Purification and Partial Characterization of a DNA 3′-Phosphatase from *Schizosaccharomyces pombe*†

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ABSTRACT: Cells that depend on oxygen for survival constantly produce reactive oxygen species that attack DNA to produce a variety of lesions, including single-strand breaks with 3′-blocking groups such as 3′-phosphate and 3′-phosphoglycolate. These 3′-blocking ends prevent the activity of DNA polymerase and are generally removed by DNA repair proteins with 3′-diesterase activity. We report here the purification and partial characterization of a 45 kDa protein from *Schizosaccharomyces pombe* total extract based on the ability of this protein to process bleomycin- or H_2O_2-damaged DNA in vitro to allow DNA repair synthesis by DNA polymerase I. Further analysis revealed that the 45 kDa protein removes 3′-phosphate ends created by the *Escherichia coli* fpg AP lyase following the incision of AP site but is unable to process the 3′-α,β unsaturated aldehyde generated by *E. coli* endonuclease III. The protein cannot cleave DNA bearing AP sites, suggesting that it is not an AP endonuclease or AP lyase. We conclude that the 45 kDa protein purified from *S. pombe* is a DNA 3′-phosphatase.

Reduction of molecular oxygen during aerobic metabolism generates reactive oxygen species (ROS), which can damage a variety of macromolecules, including DNA (*1*−*4*). ROS-induced damage to DNA encompasses a large number of lesions that include modified bases, apurinic/apyrimidinic (AP) sites, and single-strand breaks containing blocked 3′-ends such as 3′-phosphate (*5*, *6*). Both AP sites and blocked 3′-termini can be efficiently repaired by the dual function enzyme called AP endonuclease/3′-diesterase (*7*, *8*). This enzyme incises AP sites and removes 3′-blocking groups to produce 3′-hydroxyl ends for DNA repair synthesis by DNA polymerases (*7*, *8*).

Two distinct AP endonuclease/3′-diesterases, belonging to the endonuclease IV (endo IV) and the exonuclease III (exo III) family of DNA repair enzymes, respectively, exist in nature (*8*, *9*). To date, genes encoding homologues of endo IV have been found in *E. coli*, the budding yeast *Saccharomyces cerevisiae*, the fission yeast *S. pombe*, and the nematode *Caenorhabditis elegans*, but not in plants or mammalian systems (*10*, *11*). In contrast to the endo IV homologues, the exo III-related members are more highly conserved throughout evolution (*9*). *Escherichia coli* lacking both endo IV and exo III are hypersensitive to a variety of DNA damaging agents, including methyl methane sulfonate (MMS) and H_2O_2 (*12*). While MMS generates AP sites, H_2O_2 produces predominantly DNA single-strand breaks terminated with 3′-phosphate (*5*, *6*). In *S. cerevisiae*, deficiency in the *E. coli* counterparts of endo IV and exo III, respectively, Apn1 and Apn2, also causes severe sensitivity to MMS and H_2O_2 (*5*, *13*, *14*). These drug sensitivities are the direct result of the yeast *apn1 apn2* double mutant inability to repair damaged DNA (*13*, *14*).

To date, no rigorous studies have been performed to establish if endo IV- or exo III-related proteins play a similar role in DNA repair in *S. pombe* or *C. elegans*. In the case of *S. pombe*, earlier attempts failed to reveal endo IV or the exo III-related AP endonuclease activities in total extracts when using a double-stranded oligonucleotide containing a single AP site as a substrate (*10*). This finding was rather surprising, as AP endonuclease activity is ubiquitous and can be readily detected in total extracts derived from a number of organisms, including *E. coli*, *S. cerevisiae*, and humans (*7*, *8*). The inability to detect AP endonuclease activity in *S. pombe* extracts could be attributed to enzyme instability, limited protein expression, and/or substrate type (*10*). Another possibility is that the *S. pombe*-related AP endonucleases are inactive, but if so, how would *S. pombe* repair AP sites? One feasible explanation would involve cleavage of the AP site by AP lyases, leaving a blocked 3′-termini which then needs further processing (*15*). We therefore set out to determine if 3′-diesterase activities are present in the total extracts of *S. pombe* by using as the substrate chromosomal DNA bearing single-stranded breaks terminated with 3′-blocking ends (*12*). On the basis of this assay, we purified a 45 kDa protein that possesses the ability to remove 3′-phosphate from damaged DNA, but not if the DNA contains the 3′-blocking group α,β unsaturated aldehyde. We discuss the possibility that this DNA 3′-phosphatase is likely the homologue of human polynucleotide kinase, which was recently reported to be involved in DNA repair and found to be associated with proteins of the base-excision repair pathway (*16*, *17*).

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[1] Abbreviations: AP sites, apurinic/apyrimidinic sites; endo IV, endonuclease IV; exo III, exonuclease III; MMS, methyl methane sulfonate.

