Schizosaccharomyces pombe Aps1, a Diadenosine 5′,5‴-P^1,P^6-Hexaphosphate Hydrolase That Is a Member of the Nudix (MutT) Family of Hydrolases: Cloning of the Gene and Characterization of the Purified Enzyme[†,‡]

Stephen W. Ingram, Scott A. Stratemann, and Larry D. Barnes*

Department of Biochemistry, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284-7760

Received December 16, 1998; Revised Manuscript Received January 22, 1999

ABSTRACT: The fission yeast *Schizosaccharomyces pombe* contains a gene on chromosome I that encodes a hypothetical nudix hydrolase, YA9E. The gene, designated *aps1*, has been cloned and the protein has been purified from *Escherichia coli* with a yield of 10 mg of Aps1/L of culture. Aps1, composed of 210 amino acids with a calculated molecular mass of 23 724 Da, behaves as a monomer with a sedimentation coefficient of 1.92 S as determined by analytical ultracentrifugation. The effective hydrodynamic radius is about 29 Å as determined by both analytical ultracentrifugation and gel-filtration chromatography. Aps1, whose expression was detected in *S. pombe* by Western blotting, is an enzyme that catalyzes the hydrolysis of dinucleoside oligophosphates, with Ap_6A and Ap_5A being the preferred substrates. The major reaction products are ADP and p_4A from Ap_6A and ADP and ATP from Ap_5A. Values of K_m for Ap_6A and Ap_5A are 19 μM and 22 μM, respectively, and the corresponding values of k_{cat} are 2.0 s^{-1} and 1.7 s^{-1}, respectively. The enzyme has limited activity on Ap_4A and negligible activity on Ap_3A, ADP-ribose, and NADH. Aps1 catalyzes the hydrolysis of mononucleotides with decreasing activity in order from p_5A to AMP. Optimal activity with Ap_6A as substrate is observed at pH 7.6 and in the presence of 0.1−1 mM $MnCl_2$. Aps1 is the first nudix hydrolase isolated from *S. pombe*, and it is the first enzyme identified with this specific substrate specificity and reaction products.

The nudix[1] family, also called the mutT family, consists of over 100 putative proteins. These proteins are widely distributed with members in all three kingdoms (*1*) and are characterized by the sequence motif **G**X_5**E**X_7**REU**X**EE**X**GU**, where U is one of the bulky hydrophobic amino acids I, L, or V (*2*, *3*). Nudix proteins shown to be enzymes have been proposed to degrade compounds that are either deleterious to the cell or are normal metabolites present in excessive concentrations (*3*). Bessman et al. (*3*) proposed the name "nudix" hydrolases based on observations at that time that they catalyze a reaction where the substrate is a nucleoside diphosphate attached to some other moiety X. However, Safrany et al. (*4*) have recently described an enzyme, a diphosphoinositol polyphosphate phosphohydrolase (DIPP), with a nudix motif that catalyzes the hydrolysis of diphosphoinositol polyphosphates.

Several nudix hydrolases are known to degrade Ap_nA (n = 3−6). Nudix hydrolases from human (*5*), pig (*6*), lupin (*7*), and barley (*8*) are classified as Ap_4A hydrolases. A tomato Ap_4A hydrolase, which has been partially sequenced and shows similarity to the human enzyme in the region of the signature motif, is probably a nudix hydrolase (*9*). Ap_3A is a better substrate than Ap_4A for the enzyme from *Escherichia coli*, but this enzyme is almost as active on ADP-ribose and NADH as on Ap_3A (*10*). A nudix hydrolase from budding yeast shows a preference for Ap_6A as substrate (*11*).

Ap_3A and Ap_4A are generally present at basal concentrations of 10 nM−1 μM in prokaryotic and eukaryotic organisms (*12*). There are no reports in the literature on whether Ap_5A or Ap_6A exists in any single-cell organism, but they are both present in higher eukaryotes in platelets (*13*) and adrenal chromaffin cells (*14*), and Ap_5A is present in synaptic vesicles (*15*). Ap_3A and Ap_4A are synthesized in a side reaction catalyzed by some aminoacyl-tRNA synthetases during aminoacylation of cognate tRNA (reviewed in ref *16*). Although Ap_5A and Ap_6A could potentially be synthesized by aminoacyl-tRNA synthetases with p_4A and p_5A as substrates, respectively, such syntheses have not been demonstrated *in vivo*.

Dinucleoside oligophosphates have been implicated in a number of intracellular processes including signaling stress responses and DNA replication, but their function(s) remain to be definitely established. Recent research in this area has focused on a possible role for diadenosine oligophosphates in cell proliferation. This emphasis is due, in part, to three

[†] This research was supported in part by NSF Grant MCB-9604124 to L.D.B. S.A.S. is the recipient of a student research fellowship from the American Association for Dental Research.

[‡] GenBank accession no. AF125215.

* To whom correspondence should be addressed: Telephone (210) 567-3730; FAX (210) 567-6595; e-mail barnesl@uthscsa.edu.

[1] Abbreviations: Ap_nA, diadenosine 5′,5‴-P^1,P^n-oligophosphate, (n = 3−6); p_4A, adenosine tetraphosphate; p_5A, adenosine pentaphosphate; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; nudix, nucleoside diphosphate X; Aps1, $Ap_{six}A$ hydrolase 1; HIT, histidine triad; Fhit, fragile histidine triad; GAFH, GalT, Ap_4A phosphorylase, Fhit, Hint; PCR, polymerase chain reaction; IPTG, isopropyl β-D-thiogalactopyranoside; LB, Luria broth; TB, Terrific broth; dNTP, deoxynucleoside triphosphates; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; DIPP, diphosphoinositol polyphosphate phosphohydrolase.

10.1021/bi982951j CCC: $18.00
Published on Web 03/04/1999

recent findings. One is that Fhit, a putative tumor suppressor deleted in many types of tumors (*17*), acts as an Ap_3A hydrolase *in vitro* (*18*). Second, characterization of two mutant strains of *E. coli* suggests Ap_4A affects the timing of cell division. Both strains, one of which is mutated in an Ap_4A hydrolase and the other in a glycyl-tRNA synthetase involved in Ap_4A synthesis, exhibit the same phenotype and similar increases in intracellular Ap_4A concentration. Nishimura (*19*) suggested the increase in intracellular Ap_4A concentration may have caused the cell division phenotype. Third, the Ap_3A/Ap_4A ratio changes in opposite directions in human cells induced to differentiate compared to those induced to enter apoptosis (*20*). In higher eukaryotes dinucleoside oligophosphates function as extracellular signals, with Ap_nA ($n = 3-6$) involved in vascular tonus and Ap_nA ($n = 5, 6$) acting as neurotransmitters. These proposed intracellular and extracellular functions have been reviewed extensively (*21*−*27*).

