at 0°C, to 50 μl of cellulose phosphate (Whatman, P11) equilibrated with 50 mM glycylglycine-KCl (pH 7.0)–1 mM EDTA–1 mM DTT–15% glycerol–0.1% Emulphogene. After removal of the supernatant solution, the matrix was washed six times at 4°C with 1 ml of equilibration buffer and then eluted with 3 column volumes each of 300 mM KCl and then 400 mM KCl in equilibration buffer. The fractions constituting the eluted peak of radioactivity (shown by SDS-PAGE to be [^{32}P]PPK) were stored at −80°C.

Samples containing [^{32}P]PPK were subjected to chemical and/or heat treatments and then mixed with SDS-PAGE solubilization buffer and loaded onto 15% polyacrylamide gels. After electrophoresis, the gels were immediately dried under vacuum and subjected to autoradiography. Regions of the gel corresponding to radioactive bands were excised for quantitation of the ^{32}P label. It was found that ATP migrates at the bromophenol blue dye front while P_i migrates before the dye front, presumably at the buffer front. Hence, bands excised from the gel at the 72-kDa position, the dye front, or the buffer front were assumed to correspond to PPK, peptides, and amino acids or P_i. The degree of dephosphorylation was measured as activity in the band at the buffer front divided by the total activity.

Analysis of [^{32}P]polyphosphate by PAGE. For separation of the reaction products by PAGE, 15% polyacrylamide gels (5% cross-linked) were prepared as described by Clark and Wood (5) but with a buffer containing 86.3 mM taurine, 0.15 mM EDTA-Na_2, and 7 M urea.

RESULTS

Purification of the PPK. After fractionation of the cells, the PPK activity was found almost entirely in the isolated membranes. Upon treatment of the membranes with the detergent Emulphogene plus $MgCl_2$, more than half of the

TABLE 1. Preparation of membranes from *N. meningitidis* and detergent extraction

Fraction	Vol (ml)	Protein (mg)	Activity[a]	Sp act
Whole cells[b]	130	3,350	12,900	3.85
Periplasm	108	238	63	0.26
Cytoplasm	100	640	331	0.52
Membranes	125	2,410	27,300	11.3
E. Sn1a[c]	103	1,230	742	0.60
E. Sn1b	101	141	151	1.07
E. Sn2a	97	136	10,900	80.1
E. Sn2b	99	79	5,260	66.6
E. Sn2c	100	50	199	3.98
Insoluble pellet	50	625	11,600	18.6

[a] Units of activity are nanomoles of [^{32}P]phosphate incorporated into polyphosphate per minute.

[b] It is probable that the PPK activity in the whole cells was underestimated by this assay.

[c] E. Sn1a and E. Sn1b, the first and second, respectively, of two extractions with Emulphogene-$MgCl_2$; E. Sn2a, E. Sn2b, and E. Sn2c, the first, second, and third, respectively, of three extractions with Emulphogene-EDTA.

protein was solubilized, while the PPK remained in the membrane fraction. In the presence of Emulphogene plus EDTA, the enzyme was extracted in a fraction containing relatively little protein (Table 1; Fig. 1). While the particulate nature of the whole cells and membrane fractions complicated interpretation of their measured PPK activities, it appeared that around 60% of the activity was extracted from the membranes by the detergent treatment. Whether the unextracted activity represents PPK in another form or some other activity incorporating label into an insoluble or CTAB-precipitable form is a matter for further study.

DEAE chromatography of the detergent-EDTA extract resulted in a broad peak of activity (Fig. 2), which was eluted from the column between 100 and 200 mM KCl. The six 5-ml fractions corresponding to the center of the activity peak were taken for the next stage of purification.

Affinity chromatography was first attempted by using ATP linked (by ribose hydroxyls) to agarose beads, i.e., a substrate affinity column. However, the yields were low since not all of the activity bound initially to the column and much was eluted during the washing steps. On the basis of the finding that the PPK activity assay was inhibited by low concentrations of ADP, affinity chromatography was performed with ADP-agarose. This procedure resulted in a remarkable purification of the kinase. Although some activity leaked from the column during the washing stage, most (78%) of the activity came off the column upon addition of the ATP eluent (Fig. 3a). SDS-PAGE analysis showed the purification of a protein with an apparent M_r of 72,000, the intensity of the stained band corresponding to the amount of activity in each fraction (Fig. 3b). The purified PPK could be stored at −20°C for at least 4 months without significant loss of activity.