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MATERIALS AND METHODS

Strains and Media. The *S. pombe* strains used in this study were the wild-type SP223 (*h*$^-$ *ura4-294 leu1-31 ade6-216*) and SP358 (*h*$^-$ *leu1-32 ura4-D18 ade6-210*), generously provided by Dr. Howard Lieberman, Columbia University, U.S.A., and Paul Young, Queens University, Canada, respectively. The strains were grown in a liquid medium made up of yeast extract and supplemented with 20 μg/mL adenine (*10*). The *E. coli* strain BW528 (Δ(*xth-pnc*), *nfo1::kan*)), kindly provided by B. Weiss (Emory, Atlanta, GA), was grown in Luria broth as previously reported (*12*).

Purified Enzyme and Assays. E. coli endonuclease IV (stock of this laboratory) was purified according to Yang et al., 1999. Purified fpg and endonuclease III were generously provided by Dr. Jacques Laval (Institute Gustave, Paris, France) and Dr. Melamede (University of Vermont, VT). The activity of each enzyme was determined by monitoring the ability to cleave AP sites introduced into plasmid DNA. This AP-site plasmid was prepared by incubating 2 μg of the superhelical form of purified pBluescript S/K plasmid in the presence of 10 mM citric acid (pH 5.0) and 100 mM NaCl, followed by heat treatment at 70 °C for 15 min (*18*). This treatment introduces at least one AP site per plasmid molecule. The fpg enzyme cleaves the AP site to produce 3′-phosphate (*19*), while endonuclease III cleaves the AP site to produce the α,β-unsaturated aldehyde product, 4R-4-hydroxy-*trans*-2-pentenal, which is efficiently removed by endonuclease IV (*20*). All assays were performed in a buffer containing 50 mM HEPES−KOH (pH 7.6), 50 mM KCl, 1 mM EDTA, 100 μg of BSA, and 10% glycerol.

3′-Diesterase Assay. The 3′-diesterase activity was assayed as previously described by Masson and Ramotar (1997) using chromosomal DNA isolated from either bleomycin- or H_2O_2-treated *E. coli* strain BW528, which lacks the DNA-repair enzyme exonuclease III and endonuclease IV (*12*, *15*). A typical assay contained 200 ng of damaged DNA and either purified protein or total extract (ranging from 0.5 to 5 μg of cellular protein) in a total of 15 μL of reaction mixture (50 mM HEPES−KOH, pH 7.4, 50 mM KCl, 50 μg/mL BSA, and 10 mM $MgCl_2$). The assay mixture was incubated for 20 min at 37 °C, followed by heat inactivation at 70 °C for 5 min. The sample was chilled on ice, and 225 μL of chilled DNA polymerase I reaction mix (25 mM HEPES−KOH, pH 7.4, 25 mM KCl, 50 μg of BSA, 10 mM $MgCl_2$, 100 mM each of dATP, dCTP, dGTP, and [*methyl*-^{3}H]-dTTP (91 Ci mmol$^-$, Amersham) at a specific activity of 1260 cpm pmol^{-1}; DNA polymerase I (0.5 U; Pharmacia)) was added. The tube was incubated at 37 °C, and at various times (0−12 min), aliqouts of 40 μL were withdrawn into prechilled tubes on ice. The extent of [*methyl*-^{3}H]dTMP incorporation into the damaged DNA was determined by precipitation with trichloroacetic acid (*12*). Linear incorporation of [*methyl*-^{3}H]dTMP was used to calculate the amount of 3′-diesterase activity in the extract that acted on the damaged DNA. Untreated DNA was used as a control. One unit of 3′-diesterase is defined as the amount (pmol) of [*methyl*-^{3}H]dTMP incorporated into the damaged DNA by DNA polymerase I per min by 1 μg of protein extract.