Genome sequencing of the fission yeast *Schizosaccharomyces pombe* has identified a gene on chromosome I that encodes a hypothetical nudix hydrolase, YA9E. Here we describe the cloning of the gene, its expression in *E. coli*, and characterization of the purified protein. This protein, designated Aps1 ($Ap_{six}A$ hydrolase 1), catalyzes the hydrolysis of Ap_nA ($n = 4-6$), with Ap_6A and Ap_5A being the preferred substrates. This is the only characterization to date of any nudix hydrolase from fission yeast and the first of an enzyme with this substrate specificity and reaction products.

EXPERIMENTAL PROCEDURES

Materials. Dinucleoside oligophosphates (except Ap_6A, Ap_4G, Ap_4C, and Ap_4U), mononucleotides (except p_5A), ADP-ribose, and other biochemicals were purchased from Sigma. Pyruvate kinase and lactate dehydrogenase also were from Sigma. Hydroxyapatite, high-resolution grade, was from Calbiochem. DEAE-cellulose (microgranular DE-52) was purchased from Whatman. The MonoQ HR5/5 FPLC column, carboxymethyl-Sepharose, Sephadex G75 superfine, and DEAE-Sephacel resins were manufactured by Pharmacia LKB. Ap_6A was synthesized from ATP by reaction with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide in Hepes buffer, pH 6.5, and $MgCl_2$ as described by Ng and Orgel (*28*). Ap_4C and Ap_4U were synthesized as described previously (*29*). Synthesized dinucleotides were purified by anion-exchange chromatography and analyzed for purity by HPLC (*29*). Ap_4G was kindly provided by A. Shatkin. p_5A was synthesized as described by Ortiz et al. (*30*).

Cloning of the aps1 Gene. PCR primers 5′-tgagaattctcatttaaatatcaccgca-3′ and 5′-tagcaagcttgaactgctggaatatatgct-3′ (*Eco*RI and *Hin*dIII sites are underlined) were used to amplify a fragment containing the *aps1* gene from *S. pombe* 972h$^-$ genomic DNA (*31*). The PCR product was digested with *Eco*RI and *Hin*dIII, gel-purified, and cloned into gel-purified, *Eco*RI/*Hin*dIII-digested pUR18 plasmid (*32*). This vector was named pAPS1. The PCR-amplified fragment in this plasmid was sequenced with PE Applied Biosystems BigDye terminator cycle sequencing reagents on an Applied Biosystems 373 automated fluorescent sequencer (Foster City, CA). About 300 base pairs 5′ and 3′ of the open reading frame of *aps1* were cloned with the gene to assist in expression of the gene in *S. pombe* and construction of a vector for gene disruption in future experiments.

Expression of the aps1 Gene. The open reading frame of *aps1* was subcloned into pSGA02 (*33*) by digestion of pAPS1 and pSGA02 with *Nco*I and *Nde*I and ligation of the appropriate fragments. The resulting plasmid was named pSGAPS1. pSGAPS1 was transformed into *E. coli* SG100 (*33*) and a single colony was used to inoculate a 50 mL LB starter culture. This culture was used to inoculate 2 L of TB medium to an initial OD_{600} of 0.05. At an OD_{600} of 1.0, the culture was induced by adding IPTG to 1 mM. The culture was grown 16 h at room temperature (∼25 °C) and the cells were pelleted at 4 °C (all remaining steps were done at 4 °C) by centrifuging at 7000*g* for 30 min. Cells were washed in buffer A (50 mM Hepes, pH 7.5, and 10% glycerol), pelleted as before, and stored at −80 °C until used for purification of Aps1.

Assay of Enzymic Activity. The purification of Aps1, determination of pH optimum, and the effect of divalent cations were monitored by assaying Ap_6A hydrolase activity with a coupled enzyme assay. The assay was based on the hydrolysis of Ap_6A to ADP and p_4A, use of ADP as a substrate for pyruvate kinase, and oxidation of NADH by lactate dehydrogenase as detected by the change in absorbance at 340 nm. Controls were done for each condition to ascertain that the measured effects were on Aps1 and not the coupling enzymes. Samples were assayed at 37 °C in 1 mL of 50 mM Hepes, pH 7.6, 1 mM $MnCl_2$, 0.5 mM phosphoenolpyruvate, 0.25 mM NADH, and 50 μM Ap_6A with 10 units of lactate dehydrogenase and 10 units of pyruvate kinase. The pH optimum was determined by assaying enzymic activity at pH 6.1 (Mes buffer), 6.6, 7.1, 7.6 (Hepes buffer), 8.1, and 8.7 (Bicine buffer). Divalent cations, $MgCl_2$, $MnCl_2$, $CaCl_2$, and $CoCl_2$, were assayed at 0.1, 0.5, 1, 5, and 10 mM concentrations. Because of background oxidation of NADH, activity of the crude supernatant fraction was determined by subtracting the rate of NADH oxidation in the absence of Ap_6A from the rate in the presence of Ap_6A.

HPLC analysis was used to determine the substrate specificity of purified Aps1 and to identify reaction products. Potential substrates, each at 100 μM, were incubated in the absence or presence of 0.6 μg of Aps1 in 300 μL of 50 mM Hepes, pH 7.6, 1 mM $MnCl_2$, and 100 μg/mL BSA at 37 °C for 20 min. The reaction was stopped by freezing on dry ice, and 250 μL of the reaction was injected onto a MonoQ column and analyzed with a Hewlett-Packard HP1090 HPLC. Nucleotides were eluted with a linear gradient of ammonium bicarbonate, pH 8.5, from 0.2 to 1 M (*34*). Nucleotides were detected at 254 nm and were identified by retention time. Peak areas were measured with the HP Chemstation, and the percent hydrolysis of a substrate was calculated by dividing the peak areas of the substrate in the absence and presence of Aps1.