A further elution with 1 mM ADP resulted in the release of a small amount of a protein with an apparent M_r of around 45,000. These fractions were without PPK activity.

The progress of the purification is summarized in Table 2. Assuming an M_r of 72,000 for the kinase, and also that the activity of the enzyme was not affected by the purification procedure, the copy number could be calculated to be around 500 per cell in the starting culture.

It was discovered subsequently that the enzyme could be extracted from the meningococcal membranes by suspension in glycylglycine containing 400 mM KCl to the same extent as with Emulphogene-EDTA. The purification procedure described above could then be followed, resulting in the isolation of the same protein, as judged by SDS-PAGE. However, the yields of PPK from the DEAE-Sepharose and the ADP-agarose chromatographies were considerably lower in the absence of Emulphogene.

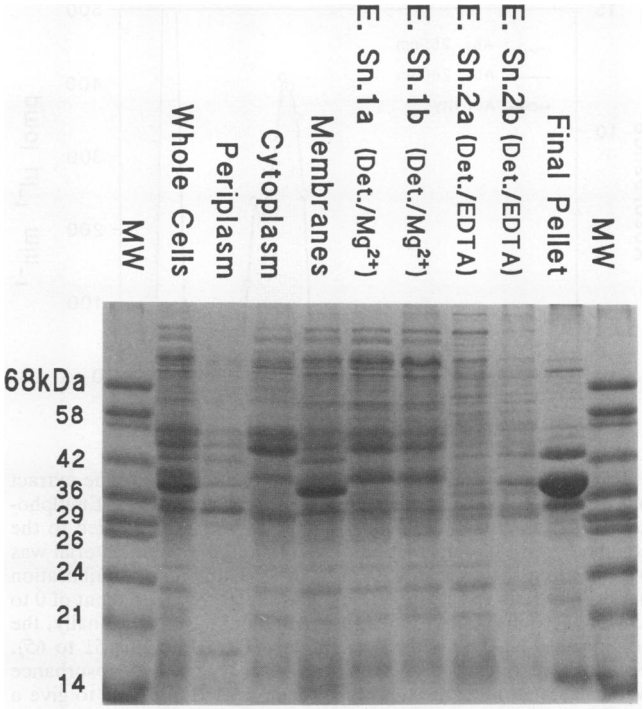

FIG. 1. SDS-PAGE analysis of fractionation of *N. meningitidis*. Lane MW contained 1.5 μg of each of the molecular weight markers; lanes E. Sn1a and E. Sn1b contained supernatant from the first and second extractions, respectively, of membranes with Emulphogene and $MgCl_2$; E. Sn2a and E. Sn2b contained supernatant from the first and second extractions, respectively, with Emulphogene and EDTA. Amounts of protein were 10 μg for the samples of periplasm and of E. Sn2b and 20 μg for each of the others. Molecular mass markers were bovine serum albumin (68 kDa), catalase (58 kDa), alcohol dehydrogenase (42 kDa), lactic dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), α-chymotrypsinogen A (26 kDa), trypsin (24 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14 kDa). Proteins were visualized by staining with Coomassie blue.

pH optimum of reaction: dependence on cations and anions. As the pH of the standard assay was varied, the enzymatic activity showed a broad peak between pH 6.2 and 7.2. The enzyme produced polyphosphate only from ATP, being unable to utilize CTP, UTP, or GTP (determined by the metachromatic assay). The purified enzyme was found to have a dependence on both magnesium and phosphate for production of polyphosphate. The optimum concentrations of these cofactors were around 10 mM KP_i and 10 mM $MgCl_2$ at pH 7.0.

In experiments using 3 mM KP_i, the enzyme was able to utilize manganese or cobalt as a cofactor in place of magnesium, although with efficiencies only 1/4 and 1/10, respectively, of that with Mg^{2+}. Calcium or zinc as the sole divalent cation did not stimulate enzymatic activity.