Purification of the S. pombe DNA 3′-Phosphatase. A 50 mL inoculum of *S. pombe* strain SP223 was grown in YEA medium to stationary phase at 30 °C by shaking in an orbital shaker at 250 rpm. The cells were subcultured into 5 L of YEA medium and allowed to grow until an OD600 of ∼1.2. Cells were centrifuged at 500 g for 5 min, and the total pellet (20 mL) was washed once with 20 mM potassium phosphate buffer pH 7.0 and stored frozen at −80 °C overnight. The cell pellet was thawed on ice for 2 h, followed by addition of an equal volume (20 mL) of ice cold extraction buffer (50 mM Tris−HCl, pH 7.5, 30 mM KCl, 10% gylcerol, 1 μg/mL each of aprotinin, leupeptin, chymostatin, and TPCK, and 1 mM each of PMSF and benzamidine). The crude extract was prepared by using a bead beater (Biospec Products, Bartlesville, OK) as previously described for preparing *S. cerevisiae* extract with 0.5 mm Zirconium beads (*21*). The cells were lysed by spinning for 20 s followed by cooling for 2 min, and this was repeated 12 times. The crude extract was sedimented at 20 000 g for 20 min to yield the total extract with a protein concentration of ∼25 mg/mL. The total extract was adjusted to 0.5 M NaCl, and polymin P was added to 0.1%. The sample was stirred for 20 min and sedimented at 20 000 g/20 min. The supernatant was collected, and chilled ammonium sulfate was added (0.5 g/mL) gradually with stirring. After solubilization of the ammonium sulfate, the sample was stirred for an additional 20 min, followed by centrifugation at 20 000 g/20 min. The pellet was resuspended in 4 mL of yeast extraction buffer and dialyzed against 100 volumes of buffer A (50 mM Tris−HCl, pH 7.5, 25 mM NaCl, 10% gylcerol, 0.05 μg/mL each of aprotinin, leupeptin, chymostatin, and TPCK, and 0.05 mM each of PMSF and benzamidine) for 2 × 3.0 h. The fraction was heated 60 °C/10 min and then recentrifuged at 20 000 g/20 min. This step resulted in 40% loss in activity, but with nearly 100-fold purification. The supernatant was next applied onto a 20 mL × 2 cm DEAE-Sepharose column (Pharmacia) equilibrated with buffer A at a flow rate of 1 mL/3 min. After application of the sample, the column was washed with buffer A and eluted with a 300 mL linear NaCl (50 to 800 mM) gradient (fraction size was 5 mL). Two peaks of activity were detected, the major activity (DEAE-I) eluting at ∼300 mM and the minor activity (DEAE-II) eluting at ∼600 mM. The major activity was pooled from five fractions (∼25 mL) and reprecipitated with ammonium sulfate as above. The dialyzed DEAE-I fraction was applied onto a 5 mL single-stranded DNA agarose column and eluted with a 75 mL NaCl (0.1 to 0.8 M) linear gradient in buffer A (fraction size 2 mL). The activity eluted at ∼0.3 M NaCl, which was pooled (∼10 mL) and dialyzed against buffer A. The dialyzed sample was applied onto a MonoQ (5 mL) column, and the flow through fractions containing the activity was collected and directly applied onto a 5 mL MonoS column equilibrated with buffer A. The column was washed and developed with a 60 mL NaCl (0.05 to 1.0 M) linear gradient in buffer A (fraction size 2 mL). The activity was recovered at ∼0.5 M NaCl, and 1 mL samples of various fractions were concentrated by centricon (10-NMWL, Millipore, Bedford, MA) to ∼100 μL and analyzed by 12% SDS gel, followed by staining with silver nitrate.

Renaturation and Excision of Proteins from SDS Gels. Following 12% SDS polyacrylamide gel electrophoresis, the gel was washed for 3 × 45 min with 200 mL of buffer A containing 1% Trition X-100 by gently shaking on an orbital shaker (*12*). A duplicate lane was removed and stained by silver to locate the corresponding polypeptide bands. The

polypeptides were excised with a razor blade, crushed into small pieces, and placed into 100 μL of buffer A and left at 4 °C overnight. Enzymatic assay was performed with the suspension.

5′-Kinase Assay. The oligonucleotide-based assay for 5′-phosphorylation activity was performed as previously reported, except with slight modification (*22*). In brief, enzyme preparation (1 μL) was added to a 10 μL reaction mixture containing 100 mM Mes, pH 5.5, 10 μM oligo (34 mer), 0.4 μCi [γ-^{32}P]ATP (3000 Ci/mmol, Amersham), 20 μM ATP, 1 μg nuclease-free BSA, 10 mM $MgCl_2$, and 2 mM DTT. The reaction was incubated for 20 min at 37 °C, followed by the addition of EDTA to 25 mM, DNA-sequencing stop solution, and electrophoresis on 20% acrylamide/7M urea gel. The sequence of the 34-mer oligo used in the 5′-DNA kinase was 5′-OH−CTGGAAGTTTGTGGG-CTGGAACGTGGCAGGGCTC.

Immunoblotting. The purified protein (50 ng) was loaded on a 12% SDS−PAGE gel, transferred onto Nitrocellulose membrane, and probed with either preimmune sera or anti-Pnk1 polyclonal antibodies (dilution of 1:1000) raised against purified recombinant *S. pombe* Pnk1 (*23*). The blot was developed using Renaissance Western Blot chemiluminescence reagent Plus (NEN Life Science Products, U.S.A.). Protein molecular weight standards (BenchMark, GIBCO) were used as a size marker.

RESULTS

The *E. coli* strain BW528 lacks the DNA repair enzymes endo IV and exo III and therefore accumulates unrepaired lesions, such as single-strand breaks with blocked 3′-ends, when challenged with DNA damaging agents (including the antitumor drug bleomycin) (*5*, *12*). As such, we isolated bleomycin-damaged chromosomal DNA from this mutant and used it as a substrate to monitor for the presence of 3′-repair diesterase activity in total extracts derived from *S. pombe*. In this assay, removal of the preexisting blocked 3′-ends in the damaged DNA can be readily assessed by following [*methyl*-^{3}H]dTMP incorporation by DNA polymerase I (*12*). Preincubation of the bleomycin damaged DNA with *S. pombe* total extract (5 μg) permitted the time-dependent incorporation of [*methyl*-^{3}H]dTMP by DNA polymerase I (Figure 1A). When the damaged DNA was preincubated with 5-fold less of *S. pombe*, total extract incorporation of [*methyl*-^{3}H]dTMP was reduced (Figure 1A). Only background incorporation of [*methyl*-^{3}H]dTMP was observed if the DNA was pretreated with heated extract (Figure 1A). The background level of [*methyl*-^{3}H]dTMP incorporation detected in the substrate DNA, in the absence of protein extract, was previously reported and likely due to endogenous processing of some lesions by other weak 3′-diesterases present in the *E. coli* mutant (*12*). As expected, preincubation of the bleomycin damaged chromosomal DNA with purified *E. coli* endo IV (10 ng) showed a substantial level of [*methyl*-^{3}H]dTMP incorporation by DNA polymerase I (Figure 1A). The data suggest that total extracts derived from *S. pombe* contain an activity capable of processing bleomycin-induced DNA lesions.