HPLC analysis was also used to determine the time course of Ap_5A and Ap_6A hydrolysis by Aps1. Aps1 (5.2 μg) was incubated with 50 mM Hepes, pH 7.6, 1 mM $MnCl_2$, 100 μg/mL BSA, and 100 μM substrate at 37 °C in a total volume of 1.3 mL. Aliquots (120 μL) were withdrawn at the indicated times and quick-frozen on dry ice, and 95 μL of the withdrawn sample was injected onto a MonoQ column.

Nucleotides were separated and quantified as described above.

HPLC analysis was also used to measure the K_m and V_{max} values of Aps1 for Ap_6A and Ap_5A. Reactions were incubated for either 1.5 or 3 min at 37 °C in 300 μL of 50 mM Hepes, pH 7.6, 1 mM $MnCl_2$, 100 μg/mL BSA, and 1 ng/μL purified enzyme. Substrate concentration was varied from 2.0 μM to 200 μM. Rates calculated at each concentration were corrected for hydrolysis exceeding 10% of the substrate by the method of Lee and Wilson (*35*). Data were analyzed by use of Microsoft Excel software with a direct fit to the Michaelis−Menten equation.

Determination of the N-Terminal Sequence. Sixty picomoles of the hydroxyapatite fraction of Aps1 was subjected to protein sequence determination on an Applied Biosystems 477A sequencing system as described by Matsudaira (*36*).

Determination of Hydrodynamic Properties. The effective hydrodynamic radius (Stokes' radius) of Aps1 was determined by gel-filtration chromatography on Sephadex G75 superfine resin. The column was calibrated with proteins of known effective hydrodynamic radius using the values and procedure reported by Ackers (*37*, *38*). Hydrodynamic properties of Aps1 also were determined by analytical ultracentrifugation (*39*). Sedimentation velocity and sedimentation equilibrium analyses of Aps1 were performed in a Beckman XL-A analytical ultracentrifuge equipped with scanner optics. Centrifugation was at 60 000 rpm for 4.5 h for sedimentation velocity. Centrifugation was at 26 500 rpm for 28 h, at 29 600 rpm for 18 h, and at 32 500 rpm for 12 h for sedimentation equilibrium. The analyses were done at different protein concentrations in 100 mM Hepes, pH 7.5, at 20 °C, and sedimentation of the protein was monitored at 280 nm. The partial specific volume of Aps1 was calculated as 0.732 cm^3/g from the amino acid composition. Data were analyzed by the method of van Holde and Weischet (*40*) with Ultrascan software (B. Demeler, University of Texas Health Science Center at San Antonio). Values of the sedimentation coefficient were determined at the 0.5 fraction in the boundary distribution plot (see Figure 3). The frictional coefficient, the diffusion coefficient, and the effective hydrodynamic radius were calculated from the Svedberg equation, Fick's first law, and Stokes' law, respectively (*41*).

Preparation of Antisera to Aps1. Rabbit polyclonal antibodies to Aps1 were prepared by Cocalico Biologicals, Inc. (Reamstown, PA). Preparation and affinity purification of the antibodies were done as described by Harlow and Lane (*42*).

Miscellaneous. Protein samples were subjected to electrophoresis on 12.5% or 15% (w/v) polyacrylamide gels under denatured protein conditions using the discontinuous buffer system described by Laemmli (*43*) and modified by Studier (*44*). Gels were stained in Coomassie blue and destained in 10% (v/v) acetic acid. Molecular mass markers were from Sigma (Sigma Mark VII-L, SDS 7). For Western blot analysis, proteins in SDS gels were transferred electrophoretically to nitrocellulose (*45*) and probed with affinity-purified, rabbit polyclonal antibodies to Aps1. Goat anti-rabbit antibody coupled to horseradish peroxidase was used to detect immunopositive proteins by chemiluminescence using ECL Plus reagent (AmershamPharmacia).

Protein mass was measured as described by Lowry et al. (*46*) after dilution of samples into 1% (w/v) SDS. Absorbance values of samples were corrected for absorbance due to glycerol and to Hepes. Bovine serum albumin was used as a protein standard. The value of the extinction coefficient of Aps1 was calculated as 26 470 M^{-1} cm^{-1} or 1.12 mL mg^{-1} cm^{-1} based on the amino acid composition as described by Pace et al. (*47*). The concentration of purified Aps1 samples was determined from absorbance at 280 nm and the extinction coefficient.

Standard molecular biological procedures were done as described by Sambrook et al. (*48*). All experiments were done at least twice with appropriate controls, and each reaction was done in duplicate in assays of enzyme activity.

RESULTS

Gene Cloning. The intronless *aps1* gene, located on *S. pombe* chromosome 1 (GenBank accession number AF125215), was cloned as described under Experimental Procedures. The sequence of the cloned fragment was determined and found to agree exactly with the sequence entered in GenBank. The *aps1* gene encodes a protein of 210 amino acids with a deduced molecular mass of 23 724 Da and a theoretical p*I* of 9.49.

Purification of Aps1. The cell pellet, 28 g, was suspended in 84 mL (3 vol of buffer:1 wt of cells) of breaking buffer (buffer A containing 5 μg/mL leupeptin, 5 μg/mL pepstatin A, and 0.5 mM phenylmethanesulfonyl fluoride), sonicated with a Misonix Ultrasonix Processor XL sonicator using a macrotip probe on a setting of 6 for 3 × 30 s with 30 s of cooling between each sonication. The resulting homogenate was centrifuged for 30 min at 43000*g* to yield a crude supernatant fraction. The crude supernatant fraction was dialyzed in buffer A containing 0.5 mM PMSF.

The dialyzed crude supernatant fraction was added to a 2.6 × 39 cm column of carboxymethyl-Sepharose resin equilibrated in buffer A. The column was eluted with a 150−300 mM gradient of NaCl in buffer A. Fractions eluting in the gradient that contained Ap_6A hydrolase activity were pooled, concentrated under N_2 in an Amicon unit with a PM-10 membrane, dialyzed into buffer B (10 mM sodium phosphate, pH 7.5, and 10% glycerol) and applied to a 1.5 cm × 19 cm column of hydroxyapatite resin equilibrated in buffer B. The protein was eluted with a gradient of 75−200 mM sodium phosphate, pH 7.5, and 10% glycerol. Active fractions were pooled, concentrated as above, and dialyzed in 10 mM Hepes, pH 7.5. The enzyme was stored either in 10 mM Hepes, pH 7.5, or in 50 mM Hepes, pH 7.5, and 10% glycerol at −80 °C. An SDS gel illustrating the purification is shown in Figure 1, panel A. In three different preparations of Aps1, the specific activity of the hydroxyapatite fraction was 7−9 times the specific activity of the crude supernatant fraction indicating that Aps1 was 11−14% of the crude supernatant protein in this *E. coli* expression system. The typical yield was 10 mg of purified Aps1/L of *E. coli* culture.