Assays for polyphosphate production were carried out at 10 mM $MgCl_2$ and a range of concentrations of various anions. The enzyme showed a distinct preference for phos-

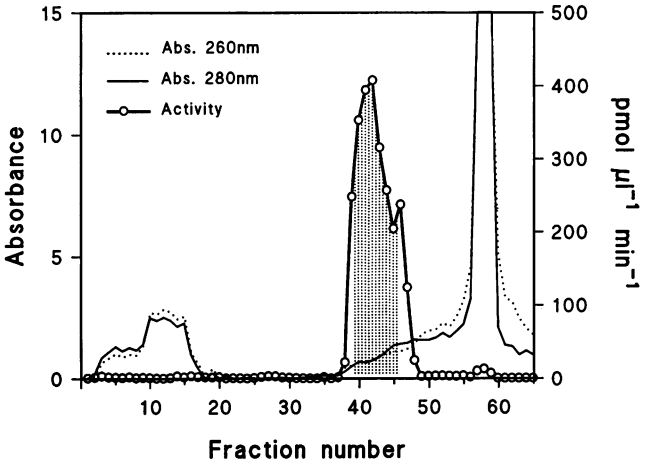

FIG. 2. Ion-exchange chromatography of the membrane extract on DEAE-Sepharose. The dialyzed membrane extract (Emulphogene extracts 2a and 2b; final volume, 137 ml) was applied to the column (1.5 by 15 cm; fractions 1 to 14), and unbound material was washed from the column with an additional 160 ml of equilibration buffer (fractions 15 to 30). Elution was effected by a gradient of 0 to 250 mM KCl in 100 ml of the buffer (fractions 31 to 50). Finally, the column was washed with 75 ml of 1 M KCl (fractions 51 to 65). Fractions were assayed for their A_{280} (where necessary, absorbance was calculated after the liquid had been diluted in buffer to give a reading below 2) and for PPK activity by the ^{32}P incorporation assay. The six fractions constituting the center of the activity peak (shaded) were taken for the next stage of purification. Abs., absorbance.