To exclude the possibility that the [*methyl*-^{3}H]dTMP incorporation was not due to endonuclease incision of the damaged DNA by the *S. pombe* extract, thus leading to the formation of 3′-hydroxyl group, we examined the extent of incorporation into undamaged DNA (Figure 1B). No significant [*methyl*-^{3}H]dTMP incorporation was detected into the undamaged DNA preincubated with the *S. pombe* extract (Figure 1B). Thus, these data are consistent with the *S. pombe* extract containing an activity that acts on damaged DNA to produce 3′-hydroxyl ends for DNA repair synthesis.

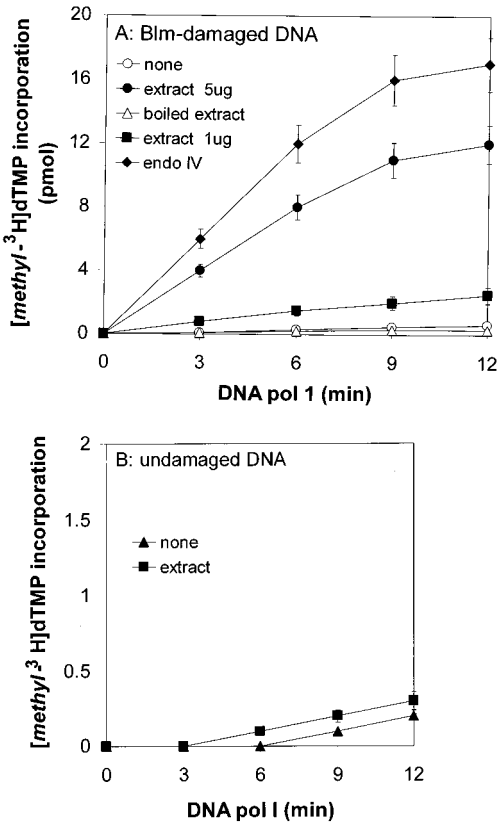

FIGURE 1: Effect of *S. pombe* total extract on the level of [*methyl*-^{3}H]dTMP incorporation into bleomycin-damaged and undamaged DNA by DNA polymerase I. Panel A: bleomycin-damaged chromosomal DNA was isolated from an *E. coli* mutant lacking DNA repair activities and pretreated without or with *S. pombe* extract (5 μg and 1 μg) for 20 min before monitoring [*methyl*-^{3}H]-dTMP incorporation. In the case of heat treatment, the extract was heated to 95 °C for 5 min before pretreating the DNA. In the control experiments, the DNA was pretreated with 10 ng of purified endonuclease (endo IV). Panel B: undamaged chromosomal DNA was isolated from the *E. coli* mutant with no prior exposure to bleomycin. Pretreatment of the undamaged DNA with *S. pombe* extract (5 μg) was the same as that in panel A, before monitoring [*methyl*-^{3}H]dTMP incorporation.

Since bleomycin introduces a variety of lesions into DNA, such as 3′-phosphate, 3′-phosphoglycolate, AP sites, and double-strand breaks (*24*), we examined if the *S. pombe* extract can also repair damaged DNA isolated from strain BW528 treated with H_2O_2. H_2O_2 is known to produce predominantly single-strand breaks terminated with 3′-phosphate (*6*). The *S. pombe* extract was also proficient in stimulating [*methyl*-^{3}H]dTMP incorporation into the H_2O_2-damaged DNA (Figure 2). Interestingly, the extent of incorporation was consistently much greater with H_2O_2-damaged DNA, as compared with that from the bleomycin-damaged DNA. It is noteworthy that bleomycin- or H_2O_2-damaged DNA was isolated from strain BW528 treated with equitoxic doses of the DNA damaging agents (*12*, *16*). Since the same amount of damaged DNA was used in the assays,

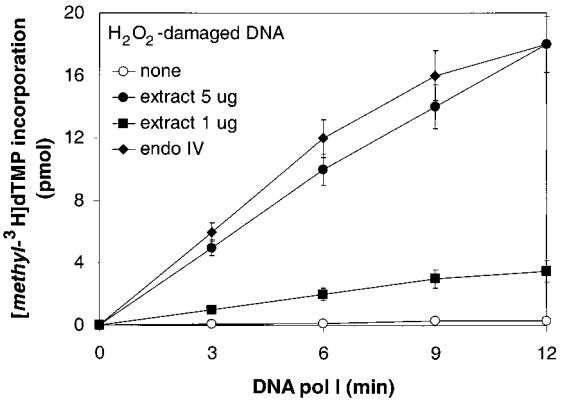

FIGURE 2: Effect of *S. pombe* total extract on the level of [*methyl*-^{3}H]dTMP incorporation into H_2O_2-damaged DNA by DNA polymerase I. H_2O_2-damaged chromosomal DNA was isolated from the *E. coli* mutant and subjected to pretreatment with *S. pombe* extract or purified endonuclease IV, as in Figure 1.

it would appear that H_2O_2-induced lesions are processed more efficiently than the bleomycin-induced lesions by the *S. pombe* extract.