During development of the purification procedure we observed that the majority of the Ap_6A hydrolase activity did not bind to carboxymethyl-Sepharose resin. This was unexpected since the theoretical p*I* for Aps1 is 9.49. The protein that did not bind to the carboxymethyl-Sepharose

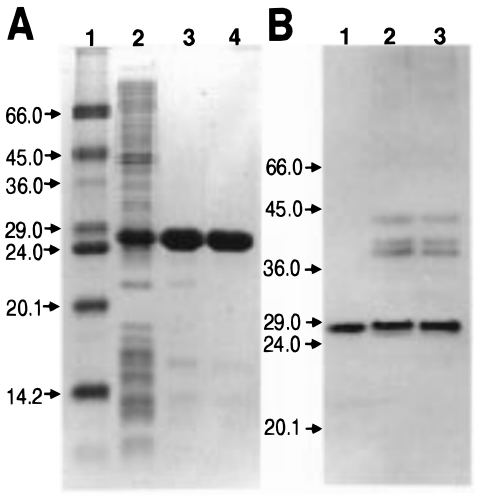

FIGURE 1: Purification of Aps1 from *E. coli* SG100 cells and expression of Aps1 in *S. pombe*. Panel A shows a Coomassie blue-stained SDS−15% polyacrylamide gel that illustrates the purification of Aps1 (apparent molecular mass = 26.8 kDa). Lane 1 = 2.5 μg of molecular mass standards; lane 2 = 20 μg of crude supernatant from *E. coli* SG100 cells expressing Aps1; lane 3 = 10 μg of carboxymethyl-Sepharose fraction; and lane 4 = 10 μg of hydroxyapatite fraction. Panel B is a Western blot from an SDS−12.5% polyacrylamide gel showing expression of Aps1 in *S. pombe*. Lane 1 = 10 ng of purified Aps1; lane 2 = 200 μg of *S. pombe* DEAE-cellulose flowthrough fraction; lane 3 = 5 ng of purified Aps1 (hydroxyapatite fraction) + 100 μg of *S. pombe* DEAE-cellulose flowthrough fraction.

Table 1: Substrate Specificity of *S. pombe* Aps1 Protein[a]

substrate (100 μM)	relative hydrolysis[b] (%)	reaction products
Ap_6A	100.0	p_4A, ATP, ADP, AMP
Ap_5A	88.7	p_4A, ATP, ADP, AMP
Ap_4A	28.5	ATP, ADP, AMP
Ap_3A	<1.0	ADP, AMP
p_5A	84.1	p_4A, ATP, ADP, AMP
p_4A	29.3	ATP, ADP, AMP
ATP	4.4	ADP, AMP
dATP	3.5	dADP, dAMP
ADP	<1.0	ND[c]
AMP	<1.0	ND
GTP	7.7	GDP, GMP
dGTP	5.8	dGDP, dGMP
Ap_4G	35.9	GTP, GDP, GMP, ATP, ADP, AMP
Ap_4U	8.7	ATP, ADP, AMP, UTP, UDP, UMP[d]
Ap_4C	15.8	ATP, ADP, AMP, CTP, CDP, CMP[d]
Gp_4G	45.0	GTP, GDP, GMP
Gp_5G	19.4	p_4G,[e] GTP, GDP, GMP
NADH	<1.0	ND
ADP-ribose	<1.0	ND
γS-ATP	<1.0	ADP

[a] Assay conditions are described in Experimental Procedures. [b] Percent hydrolysis was calculated by dividing the peak areas in the presence and absence of enzyme. Data are representative values of a minimum of two assays with an experimental difference of 10% or less. [c] ND = not detected. [d] UMP, UDP, UTP, CMP, CDP, and CTP were not resolved during the chromatography and are presumed products. [e] No p_4G standard was available. Identification of this product is based on relative retention time.

resin appeared to contain Aps1 on the basis of the banding pattern on an SDS gel. However, this fraction was very impure, had a high absorbance at 260 nm, had an apparent molecular mass greater than 75 kDa based upon gel-filtration chromatography on Sephadex G75, and eluted in multiple peaks of Ap_6A hydrolase activity from DEAE-Sephacel and hydroxyapatite resins. This peak of activity, which was not pursued further due to its impurity and peculiar behavior on chromatographic resins, may represent a portion of the expressed Aps1 that is interacting with nucleic acids, thus preventing its binding to carboxymethyl-Sepharose. Several nudix hydrolases are thought to bind nucleic acid when expressed in *E. coli* (*1*, *10*, *49*).

Determination of the N-Terminal Sequence. Determination of the sequence of the first 10 N-terminal residues, MLENNGSVIL, of Aps1 indicated that the N-terminus was not blocked and no other N-terminal residues were detected. The sequence corresponded exactly to the sequence predicted by the nucleotide sequence of *aps1*, thereby confirming that the purified protein was Aps1.

Expression of Aps1 in S. pombe. To detect expression of Aps1 in wild-type *S. pombe* strain 972h$^-$, a crude supernatant fraction was subjected to chromatography on DEAE-cellulose. The flowthrough fraction from the DEAE-cellulose column was subjected to SDS−polyacrylamide gel electrophoresis and Western blot analysis using affinity-purified antibody to Aps1. The Western blot is shown in Figure 1, panel B.