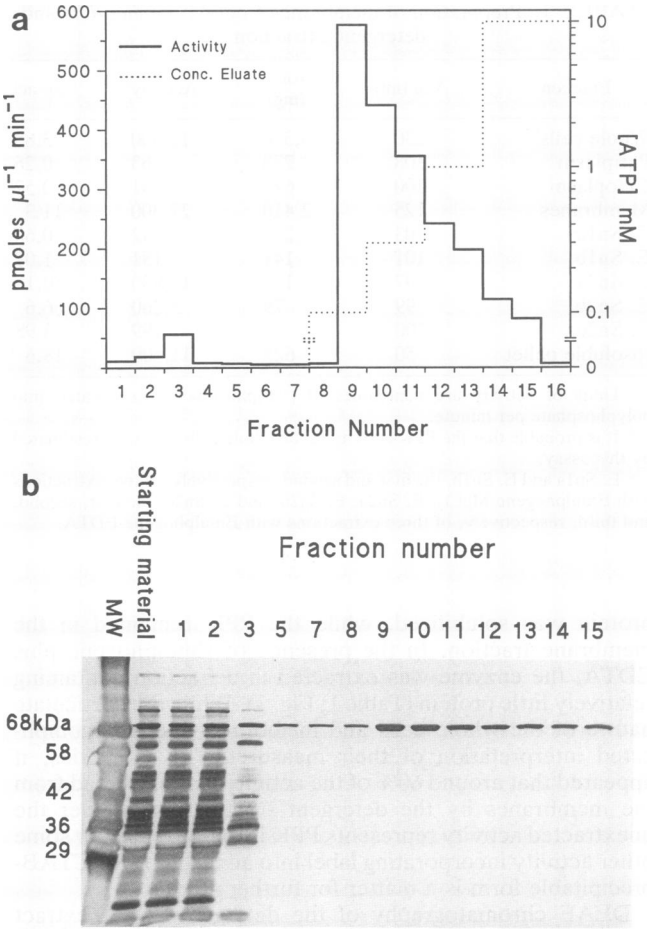

FIG. 3. (a) Purification of PPK by affinity chromatography on ADP-agarose. The pooled fractions constituting the peak of activity from the DEAE column, diluted twofold with affinity chromatography starting buffer (50 mM glycylglycine-KCl [pH 7.0], 10 mM $MgCl_2$, 1 mM DTT, 0.1% Emulphogene, 15% glycerol), were applied to the 3-ml column in a volume of 60 ml. The column was washed with 6 column volumes of starting buffer and then eluted with 2 column volumes each of 0.1, 0.3, 1.0, and 10 mM ATP in the same buffer. The dotted line represents the concentration of ATP in the buffer applied to the column, while the solid line shows the PPK activity in the eluted fractions. (b) SDS-PAGE analysis of affinity purification of PPK on ADP-agarose. The lane designated MW contained 2.5 µg of each of the molecular weight markers. Other lanes contained 10 µl of the starting material, column washes, or eluted fractions. Proteins were visualized by silver staining.

phate over the other anions tested (Fig. 4). Potassium acetate (pH 7.0) or chloride was incapable of stimulating the enzymatic activity. The optimum concentration of potassium phosphate was between 3 and 10 mM. Sulfate or arsenate was considerably less efficient at stimulation of enzymatic activity. Sodium polyphosphate with an average chain length of 64 (1 mM with respect to phosphate; Sigma) stimulated enzymatic activity in the absence of P_i, producing 40% of the activity measured in 10 mM KP_i.

The kinase activity decreased to some extent with increasing salt (NaCl or KCl) concentration. Activities at 250 and 400 mM were reduced to 50 and 15%, respectively, of the value at 50 mM (standard assay conditions).

PPK activity was inhibited more than 95% in the presence of 0.5 mM cadmium chloride, lead acetate, or zinc chloride and by preincubation with 0.1 mM sodium parachloromercuribenzoate. At 1 mM ATP, the enzyme activity was inhibited 66% by 50 µM, 90% by 100 µM, and 100% by 200 µM ADP.

Kinetics of PPK. The plot of reaction velocity against substrate (ATP) concentration gave a curve suggesting a sigmoidal relationship (Fig. 5). Extrapolation of the nonlinear Lineweaver-Burk plot for ATP concentrations above 300 µM gave a K_m of 1.5 mM and a turnover number of around 47 ATP molecules per polypeptide per s.

Visualization of the product. When enzymatically synthesized [^{32}P]polyphosphate was analyzed by PAGE, the product migrated near the top of the gel, suggesting a length in excess of 200 residues. Treatment in 80 mM HCl at 100°C for 40 min caused almost complete hydrolysis to P_i. Hydrolysis in 1 M NaOH at 100°C for 5 or 10 min gave a ladder of discrete bands corresponding to polyphosphates differing in chain length by one phosphate residue (32). For all of the reaction times tested (down to 30 s at 4°C) at which the kinase produced detectable product, the polyphosphate migrated with the same high M_r; no evidence was seen of polyphosphate molecules of intermediate lengths.

Analysis of [^{32}P]PPK. Labelled PPK, purified by cellulose phosphate chromatography, was mixed with loading buffer and subjected to SDS-PAGE. The labelled band comigrated with PPK which had been solubilized by boiling in the loading buffer.

The enzyme underwent gradual dephosphorylation in the equilibration buffer at 21°C, the degree of phosphate liberation being 20% for enzyme subjected to SDS-PAGE immediately after removal from a stock solution stored at −80°C, and 30, 40, and 50% after 10, 20, and 40 min. Incubation in buffer at 56 or 95°C for 20 min resulted in 80 or 100%

TABLE 2. Purification of PPK from *N. meningitidis*

Fraction	Vol (ml)	Protein (mg)	Activity[a]	Sp act	Net yield (%)	Purification factor
Cells in Tris-EDTA-sucrose	130	3,350				1.0
Membranes in Emulphogene-$MgCl_2$	125	2,413	27,300	11.3		1.37[b]
Emulphogene-EDTA extracts	196	215	16,100	74.9	59.1	9.08
DEAE peak fractions	30	19.5	7,820	401	28.7	48.6
ADP eluate	18.5	0.546	6,070	11,100	22.3	1,350

