The Drosophila Ribosomal Protein S3 Contains a DNA Deoxyribophosphodiesterase (dRpase) Activity

(Received for publication, January 29, 1997, and in revised form, May 19, 1997)

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The Drosophila ribosomal protein S3 has been previously demonstrated to cleave DNA containing 8-oxoguanine residues and has also been found to contain an associated apurinic/apyrimidinic (AP) lyase activity that cleaves phosphodiester bonds via a β, δ-elimination reaction. The activity of this protein on DNA substrates containing incised AP sites was examined. A glutathione S-transferase fusion protein of S3 was found to efficiently remove sugar-phosphate residues from DNA substrates containing 5′-incised AP sites as well as from DNA substrates containing 3′-incised AP sites. Removal of 2-deoxyribose-5-phosphate as 4-hydroxy-2-pentenal-5-phosphate from a substrate containing 5′-incised AP sites occurred via a β-elimination reaction, as indicated by reaction of the released sugar-phosphate products with sodium thioglycolate. The reaction for the removal of 4-hydroxy-2-pentenal-5-phosphate from the substrate containing 3′-incised AP sites was dependent on the presence of the Mg2+ cation. These findings suggest that the S3 ribosomal protein may function in several steps of the DNA base excision repair pathway in eukaryotes and may represent an important DNA repair function for the repair of oxidative and ionizing radiation-induced DNA damage.

Cellular DNA is constantly exposed to both exogenous and endogenous agents that can produce oxidative base damage (1). To remove this damage and restore the integrity of the DNA, specific DNA repair pathways have evolved (1, 2). The major pathway for the removal of oxidative base damage is the DNA base excision repair pathway, found in prokaryotes and eukaryotes (3). In this pathway, oxidized DNA bases are removed by specific DNA glycosylases, leaving apurinic/apyrimidinic (AP) sites in the DNA (1, 4). These AP sites are then acted on by specific DNA glycosylases, leaving apurinic/apyrimidinic base excision repair pathway, found in prokaryotes and eukaryotes (4). Removal of the sugar-phosphate products allows the subsequent restoration of nucleotides by DNA polymerases and the re-formation of intact phosphodiester bonds by DNA ligases.

Several of the enzymes involved in DNA base excision repair have been found to be multifunctional proteins. For example, the Escherichia coli Fpg protein, the product of the mutM gene, is a DNA glycosylase that removes 8-oxoguanine and formamidopyrimidines from DNA and also has an associated AP lyase activity as well as an activity that excises 2-deoxyribose-5-phosphate at 5′-incised AP sites via a β-elimination reaction (7, 10). Recently, the Drosophila ribosomal protein S3 has been shown to have an enzymatic activity that cleaves DNA containing 8-oxoguanine residues and an associated AP lyase activity that cleaves phosphodiester bonds via a β, δ-elimination reaction (11). This protein was able to rescue the H2O2 sensitivity of an E. coli mutM strain and completely abolish the mutator phenotype of mutM caused by 8-oxoguanine-mediated G→T transversions (11).

The similarity in DNA repair activities of the E. coli Fpg protein and the Drosophila S3 protein suggested the possibility that the S3 protein may contain an integral dRpase activity. We investigated the dRpase activities of a glutathione S-transferase fusion protein of S3 (GST-S3) on DNA substrates containing either 5′- or 3′-incised AP sites. The GST-S3 fusion protein, and not GST alone, was able to remove sugar-phosphate products from both types of substrates. An unsaturated sugar-phosphate product is released from the substrate containing 5′-incised AP sites by β-elimination. The removal of trans-4-hydroxy-2-pentenal-5-phosphate from the substrate containing 3′-incised AP sites is via a hydrolytic reaction requiring Mg2+. These S3-associated dRpase activities suggest that this ribosomal protein may be active in several steps of the DNA base excision repair pathway.

EXPERIMENTAL PROCEDURES

Enzymes and Reagents—Bacteriophage M13mp18 single-stranded DNA and a M13 24-mer sequencing primer (1–47) were purchased from U. S. Biochemical Corp. The large fragment (Klenow) of DNA polymerase I was purchased from Boehringer Mannheim. E. coli uracil-DNA glycosylase was purchased from Epicentre Technologies, and endonuclease IV was prepared as described previously (12). Endonuclease III was a generous gift of Dr. Richard Cunningham (State University of New York, Albany, NY). Sodium thioglycolate and spermidine were obtained from Sigma.

Overexpression and Purification of GST-S3—Drosophila ribosomal gene S3 was overexpressed as a GST fusion construct in a bacterial strain (mutM::mini-tet) (13) that is defective for one of the major EDTA-resistant activities in E. coli for the removal of DNA 5′-terminal deoxi-
yribose phosphates (7). The conditions for overexpression and purification of GST and GST-S3 were identical to those reported previously (11).