Purification of the Protein that Activates H_2O_2-Damaged DNA for Repair Synthesis. To purify the activity responsible for stimulating [*methyl*-^{3}H]dTMP incorporation into H_2O_2-damaged DNA, we subjected the *S. pombe* total extract to chromatography on several columns. The total extract was subjected to ammonium sulfate precipitation and heat denaturation at 60 °C/10 min, and the clarified supernatant was subjected to chromatography on DEAE−Sepharose (Materials and Methods). This chromatography step produced two peaks of 3′-diesterase activity, a major activity (DEAE-I) that eluted at 0.3 M NaCl and a minor activity (DEAE-II) that eluted at a higher NaCl concentration (0.6 M). No further analysis was performed with this minor activity. The DEAE-I fraction was subjected to chromatography on single-stranded DNA agarose, and the activity eluted at 0.3 M NaCl. The eluted fraction from the DNA agarose column did not bind to MonoQ but bound to MonoS and eluted as a symmetrical peak with a NaCl concentration of 0.5 M (Figure 3A). Various fractions from the MonoS column were concentrated and analyzed by silver-stained gels (Figure 3B). A major 45 kDa polypeptide was found in the fraction (Figure 3B, lane 5) corresponding to the peak with the highest activity (Figure 3A, fraction number 23). Since this fraction also contained some minor polypeptides (not very visible after photography), we examined if the 45 kDa polypeptide had associated activity. The 45 kDa polypeptide was excised from a 12% SDS polyacrylamide gel after several washings with 1% Triton X-100 to aid renaturation of the protein, and it was monitored for 3′-diesterase activity. The excised polypeptide exhibited only a weak ability to process the H_2O_2-damaged DNA, requiring nearly 20 times more protein to show the same level of [*methyl*-^{3}H]dTMP incorporation as the undenatured protein (Figure 3C). In control experiments, two minor polypeptides excised from the gel showed no [*methyl*-^{3}H]dTMP incorporation into the damaged DNA (data not shown). The present findings strongly suggest that the 45 kDa protein has associated 3′-diesterase activity that can process H_2O_2-damaged DNA.

Purified Protein Activity is Stimulated by Mg^{2+}. Throughout the purification scheme, $MgCl_2$ was included in the assay