[a] Units of activity are nanomoles of [^{32}P]phosphate incorporated into polyphosphate per minute.
[b] Since the whole cells gave anomalous results in the activity assays (see Table 1), it was assumed that the activity present in the original culture was equal to the sum of the activities of the cellular fractions (periplasm, cytoplasm, and membrane).

dephosphorylation, respectively. The [^{32}P]PPK was stable to pH 13.2 and to 1 M NaOH at 21°C. Boiling of the labelled enzyme in 1 M NaOH resulted in a smear of radioactivity on SDS-polyacrylamide gels corresponding to a molecular mass between 5 and 10 kDa and a band migrating at the dye front, these being presumably the products of partial alkaline hydrolysis of the polypeptide. However, no release of P_i was observed. In contrast, treatment with dilute acid (pH 0.8) at 21°C gave 60 to 70% hydrolysis after 5 min, and the enzyme was more than 90% dephosphorylated after 20 min in 1 M HCl. Treatment with 50 mM or 1 M hydroxylamine-HCl (pH 6.8) resulted in 80 or 100% dephosphorylation. These properties were consistent with an N-phosphate linkage (6, 34). The enzyme could be dephosphorylated by boiling in 2.5 M NaOH, with a half-life around 20 min suggesting that the phosphorylated residue was phosphoarginine.

Amino acid sequence of the kinase. Analysis of the N terminus and two internal peptides produced by lysine endopeptidase gave the amino acid sequences shown in Fig. 6. Some homology was found between the N-terminal sequence and that of the sequence determined for the PPK from *Escherichia coli* (2). No significant homologies were seen between any of the peptides and other protein sequences submitted to the GenBank data base.

DISCUSSION

Polyphosphate has been detected in many microorganisms and is commonly present as intracellular inclusions of metachromatic material (36, 37). Neisseriae accumulate at least 10% of their phosphate as polyphosphate (27), and in the case of the strains studied, about half of this was located extracellularly. Interest in the mechanism of neisserial PPK, its comparison with other purified enzymes, and the possible significance of a polyphosphate capsule for bacterial survival in their human host led to the investigation of enzymes responsible for polyphosphate synthesis in the organisms.

PPK was purified from *N. meningitidis* BNCV to give a protein with an apparent M_r of 72,000 on SDS-PAGE. Other purified PPKs had the apparent molecular weights shown in Table 3, which summarizes some of the properties of microbial PPKs. The K_m, the turnover number, and the amount of enzyme per meningococcal cell, calculated from the efficiency and yield of the purification, are similar to those published for *E. coli* (1). However, most of the enzymes in Table 3 were only partially purified, and there is not suffi-

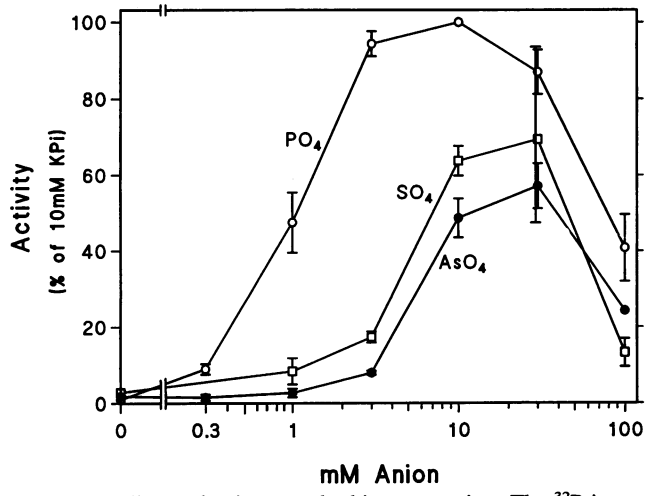

FIG. 4. Effects of anions on the kinase reaction. The ^{32}P incorporation assay was carried out with a buffer containing 40 mM glycylclycine-KCl (pH 7.0), 10 mM $MgCl_2$, 1 mM ATP, 15% glycerol, and a range of concentrations of the anions under investigation (potassium salts at pH 7.0). Measured activities are expressed as percentages of the activity obtained in a control assay containing 10 mM potassium phosphate. Each point represents the mean of at least three calculated percentages ± the standard deviation. Results obtained with ammonium sulfate were very similar to those obtained with potassium sulfate and for the sake of clarity are not shown.