Substrate Specificity. The relative substrate specificity of Aps1 with a variety of substrates is shown in Table 1. The preferred substrate is Ap_6A, although Ap_5A is almost as good. The relative activity of Aps1 on Ap_nA ($n = 3-6$) decreases with decreasing phosphate chain length such that there is no detectable activity with Ap_3A. The same trend is also observed in adenine mononucleotides (p_nA, $n = 1-5$) with no detectable activity on ADP or AMP. The enzyme is slightly less active on dATP and dGTP than on ATP and GTP, respectively. Ap_4G and Gp_4G are both preferred over Ap_4A, indicating the enzyme prefers dipurine nucleoside tetraphosphates that contain a guanine. However, the preference for guanine was not seen in the dipurine nucleoside pentaphosphates as Gp_5G is cleaved to 21% the extent of Ap_5A. The enzyme cleaves Ap_4A and Ap_4G to a greater extent than both Ap_4U and Ap_4C, indicating a preference for dinucleoside tetraphosphates lacking pyrimidines. The enzyme showed no detectable activity on ADP-ribose or NADH.

Hydrolysis of Ap_6A and Ap_5A and Identification of Reaction Products. Since Ap_6A and Ap_5A were the two major substrates based on relative hydrolysis, the reaction was further characterized with respect to these two substrates. Figure 2 shows a time course for Aps1-catalyzed hydrolysis of Ap_6A and Ap_5A. The major products from Ap_6A hydrolysis (Figure 2A) are p_4A and ADP. ATP is a minor product resulting from a direct, symmetrical cleavage of Ap_6A. This conclusion is based on the following observations. Some ATP generated in the reaction comes from hydrolysis of p_4A. However, the amount of ATP generated by this reaction cannot account for the mass of ATP produced. The amount of ATP arising from p_4A hydrolysis is equal to the difference in the amount of p_4A and ADP present (since ADP is a stable product of Ap_6A hydrolysis). The total amount of ATP present in the reaction is always greater than the amount of ATP arising from p_4A hydrolysis, hence the conclusion that some Ap_6A is degraded directly to ATP. No p_5A was detected from the degradation of Ap_6A, and based on the limits of detection in our HPLC system, the concentration of p_5A was less than 1.5% the initial Ap_6A concentration at

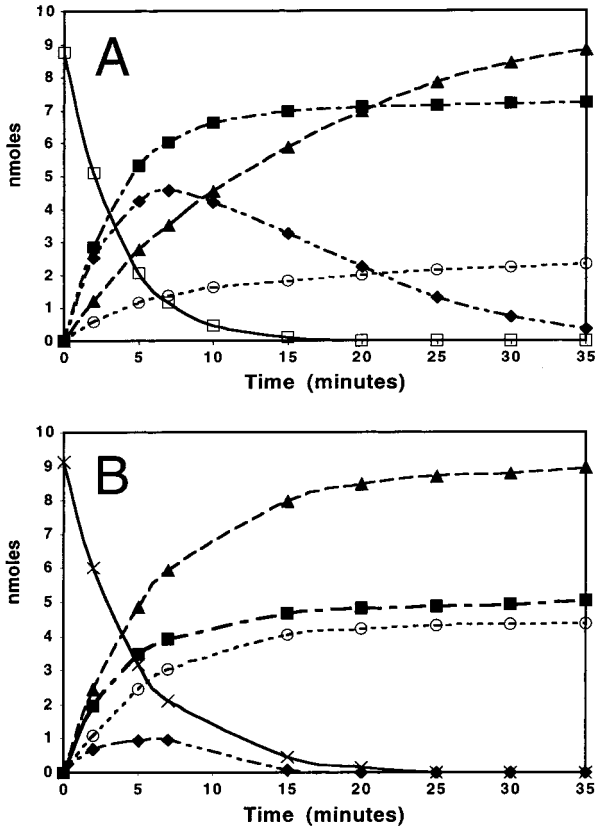

FIGURE 2: Time course of Ap_6A (panel A) and Ap_5A (panel B) degradation by Aps1. (○) AMP; (■) ADP; (▲) ATP; (◆) p_4A; (×) Ap_5A; and (□) Ap_6A. Aps1 (5.2 μg) was incubated with 50 mM Hepes, pH 7.6, 1 mM $MnCl_2$, 100 μg/mL BSA, and 100 μM substrate at 37 °C in a total volume of 1.3 mL. Aliquots (120 μL) were withdrawn at the indicated times and frozen on dry ice, and 95 μL of the withdrawn sample was injected onto a MonoQ column. Nucleotides were separated and quantified as described in Experimental Procedures.

every time point. Since there is undetectable hydrolysis of Ap_6A to AMP and p_5A, most of the AMP present in this reaction must arise from the hydrolysis of the initial reaction products. The major products from hydrolysis of Ap_5A are ATP and ADP (Figure 2B). However, minor products, p_4A and AMP, are also produced from Ap_5A (Figure 2B).

Kinetic Properties. A profile of Aps1 Ap_6A-degrading activity in the presence of 100 μM substrate, 50 mM Hepes, pH 7.6, and at 37 °C as a function of divalent cation concentration for $MgCl_2$, $CoCl_2$, and $CaCl_2$ exhibits a rapid increase in activity from 0 to 0.1 mM with a sharp decrease at 0.5 mM. The optimal divalent cation, $MnCl_2$, displays a broader range of stimulation with maximum activity at 0.1−1.0 mM and a gradual decrease to about $^1/_3$ maximum activity at 5 mM. Relative velocities at 0.1 mM $MnCl_2$, $CoCl_2$, $CaCl_2$, and $MgCl_2$ were 1:0.45:0.45:0.29, respectively. The activity in the absence of divalent cation was 20% the activity with 0.5 mM $MnCl_2$, and the activity in the presence of 5 mM EDTA was 3.8% that with 0.5 mM $MnCl_2$.

A profile of Aps1 Ap_6A-degrading activity in the presence of 0.5 mM $MnCl_2$, 100 μM substrate, and 50 mM buffer at 37 °C as a function of pH shows a gradual increase in activity from about 60% maximum activity at pH 6.1 to maximum activity at pH 7.6 with a sharp drop in activity at pH 8.1 to about 35% maximum activity.