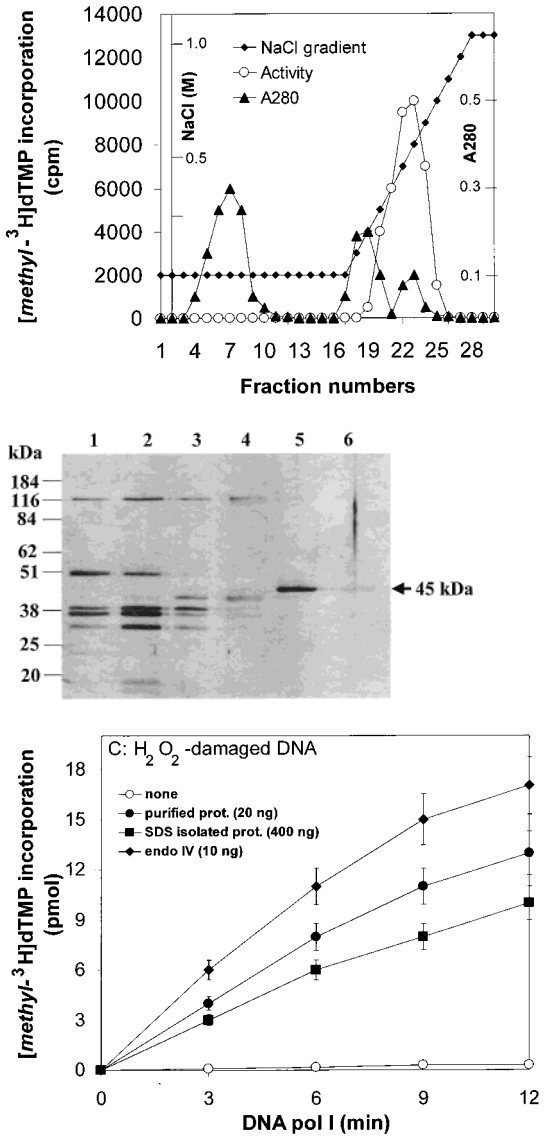

FIGURE 3: Analyses of 3′-diesterase activity and purity in various fractions derived from a mono-S column. Panel A: eluate from single-stranded DNA agarose was subjected to chromatography on a 5 mL monoS column and developed with a linear 50 mM to 1M NaCl gradient. Fraction size of 2 mL was collected and assessed for [*methyl*-^{3}H]dTMP incorporation by DNA polymerase I into H_2O_2-damaged DNA. The protein concentration in each fraction was monitored by a spectrophotometer (absorbance at 280 nm). The NaCl gradient was monitored with a conductivity meter. Panel B shows a silver-stained gel of concentrated fractions from the monoS column. Fractions 8, 10, 17, 19, 23, and 24 were concentrated by centricon and loaded on a 12% SDS-polyacrylamide gel, and following migration, the gel was stained with silver nitrate. The arrow indicates the position of the purified 45 kDa protein, and the position of the prestained bench mark protein ladder (GIBCO) is indicated (kDa). Panel C: the 45 kDa protein was excised from a SDS gel after washing with 1% Triton-X100 and monitored for the ability to allow [*methyl*-^{3}H]dTMP incorporation into H_2O_2-damaged chromosomal DNA by DNA polymerase as in Figures 1 and 2.

buffer. To determine if this divalent ion is absolutely required for the protein enzymatic activity, buffers were prepared with and without $MgCl_2$. Since DNA polymerase I activity also requires Mg^{2+}, we carried out the reaction in two steps. In the first step, the damaged DNA was pretreated with the purified 45 kDa protein in the absence or presence of $MgCl_2$ for 20 min, heated inactivated (72 °C/3 min), and followed

Table 1: Effect of Metal Ions on the Enzymatic Activity of the Purified 45 kDa Protein[a]

metal ions	concentrations (mM)	relative activity %
none	0	<5
$MgCl_2$	0.1	25
$MgCl_2$	1	60
$MgCl_2$	3	80
$MgCl_2$	5	100
$MgCl_2$	10	100
$MnCl_2$	5	60
$CuCl_2$	2	<5
$CoCl_2$	2	<5
Fe_2SO4	2	<5
$CaCl_2$	2	<5
NaCl	50	100
NaCl	250	<5

[a] Activity measurement was performed as described (Material and Methods).

by the second step, i.e., the addition of DNA polymerase I and its reaction buffer. In the absence of Mg^{2+}, the purified 45 kDa protein showed an extremely weak ability to process the damaged DNA accounting for less than 5% of the activity detected in the presence of Mg^{2+} (Table 1). Addition of increasing concentrations of Mg^{2+} sharply stimulated the protein activity, reaching maximal activity with 5 mM $MgCl_2$ (Table 1). The purified 45 kDa protein activity was also stimulated by $MnCl_2$, but this divalent ion was not as effective as Mg^{2+}. Other divalent metal ions such as Cu^{2+}, Co^{2+}, Fe^{2+}, or Ca^{2+} did not stimulate the protein's ability to process damaged DNA (Table 1). Addition of NaCl in excess of 250 mM into the buffer during preincubation of the 45 kDa protein with the damaged DNA completely abolished the ability of the protein to process DNA lesions (Table 1). The above data suggests that the 45 kDa protein is dependent upon Mg^{2+} for enzymatic activity.

Purified Enzyme Acts on 3′-Phosphate. To assess the type of lesions that can be processed by the purified enzyme, we used a plasmid DNA as substrate and monitored [*methyl*-^{3}H]dTMP incorporation (Figure 4). Treatment of the plasmid under acidic condition generates AP sites (*25*), which can be cleaved by purified *E. coli* endonuclease IV to produce the 3′-hydroxyl end to permit DNA polymerase I-stimulated [*methyl*-^{3}H]dTMP incorporation (Figure 4A,B). No incorporation was observed if the acid-treated plasmid was preincubated with the 45 kDa purified protein (Figure 4A). This finding indicates that the enzyme lacks the ability to incise AP sites.

We next examined if the 45 kDa protein can remove the 3′-blocking group left by AP lyases (*19*, *20*, *26*). In this case, we used two bacterial purified enzymes, endonuclease III and fpg, which can process preexisting AP sites to produce two distinct 3′-ends (*19*, *20*, *26*). While endonuclease III cleaves AP sites by a β-elimination reaction to produce a 3′- α,β-unsaturated aldehyde, the fpg enzyme cleaves the AP site by a β-elimination reaction followed by δ-elimination to produce a 3′-phosphate (*19*, *20*, *26*). Both the 3′- α,β unsaturated aldehyde and the 3′-phosphate can block DNA polymerase I action (*7*). Preincubation of the AP site plasmid DNA with purified endonuclease III, followed by incubation with the purified 45 kDa protein, did not allow [*methyl*-^{3}H]-dTMP incorporation by DNA polymerase I into the plasmid DNA (Figure 4A,B). However, if the AP site plasmid was incubated with purified fpg, following up by incubation with