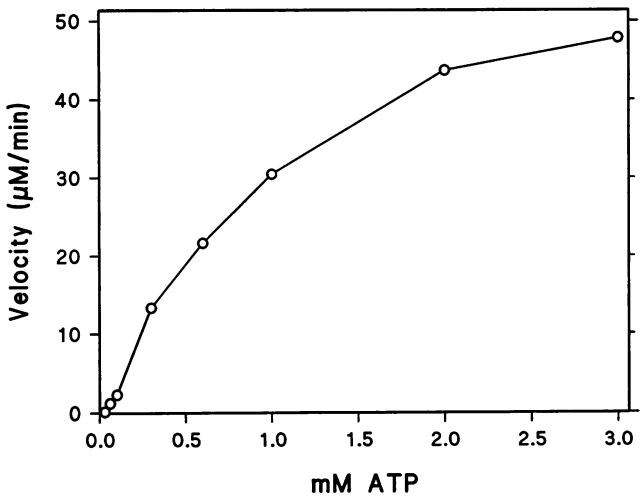

FIG. 5. Kinetics of PPK. Activity assays were performed with 1.9 μg of PPK, in volumes of 1 ml, at ATP concentrations ranging from 0.03 to 3 mM. Samples (0.1 ml) were removed, at times from 1 to 25 min, for precipitation of synthesized polyphosphate and counting of radioactivity. The data were analyzed graphically, and the initial velocities of the reactions were calculated in micromolar ATP utilized per minute.

A

N-terminal	PEQNRILXRELSLLAFNRRVLAQAEDKN
Internal 1	XPSETIADVTEAARSLIRHQYDLFNNVLQPELAREEIHFY[1]RRR
Internal 2	GPVNLVRLNAVPDLVNRPDLK

B

N. meningitidis	PEQNRILX-RELSLLAFNRRVLAQAEDKN
	: ... .:::.:.:. ::: : ::
E. coli	MGQEKLYIEKELSWLSFNERVLQEAADKS

FIG. 6. Amino acid sequence of N terminus and internal peptides of PPK. (A) Sequence determinations from neisserial PPK. Superscript 1 indicates that this cycle produced peaks for Y and F in the ratio 1:0.8. (B) Comparison of N-terminal sequences from *N. meningitidis* and *E. coli*. The *E. coli* sequence was published by Akiyama et al. (2). Standard single-letter codes for amino acids are used. X represents residues whose identity could not be determined, often cysteines. In the sequence comparison, colons designate identical amino acids; periods indicate structurally similar residues.

cient information to draw conclusions concerning structural or functional relationships within or between PPKs from the groups of organisms represented. The location of the enzyme was not investigated rigorously, although the enzyme is obviously attached to the membrane fraction by weak bonds, probably involving ionic interactions.

The dependence on divalent cations for activity of the enzyme is in keeping with all other described PPKs as is the production in vitro of exclusively long-chain polyphosphates. The effect of electrolytes on enzyme activity has been described to some extent for each species, different enzymes having different sensitivities to ammonium sulfate and to high salt concentrations.

Of particular interest is the dependence of enzymatic activity on phosphate and, in its absence, polyphosphate. Assuming an intracellular concentration of phosphate (data for *E. coli* [20]) of around 5 mM, the necessity for phosphate could reflect an allosteric activation. The ability to substitute polyphosphate for orthophosphate, however, lends support to the hypothesis that orthophosphate or polyphosphate is necessary as a primer. Experiments to test whether ^{32}P-KP$_i$ was incorporated into polyphosphate by PPK were, however, inconclusive. The stimulation by arsenate and sulfate could be due to an artificial substitution for phosphate at a site of allosteric activation. It is also possible that arsenate or sulfate could bind as substrates, in place of phosphate, at the active site of the enzyme and stimulate phosphoryl transfer from ATP. The adduct thus formed may decompose rapidly in the case of arsenate, but the phosphate moiety at the

TABLE 3. Comparison of the properties of PPK purified from several microorganisms