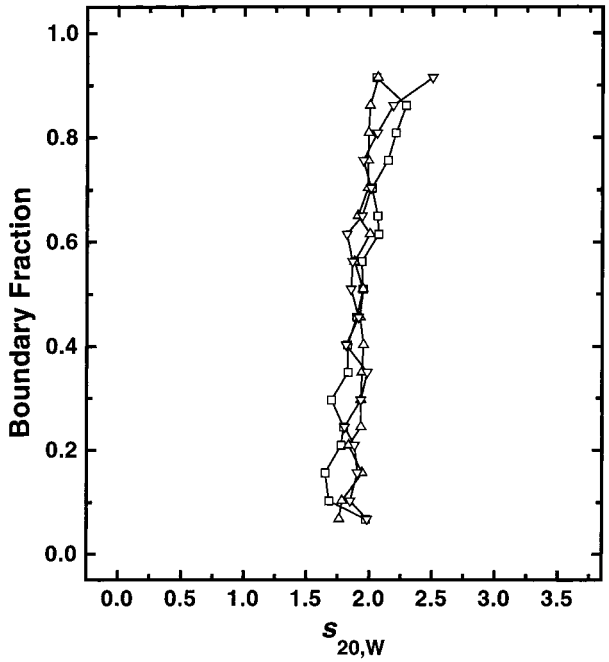

FIGURE 3: Sedimentation velocity analysis of Aps1. Aps1 was subjected to analytical ultracentrifugation at 0.3 (□), 0.4 (△), and 0.6 (▽) mg/mL in 100 mM Hepes, pH 7.5, under the conditions described in Experimental Procedures for sedimentation velocity. Shown is the integral distribution of the sedimenting boundaries as a function of $S_{20,w}$ after analysis of data by the method of van Holde and Weischet (*40*).

Measured values for the K_m for Ap_6A and Ap_5A were 19.1 ± 4.9 μM ($n = 5$) and 22.0 ± 5.2 μM ($n = 3$), respectively. The measured catalytic constant, k_{cat}, for Ap_6A was 2.0 ± 0.39 s^{-1} ($n = 5$) and for Ap_5A the value was 1.7 ± 0.17 s^{-1} ($n = 3$). Calculated specificity constants k_{cat}/K_m were 1.0 × 10^5 M^{-1} s^{-1} for Ap_6A and 7.7 × 10^4 M^{-1} s^{-1} for Ap_5A.

Hydrodynamic Properties. The value of the sedimentation coefficient, $S_{20,w}$, of Aps1 was 1.92 ± 0.06 S ($n = 3$) (Figure 3). The boundary distribution of Aps1 as a function of $S_{20,w}$ (Figure 3) indicates the apparent homogeneity of the preparation. At the protein concentrations analyzed, there was no significant dependence of the sedimentation coefficient on protein concentration. The apparent molecular mass was 21 382 ± 605 Da ($n = 3$) as determined by sedimentation equilibrium (data not shown). Comparison of this value with a molecular mass of 23 724 Da calculated from the amino acid composition indicates that Aps1 behaves as a monomer in solution. Values of the hydrodynamic parameters were as follows: diffusion coefficient, D, 7.38 × 10^{-7} cm^2/s; frictional coefficient, f, 5.48 × 10^{-8} g/s; frictional ratio, f/f_0, 1.48; and effective hydrodynamic radius, 28.2 Å, as calculated from the mean value (1.92 × 10^{-13} s) of the sedimentation coefficient, the calculated molecular mass, and the equations stated in Experimental Procedures. Chromatography of purified Aps1 on a calibrated Sephadex G-75 column yielded a value of 29.4 ± 0.2 Å ($n = 2$) for the effective hydrodynamic radius.

DISCUSSION

The *aps1* gene is the first gene cloned from *S. pombe* that encodes a nudix hydrolase. *aps1* is the second gene cloned from *S. pombe* that encodes a diadenosine oligophosphate hydrolase. The *S. pombe aph1* gene (GenBank accession

number U32615) encodes an Ap_4A asymmetric hydrolase (*50*). Aps1 is expressed in *S. pombe* (Figure 1B). The appearance of secondary bands on this Western blot indicates the possible existence of other nudix hydrolases in *S. pombe*.

S. pombe Aps1 Ap_6A hydrolase appears to be unique among the nudix dinucleoside oligophosphate hydrolases with respect to substrate specificity and reaction products. A recently described nudix hydrolase from the budding yeast *Saccharomyces cerevisiae*, designated YOR163w, is most similar to Aps1 with 43% sequence identity (*11*). YOR163w is enzymatically similar to Aps1; however, there are kinetic differences between the two enzymes. Aps1 hydrolyzes Ap_6A to p_4A and ADP as the major products with no detectable production of p_5A. YOR163w, however, hydrolyzes Ap_6A to p_4A + ADP (76%) and p_5A + AMP (24%) (*11*). Aps1 hydrolyzes Ap_5A to predominantly ATP and ADP, whereas YOR163w hydrolyzes Ap_5A to mainly p_4A and AMP (*11*). Thus, the predominant common product of cleavage of Ap_6A or Ap_5A by Aps1 is ADP, whereas for YOR163w, it is p_4A. Comparison of these results indicates predominant cleavage at a different point in the phosphate chain of dinucleotides for the two enzymes. Also, Aps1 hydrolyzes Ap_4A, whereas YOR163w exhibits no detectable activity on Ap_4A. Values of the specificity constant, k_{cat}/K_m, of Aps1 for Ap_6A and Ap_5A are 15 and 85 times larger than the corresponding values for YOR163w (*11*).

Aps1 is similar to other nudix hydrolases (*3*) in exhibiting some activity on a broad range of substrates as described in Table 1. Although Aps1 will catalyze the hydrolysis of Ap_4A, it is different from the nudix hydrolases described in the introduction that preferentially hydrolyze Ap_4A or Ap_3A. Several eukaryotic dinucleoside oligophosphate hydrolases belong to the HIT protein family rather than the nudix hydrolase family. HIT proteins have a characteristic histidine triad sequence motif, HxHxHx, where x is a hydrophobic residue (*50*, *51*). Human Ap_3A hydrolase (Fhit) (*18*), *S. cerevisiae* Ap_3A hydrolase (*52*), *Drosophila* Ap_3A hydrolase (*53*), and *S. pombe* Ap_4A hydrolase (*50*) belong to the HIT protein family. In addition, *S. cerevisiae* and *Kluyveromyces lactis* contain Ap_4A phosphorylases that have an HxHxQx motif (*54*−*56*). Both the HIT Ap_nA (n = 3, 4) hydrolases and the Ap_4A phosphorylases are protein subfamilies of a larger protein family designated GAFH (*17*). These HIT Ap_nA (n = 3, 4) hydrolases and Ap_4A phosphorylases, as well as other dinucleoside oligophosphate hydrolases (*57*), exhibit relatively low or negligible activity on Ap_6A and Ap_5A. Aps1 differs in several kinetic properties from a p_4A phosphohydrolase in *S. cerevisiae* (*58*) even though both enzymes catalyze the hydrolysis of p_4A and p_5A. Thus, Aps1 Ap_6A hydrolase appears to be unique among all the Ap_nA (n = 3−6) degradative enzymes currently known.