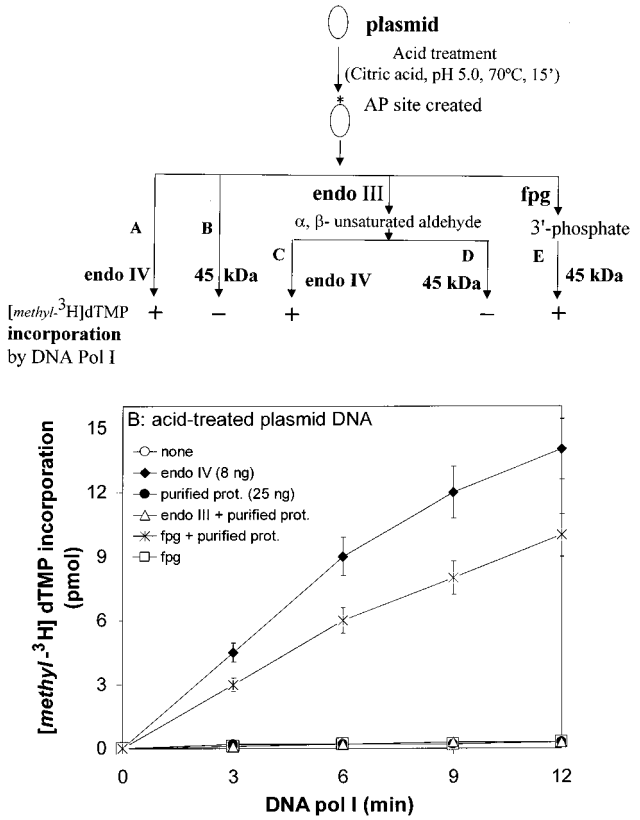

FIGURE 4: Ability of the purified 45 kDa protein to stimulate [*methyl*-^{3}H]dTMP incorporation into AP site plasmid DNA incised by the AP lyase fpg or endonuclease III. In this experiment, the pBluescript plasmid was acid-treated under conditions that allow formation of AP sites in the DNA (see Materials and Methods). The AP-site plasmid DNA was used as the substrate. Panel A depicts the various enzyme pretreatment of the AP site-plasmid DNA before monitoring for [*methyl*-^{3}H]dTMP incorporation. The plus and minus signs indicate [*methyl*-^{3}H]dTMP incorporation and no [*methyl*-^{3}H]dTMP incorporation, respectively, into the plasmid DNA. Panel B shows the actual level of [*methyl*-^{3}H]dTMP incorporation into the AP site-plasmid DNA following pretreatment with the 45 kDa purified protein alone, after pretreatment with endonuclease III followed by the 45 kDa protein, or after pretreatment with fpg followed by the 45 kDa protein.

the purified 45 kDa protein permitted a substantial level of [*methyl*-^{3}H]dTMP incorporation by DNA polymerase I into the plasmid DNA (Figure 4A,B). Like endonuclease III, fpg alone did not stimulate [*methyl*-^{3}H]dTMP incorporation by DNA polymerase I into the plasmid DNA (Figure 4B). These data establish that the 45 kDa protein is efficient at removing 3′-phosphate at the ends of damaged DNA, but not more complex 3′-ends such as 3′-α,β-unsaturated aldehyde.

Purified Enzyme Possesses 5′-Kinase Activity and Cross-Reacts with Anti-Pnk1. While this work was in progress, a recent study reported the characterization of *S. pombe* Pnk1, a counterpart of the human polynucleotide kinase hPNKP, which possesses both DNA 3′-phosphatase and 5′-kinase activity (*23*). We therefore tested if the purified protein also possessed 5′-kinase activity. To do this, we used a single-stranded 34-mer oligonucleotide with a 5′-hydroxyl end and monitored the ability of the purified protein to label the 5-end with [γ^{32}P]ATP. As shown in Figure 5A, the purified protein was capable of labeling the 34-mer oligonucleotide, and the reaction was dependent on protein concentration (lanes 2 and 3). Heat inactivation of the protein prevented labeling of the 34-mer oligonucleotide (lane 1). As a positive control, we

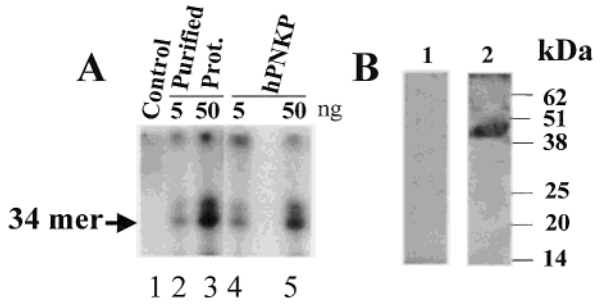

FIGURE 5: DNA 5′-kinase activity and anti-Pnk1 immunoreactivity of the purified protein. Panel A: the 5′-kinase activity of the purified protein. The protein was incubated with a 5′-OH 34-mer single-stranded oligonucleotide in the presence of [γ^{32}P]ATP and with either 5 and 50 ng of purified protein (lanes 2 and 3, respectively) or 5 and 50 ng of purified human PNKP (lanes 4 and 5, respectively). In the control (lane 1), the purified protein was heat inactivated at 95 °C/5 min. Panel B: Western blot analysis of the purified protein. Lanes 1 and 2 contained 50 ng of the purified protein and probed with either the preimmune serum (1:1000 dilution) or the anti-Pnk1 polyclonal antibodies (1:1000 dilution) raised against purified recombinant *S. pombe* Pnk1.