Organism	Mol wt	K_m (mM)	Effect of:		
			Phosphate	Polyphosphate	Sulfhydryl agents
E. coli (Kornberg et al. [18])	69,000 (tetramer)	2.0		Stimulates at low [ATP]	Used in purification
E. coli (Li and Brown [22])		1.4	Stimulates 6-fold		Used in purification
P. shermanii	83,000 (monomer)		Stimulates 10-fold	Stimulates and primes	Used in purification
C. xerosis			No stimulation	No stimulation	Used in purification
S. acidocaldarius	57,000	0.075	Inhibits at concn of >2 mM	Slight inhibition	
S. cerevisiae[a]	44,000	0.13 (ADP); very high (ATP)	Inhibits at concn of >5 mM	No stimulation	Inhibits 100% at 1 mM
N. meningitidis	72,000	1.5	Necessary if no polyphosphate	Necessary if no phosphate	No inhibition up to 5 mM

[a] Because of its high K_m with respect to that of ATP, the enzyme from *S. cerevisiae* was assayed by its ability to produce radiolabelled ATP from [^{32}P]polyphosphate.

active site could then prime synthesis of a stable polyphosphate chain.

With regard to other purified enzymes, the PPK from *P. shermanii* is stimulated by the addition to the reaction buffer of either P_i or polyphosphate (31). Li and Brown (22), when investigating a PPK from *E. coli*, noted its dependence on phosphate, although their data do not allow distinction between a stimulation and a requirement for the anion. Ahn and Kornberg (1) found that at low concentrations of ATP, a pronounced lag in the time course of polyphosphate production by PPK from *E. coli* could be obviated by the addition of a polyphosphate with a chain length of four or greater. (We found a similar stimulation of the *N. meningitidis* enzyme by polyphosphate$_{(64)}$ [data not shown]).

Ahn and Kornberg showed that the polymerization reaction catalyzed by PPK from *E. coli* proceeds through an N-linked phosphoenzyme intermediate. The neisserial PPK also appears to form a phosphoenzyme intermediate, and its base stability, acid lability, and susceptibility to hydroxylamine suggest an N-phosphate linkage (6). The rapidity of hydrolysis at acid pH is not characteristic of phosphohistidine (28), and the relative lability in base suggests that the phosphate is attached to an arginine residue. The sensitivity of the enzyme to heavy metals suggests involvement of cysteine at the active site or in enzyme stability.

Several questions remain to be answered concerning the enzymatic mechanism of neisserial PPK and of PPKs in general. To date it has been shown (for *P. shermanii*) that polyphosphate can act as a primer for polyphosphate synthesis by purified PPK. It is apparent that another means of initiation of synthesis occurs in vitro and may be necessary in vivo, but the mechanism has not been elucidated. The function of inorganic polyphosphate remains a matter of debate. The exclusive use of ATP as phosphoryl donor by the neisserial enzyme and its dependence on ATP and ADP concentrations link its activity with the energy charge of the cell, while its dependence on phosphate supports a role in the maintenance of a constant intracellular level of free phosphate.

Finally, it is noteworthy that gonococci and meningococci excrete such a large proportion of the synthesized polyphosphate to its exterior. Polyphosphate, having a high affinity for divalent and trivalent metal ions, may act as a scavenger for nutrients such as iron or as a chelator, sequestering ions necessary for complement fixation. That the polyphosphate may have some of the properties of a capsule, either alone (in *Neisseria gonorrhoeae*) or in concert with polysaccharide (in *N. meningitidis*), is suggested by its location and chemical character. It is apparent that the polyphosphate is bound by some means to the cell surface, and the negatively charged polymer is reminiscent of the group polysaccharides (3, 15, 16). Indeed, the presence of capsular material on gonococci has been a topic of debate; whereas no capsular polysaccharide has been isolated from the bacteria, a capsule-like region has been reported surrounding cells in India-ink stains (12, 14).

It is likely, then, that much could be learned about the biochemistry and pathogenesis of the neisseriae from the study of mutants unable to synthesize polyphosphate. The production of synthetic oligonucleotides based on the amino acid sequences obtained in this study has led to isolation of clones containing the *ppk* gene. Sequencing, overexpression, and inactivation of the gene will give more information on the biochemistry and physiological significance of polyphosphate production.

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