**M13 Double-Stranded DNA Containing Incised AP Sites—**A M13 DNA substrate containing [32P]-labeled AP sites was prepared essentially as described previously (7, 14). [α-32P]dUTP was prepared by deamination of [α-32P]dCTP (DuPont; 2000 Ci/mmol) as described previously (15) and incorporated into DNA in a reaction (100 μl) containing 1 μg of single-stranded M13mp18 DNA, 30 ng of 24-mer primer, 2.5 nmoles of DATP, dCTP, and dGTP, 0.25 nmoles of dUTP, 0.15 nmoles of dUTP, 15 μCi of [α-32P]dUTP, 50 mM Tris-HCl, pH 7.8, 10 mM MgCl2, 1 mM dithiothreitol, and 3 units of large fragment (Klenow) of DNA polymerase I. The reaction mixture was then incubated with an additional 2.5 nmoles of DATP, dUTP, dGTP, and dCTP and 3 units of large fragment (Klenow) of DNA polymerase I, and incubation was continued for 1 h. The reaction was stopped by the addition of 4 μl of 0.5 M Na2EDTA, followed by incubation at 65°C for 5 min. The DNA was purified from unincorporated nucleotides on a 1-ml Sephadex G-50 column, precipitated with ethanol, and then lyophilized. The [32P]dUMP-containing M13 DNA was treated with uracil-DNA glycosylase and subsequently treated with either endonuclease IV to create a substrate containing 5′-incised AP sites or with endonuclease III to create a substrate containing 3′-incised AP sites as described previously (5).

**Enzyme Assays—**DNA dRpase activity was assayed in a reaction mixture containing either the release of 2-deoxyribose-5-phosphate from a M13mp18 DNA substrate containing 5′-incised AP sites or 4-hydroxy-2-pentenal-5-phosphate from a M13mp18 DNA substrate containing 3′-incised AP sites. A typical reaction (50 μl) contained 220 fmol of M13mp18 DNA substrate containing incised AP sites, 100 ng of GST-S3 enzyme, 30 mM Hepes-KOH, pH 7.4, 50 mM KCl, 1 mM dithiothreitol, and 3 units of large fragment (Klenow) of DNA polymerase I. After incubation at 37°C for 2 h, the reaction was supplemented with an additional 2.5 μl of DATP, dGTP, and dCTP and 3 units of large fragment (Klenow) of DNA polymerase I, and incubation was continued for 1 h. The reaction was stopped by the addition of 4 μl of 0.5 M Na2EDTA, followed by incubation at 65°C for 5 min. The DNA was purified from unincorporated nucleotides on a 1-ml Sephadex G-50 column, precipitated with ethanol, and then lyophilized. The [32P]dUMP-containing M13 DNA was treated with uracil-DNA glycosylase and subsequently treated with either endonuclease IV to create a substrate containing 5′-incised AP sites or with endonuclease III to create a substrate containing 3′-incised AP sites as described previously (5).

**Sugar-Phosphate Product Analysis—**M13mp18 double-stranded DNA substrate containing 5′-incised AP sites (220 fmol; 50,000 cpm) was incubated with either 100 ng of GST-S3 enzyme or 2 mM spermidine in the absence of enzyme in a 50-μl reaction containing 100 mM sodium thioglycolate, 30 mM Hepes-KOH, pH 7.4, 50 mM KCl, 1 mM dithiothreitol, and 3 units of large fragment (Klenow) of DNA polymerase I. After incubation at 37°C for 2 h, the reaction was supplemented with an additional 2.5 μl of DATP, dGTP, and dCTP and 3 units of large fragment (Klenow) of DNA polymerase I, and incubation was continued for 1 h. The reaction was stopped by the addition of 4 μl of 0.5 M Na2EDTA, followed by incubation at 65°C for 5 min. The DNA was purified from unincorporated nucleotides on a 1-ml Sephadex G-50 column, precipitated with ethanol, and then lyophilized. The [32P]dUMP-containing M13 DNA was treated with uracil-DNA glycosylase and subsequently treated with either endonuclease IV to create a substrate containing 5′-incised AP sites or with endonuclease III to create a substrate containing 3′-incised AP sites as described previously (5).

**RESULTS**

**GST-S3 Removes a 5′-Terminal Deoxyribose Phosphate from a DNA Substrate Containing Incised AP Sites—**To examine dRpase activities associated with the *Drosophila* ribosomal protein S3, the protein was purified as a GST fusion product. The purity of this enzyme was >95%. To eliminate any possible contamination with the *E. coli* Fpg protein, the GST-S3 was isolated from an *E. coli* mutM strain (11, 13).