The newly discovered diphosphoinositol polyphosphate phosphohydrolases have not been tested for activity on any typical nudix hydrolase substrates except dATP (*4*), and none of the other nudix hydrolases have been tested on the diphosphoinositol polyphosphates. The human DIPP enzyme has higher sequence identity with Aps1 and YOR163w than with any of the other nudix hydrolases. It will be interesting to learn if the DIPP enzyme will degrade diadenosine oligophosphates in addition to the diphosphoinositol polyphosphates and if Aps1 will degrade diphosphoinositol polyphosphates.

Aps1 behaves as a monomer in solution. Its frictional coefficient is about 48% larger than the frictional coefficient of a spherical protein of the same mass. The value of its effective hydrodynamic radius, about 29 Å as determined by two different methods, in comparison to the corresponding value, 19 Å, for a spherical protein of the same mass also indicates that Aps1 does not behave hydrodynamically as a sphere. It is interesting to note that when the molecular mass for Aps1 is estimated from chromatography on the Sephadex G-75 column, Aps1 appears to be a dimer, having a calculated molecular mass of 47 200 ± 600 Da. Aps1 is an excellent example of a protein whose apparent molecular mass calculated from gel-filtration chromatography differs considerably from its actual molecular mass. Structural analysis by X-ray crystallography or NMR spectroscopy will be required to determine the actual shape of Aps1. The structure of only one nudix family member, *E. coli* 8-oxo-dGTP pyrophosphohydrolase, has been determined (*59*), and NMR spectroscopy indicates this protein has a fairly asymmetric shape, consistent with the shape one would predict for Aps1 on the basis of its Stokes' radius.

The biologic function of the Aps1 Ap_6A hydrolase remains to be determined. Neither Ap_6A nor Ap_5A has been reported in *S. pombe* or any other single-cell organism. *In vivo* synthesis of Ap_6A and Ap_5A by aminoacyl-tRNA synthetases analogous to the synthesis of Ap_4A and Ap_3A would require p_5A and p_4A, respectively, as substrates. Both p_5A and p_4A have been reported in *S. cerevisiae* during sporulation but not during vegetative growth (*60*), and the relationship of these mononucleotides to Ap_6A and Ap_5A is unknown in *S. cerevisiae*. The validity of the proposal that nudix hydrolases function to degrade deleterious substances or excessive levels of normal metabolites (*3*) remains to be substantiated for *S. pombe* Aps1. However, the presence of multiple genes within the same organism that encode different Ap_nA (n = 3−6) degradative enzymes, the distribution of Ap_nA (n = 3−6) degradative enzymes in two different families (nudix and HIT) of proteins, and the presence of such enzymes in evolutionarily diverse organisms suggest that these enzymes and the dinucleoside oligophosphates may have a significant biological function(s). Such functions in *S. pombe* may be determined by studying phenotypic changes arising from disruption or overexpression of the *aps1* and *aph1* genes.

ACKNOWLEDGMENT

We thank Angela K. Robinson for assistance in purification of Aps1 and characterization of the Aps1 antibody, Preston N. Garrison for valuable discussion, and Christin Tse for analysis of analytical ultracentrifugation data. Alexander G. McLennan kindly provided unpublished data and valuable discussion. We thank A. Shatkin for the sample of Ap_4G. The N-terminal sequence of Aps1 was determined by Peggy Rifleman in the Biopolymer Sequencing and Synthesis Facility, Department of Biochemistry, UTHSCSA. Analytical ultracentrifugation was performed by Virgil Schirf in the Center for Analytical Ultracentrifugation, Department of Biochemistry, UTHSCSA. Nucleotide sequencing was performed in the Center for Advanced DNA Technology, Department of Microbiology, UTHSCSA.