used the purified human PNKP, which we previously showed to have DNA 5′-kinase activity (lanes 4 and 5) (*16*). These data indicate that the purified protein also possesses DNA 5′-kinase activity and may be related to the recently reported recombinant *S. pombe* Pnk1 (*23*). As such, we tested if the purified protein can immuno-react with antibodies raised against a recombinant form of *S. pombe* Pnk1 (*23*). Western blot analysis revealed that the purified protein was immunoreactive with the anti-Pnk1 polyclonal antibodies (Figure 5B, lane 2), but not toward the preimmune serum (lane 1). Our data suggest that the purified protein is likely the *S. pombe* Pnk1.

DISCUSSION

We purified a 45 kDa protein from total extracts derived from the fission yeast *S. pombe*. This protein has the ability to remove 3′-phosphate from single-strand breaks of damaged DNA, but not if the single-strand breaks are terminated with a more complex 3′-end, such as 3′-α,*β* unsaturated aldehyde (*19*, *20*). The biochemical activity of the purified 45 kDa protein contrasted those reported for endonuclease IV and exonuclease III and strongly suggests that this *S. pombe* enzyme is not a member of the Endo IV or Exo III family (*8*, *9*). A unique difference between the *S. pombe* enzyme and endo IV or exo III is that the former enzyme lacks an AP endonuclease activity (*7*−*9*).

We believe that the purified 45 kDa enzyme isolated herein is the same as the *S. pombe* Pnk1 (*23*) for the following reasons. A plasmid designed to express Pnk1 produced a recombinant protein that is also 45 kDa in size. Moreover, the recombinant Pnk1 protein displays DNA 3′-phosphatase activity (*23*). The gene (accession number CAB11157) encoding Pnk1 was initially identified by database searches for homologues of the human polynucleotide kinase (hPNKP), an enzyme that possesses DNA 3′-phosphatase and 5′-kinase activities (*16*). Like hPNKP, the *S. pombe* Pnk1 also possesses 5′-kinase activity (*23*). In light of this recent report, we tested our purified 45 kDa protein for 5′-kinase activity and immunoreactivity toward *S. pombe* Pnk1. We found that the purified protein has 5′-kinase activity and it cross-reacts with the anti-Pnk1 polyclonal antibodies. Therefore, our purified protein is likely the *S. pombe* Pnk1 (*23*).

We have previously shown that hPNKP can act in vivo to directly repair H_2O_2-induced DNA lesions in an *E. coli* mutant (strain BW528) deficient in the ability to process DNA strand breaks with 3′-blocking groups (*16*). Consistent with this finding, a recent study showed that hPNKP can execute its function in the base excision DNA repair pathway (*17*). A related and independent study also documented that ZmDP2, the plant homologue of hPNKP, complements the DNA repair deficiency of strain BW528 when challenged with H_2O_2 (*27*). More direct biochemical and genetic studies clearly establish that Tpp1, the *S. cerevisiae* counterpart of hPNKP, is required to repair DNA strand breaks with blocked 3′-phosphate (*28*, *29*). These studies also revealed that yeast mutants bearing deletion of only the *TPP1* gene exhibit no sensitivity to DNA damaging agents such as H_2O_2 and bleomycin (*29*). However, when TPP1 was deleted in mutants devoid of the 3′-diesterase activities of Apn1 and Apn2 (the corresponding counterparts of *E. coli* endo IV and exo III, respectively), the resulting *tpp1 apn1 apn2* triple mutant displays exquisite sensitivity to several DNA-damaging agents, including H_2O_2, bleomycin, and camptothecin, as compare to the *apn1 apn2* double mutant (*29*). This finding underscores the fact that Tpp1 can compete with Apn1 and Apn2 to repair the same DNA lesions and that multiple enzymes exist in eukaryotic cells to repair DNA strand breaks with 3′-blocking groups (*28*, *29*). The accumulated findings clearly implicate enzymes with DNA 3′-phosphatase to have a role in DNA repair (*16*, *28*, *29*). In fact, the recent report of Meijer et al. also shows that *S. pombe pnk1* null mutant is sensitive to agents that produce single-strand breaks with blocked 3′-termini, suggesting that Pnk1 plays a role in the repair of damaged DNA (*23*).

It is noteworthy that the *S. pombe* findings contrast the observation in *S. cerevisiae* where *tpp1* single null mutants alone showed no sensitivity to DNA damaging agents as compared to the parent (*29*). It is possible that *S. pombe* Pnk1 may have evolved to play a more active role in DNA repair. This is likely since no DNA repair defective phenotypes have been found associated with deletion of the *S. pombe apn1* gene encoding a homologue of *S. cerevisiae* Apn1 (*10*). The availability of the *pnk1* null mutant makes it possible to now clearly address the lingering question of whether Apn1, as well as Apn2, contributes to the repair of oxidative DNA lesions in *S. pombe*.

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