REFERENCES

1. Sheikh, S., O'Handley, S. F., Dunn, C. A., and Bessman, M. J. (1998) *J. Biol. Chem. 273*, 20924−20928.
2. Koonin, E. V. (1993) *Nucleic Acids Res. 21*, 4847.
3. Bessman, M. J., Frick, D. N., and O'Handley, S. F. (1996) *J. Biol. Chem. 271*, 25059−25062.
4. Safrany, S. T., Caffrey, J. J., Yang, X., Bembenek, M. E., Moyer, M. B., Burkhart, W. A., and Shears, S. B. (1998) *EMBO J. 17*, 6599−6607.
5. Thorne, N. M. H., Hankin, S., Wilkinson, M. C., Nuñez, C., Barraclough, R., and McLennan, A. G. (1995) *Biochem. J. 311*, 717−721.
6. Hankin, S., Winteroe, A. K., and McLennan, A. G. (1997) *Int. J. Biochem. Cell Biol. 29*, 317−323.
7. Maksel, D., Guranowski, A., Ilgoutz, S. C., Moir, A., Blackburn, M. G., and Gayler, K. R. (1998) *Biochem. J. 329*, 313−319.
8. Churin, J., Hause, B., Feussner, I., Maucher, H. P., Feussner, K., Borner, T., and Wasternack, C. (1998) *FEBS Lett. 431*, 481−485.
9. Feussner, K., Guranowski, A., Kostka, S., and Wasternack, C. (1996) *Z. Naturforsch. 51c*, 477−486.
10. O'Handley, S. F., Frick, D. N., Dunn, C. A., and Bessman, M. J. (1998) *J. Biol. Chem. 273*, 3192−3197.
11. Cartwright, J. L., and McLennan, A. G. (1999) *J. Biol. Chem.* (in press).
12. Garrison, P. N., and Barnes, L. D. (1992) in *Ap_4A and Other Dinucleoside Polyphosphates* (McLennan, A. G., Ed.) pp 29−61, CRC Press, Inc., Boca Raton, FL.
13. Schülter, H., Offers, E., Brüggemann, G., van der Giet, M., Tepel, M., Nordhoff, E., Karas, M., Spieker, C., Witzel, H., and Zidek, W. (1994) *Nature 367*, 186−188.
14. Pintor, J., Rotllán, P., Torres, M., and Miras-Portugal, M. T. (1992) *Anal. Biochem. 200*, 296−300.
15. Pintor, J., Diaz-Rey, M. A., Torres, M., and Miras-Portugal, M. T. (1992) *Neurosci. Lett. 136*, 141−144.
16. Plateau, P., and Blanquet, S. (1992) in *Ap_4A and Other Dinucleoside Polyphosphates*, (McLennan, A. G., Ed.) pp 63−79, CRC Press, Inc., Boca Raton, FL.
17. Huebner, K., Garrison, P. N., Barnes, L. D., and Croce, C. M. (1998) *Annu. Rev. Genet. 32*, 7−31.
18. Barnes, L. D., Garrison, P. N., Siprashvili, Z., Guranowski, A., Robinson, A. K., Ingram, S. W., Croce, C. M., Ohta, M., and Huebner, K. (1996) *Biochemistry 35*, 11529−11535.
19. Nishimura, A. (1998) *Trends Biochem. Sci. 23*, 157−159.
20. Vartanian, A., Prudovsky, I., Suzuki, H., Pra, I. D., and Kisselev, L. (1997) *FEBS Lett. 415*, 160−162.
21. Ogilvie, A. (1992) in *Ap_4A and Other Dinucleoside Polyphosphates*, (McLennan, A. G., Ed.) pp 229−273, CRC Press, Inc., Boca Raton, FL.
22. Kitzler, J. W., Farr, S. B., and Ames, B. N. (1992) in *Ap_4A and Other Dinucleoside Polyphosphates*, (McLennan, A. G., Ed.) pp 135−149, CRC Press, Inc., Boca Raton, FL.
23. Remy, P. (1992) in *Ap_4A and Other Dinucleoside Polyphosphates*, (McLennan, A. G., Ed.) pp 151−204, CRC Press, Inc., Boca Raton, FL.
24. Plateau, P., and Blanquet, S. (1994) *Adv. Microbial Physiol. 36*, 82−104.
25. Baxi, M. D., and Vishwanatha, J. K. (1995) *J. Pharmacol. Toxicol. Methods 33*, 121−128.
26. Miras-Portugal, M. T., Gualix, J., and Pintor, J. (1998) *FEBS Lett. 430*, 78−82.
27. Kisselev, L. L., Justesen, J., Wolfson, A. D., and Frolova, L. Y. (1998) *FEBS Lett. 427*, 157−163.
28. Ng, K. E., and Orgel, L. E. (1987) *Nucleic Acids Res. 15*, 3573−3580.
29. Robinson, A. K., de la Peña, C. E., and Barnes, L. D. (1993) *Biochim. Biophys. Acta 1161*, 139−148.
30. Ortiz, B., Sillero, A., and Gunther-Sillero, M. A. (1993) *Eur. J. Biochem. 212*, 263−270.
31. Lee, F.-J. S. (1992) *BioTechniques 12*, 677.
32. Barbet, N., Muriel, W. J., and Carr, A. M. (1992) *Gene 114*, 59−66.
33. Ghosh, S., and Lowenstein, J. M. (1997) *Gene 176*, 249−255.
34. Guranowski, A., Brown, P., Ashton, P. A., and Blackburn, G. M. (1994) *Biochemistry 33*, 235−240.
35. Lee, H.-J., and Wilson, I. B. (1971) *Biochim. Biophys. Acta 242*, 519−522.
36. Matsudaira, P. (1987) *J. Biol. Chem. 262*, 10035−10038.
37. Ackers, G. K. (1967) *J. Biol. Chem. 242*, 3237−3238.
38. Ackers, G. K. (1970) *Adv. Protein Chem. 24*, 343−446.
39. Hansen, J. C., Lebowitz, H., and Demeler, B. (1994) *Biochemistry 33*, 13155−13163.
40. van Holde, K. E., and Weischet, W. O. (1978) *Biopolymers 17*, 1387−1403.
41. van Holde, K. E. (1971) *Physical Biochemistry*, Prentice-Hall, Inc., Englewood Cliffs, NJ.
42. Harlow, E., and Lane D. (1988) *Antibodies: A laboratory manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
43. Laemmli, U. K. (1970) *Nature 227*, 680−685.
44. Studier, F. W. (1973) *J. Mol. Biol. 79*, 237−248.
45. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350−4354.
46. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem. 193*, 265−275.
47. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) *Protein Sci. 4*, 2411−2423.
48. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning*, 2nd ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
49. Frick, D. N., and Bessman, M. J. (1995) *J. Biol. Chem. 270*, 1529−1534.
50. Huang, Y., Garrison, P. N., and Barnes, L. D. (1995) *Biochem. J. 312*, 925−932.
51. Séraphin, B. (1992) *DNA Sequence 3*, 177−179.
52. Chen, J., Brevet, A., Blanquet, S., and Plateau, P. (1998) *J. Bacteriol. 180*, 2345−2349.
53. Pekarsky, Y., Campiglio, M., Siprashvili, Z., Druck, T., Sedkov, Y., Tillib, S., Draganescu, A., Wermuth, P., Rothman, J. H., Huebner, K., Buchberg, A. M., Mazo, A., Brenner, C., and Croce, C. M. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 8744−8749.
54. Plateau, P., Fromant, M., Schmitter, J.-M., and Blanquet, S. (1990) *J. Bacteriol. 172*, 6892−6899.
55. Kaushal, V., Avila, D. M., Hardies, S. C., and Barnes, L. D. (1990) *Gene 95*, 79−84.
56. Mulder, W., Sholten, I. H. J. M., van Roon, H., and Grivell, L. A. (1994) *Biochim. Biophys. Acta 1219*, 719−723.
57. Guranowski, A. and Sillero, A. (1992) in *Ap_4A and Other Dinucleoside Polyphosphates* (McLennan, A. G., Ed.) pp 81−133, CRC Press, Inc., Boca Raton, FL.
58. Guranowski, A., Starzynska, E., Barnes, L. D., Robinson, A. K., and Liu, S. (1998) *Biochim. Biophys. Acta 1380*, 232−238.
59. Abeygunawardana, C., Weber, D. J., Gittis, A. G., Frick, D. N., Lin, J., Miller, A.-F., Bessman, M. J., and Mildvan, A. S. (1995) *Biochemistry 34*, 14997−15005.
60. Jakubowski, H. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2378−2382.

BI982951J