To determine whether GST-S3 was capable of removing a 5′-terminal deoxyribose-phosphate product, the enzyme was incubated with a M13mp18 DNA substrate containing 5′-incised AP sites produced by treatment of a [32P]dUMP-containing substrate with uracil-DNA glycosylase and *E. coli* endonuclease IV. As shown in Fig. 1A, a dRpase activity acting on a 5′-incised AP site produced 2-deoxyribose-5-phosphate by a hydrolytic mechanism or trans-4-hydroxy-2-pentenal-5-phosphate by β-elimination reaction. As seen in the time course in Fig. 2, efficient release of sugar-phosphates was catalyzed by GST-S3. This reaction was 2-fold higher than that observed with the addition of 5 mM MgCl2. Reaction of 0.2 pmol of substrate with 100 ng of purified GST did not result in the release of sugar-phosphates (data not shown). The apparent *Km* for the release of sugar-phosphate was determined by Lineweaver-Burk analysis, as shown in Fig. 3, and found to have a value of 0.07 μM; the value of *Vmax* is 0.015 pmol/min. The turnover number (*kcat*) was calculated to be 0.13/min using an enzyme preparation that was 36 h old. It has been previously demonstrated that GST-S3 loses activity rapidly upon storage (15-fold decrease within 72 h after purification) (11).

**Mechanism of Release of the Sugar-Phosphate Product from a DNA Substrate Containing 5′-Incised AP Sites—**The release of sugar-phosphate from a DNA substrate containing 5′-incised AP sites by the action of GST-S3 was not dependent on the addition of MgCl2 and suggested that this reaction may proceed via a β-elimination reaction. The *E. coli* Fpg protein removes 5′-terminal deoxyribose-phosphate groups by such a mechanism (7). To determine whether the release of sugar-phosphate occurred via β-elimination, GST-S3 was incubated with the M13 DNA substrate containing 5′-incised AP sites in the presence of sodium thioglycolate. It has been shown previously that this reagent will react with an unsaturated sugar-phosphate...
product, resulting in the formation of products with altered mobilities on anion exchange chromatography (7, 8). When the M13 DNA substrate containing 5'-incised AP sites was incubated with sodium thioglycolate and the \( \beta \)-elimination catalyst spermidine (2 mM), the unsaturated sugar-phosphate product released by spermidine treatment formed products that reacted with sodium thioglycolate, as seen in Fig. 4A. When the DNA substrate was incubated with sodium thioglycolate and GST-S3, the released products co-migrated with the products produced by spermidine treatment, as seen in Fig. 4B. These results strongly suggest that the mechanism of release of the 5’-terminal deoxyribose-phosphate by GST-S3 is via a \( \beta \)-elimination reaction.

**GST-S3 Removes Sugar-Phosphate Products from a DNA Substrate Containing 3’-incised AP Sites**—To determine whether GST-S3 was capable of removing a 3’-terminal sugar-phosphate product (trans-4-hydroxy-2-pentenal-5-phosphate) as shown in Fig. 1B, the enzyme was incubated with a M13mp18 DNA substrate containing 3’-incised AP sites produced by treatment of a [\( ^{33} \)P]dUMP-containing substrate with uracil-DNA glycosylase and E. coli endonuclease III, a known \( \beta \)-elimination catalyst (AP lyase). As seen in the time course in Fig. 5, the product trans-4-hydroxy-2-pentenal-5-phosphate was released from the substrate by GST-S3. Product identity was verified by anion exchange HPLC as shown in Fig. 6. This reaction was absolutely dependent on the addition of 5 mM MgCl\(_2\). Reaction of 0.2 pmol of substrate with 100 ng of purified GST alone did not result in the release of sugar-phosphate product (data not shown). The apparent \( K_m \) for the release of trans-4-hydroxy-2-pentenal-5-phosphate was determined by Lineweaver-Burk analysis, as shown in Fig. 7, and found to have a value of 0.075 \( \mu \)M; the value of \( V_{max} \) is 0.012 pmol/min. The turnover number (\( k_{cat} \)) was calculated to be 0.08/min using an enzyme preparation that was 36 h old.
Figure 5. Time course for the release of 4-hydroxy-2-pentenal-5-phosphate from a M13mp18 double-stranded DNA substrate containing 3'-incised AP sites. Reactions incorporated M13mp18 DNA containing 4 nM [33P]-labeled 2-deoxyribose-5-phosphate end groups. The release of 4-hydroxy-2-pentenal-5-phosphate was determined by precipitation with trichloroacetic acid in the presence of Norit charcoal. Reactions contained no enzyme (○) or 100 ng of GST-S3 (●).

Figure 6. Enzymatic release of trans-4-hydroxy-2-pentenal-5-phosphate from a M13mp18 double-stranded DNA substrate containing 3'-incised AP sites. The reaction products were resolved on an MPLC AX HPLC column. The sugar-phosphate product trans-4-hydroxy-2-pentenal-5-phosphate (4h2p5p) released by GST-S3 elutes between fractions 6 and 7 (3.5 min) under these conditions; inorganic phosphate (Pi) elutes between fractions 10 and 11 (5.5 min) (9, 16).

DISCUSSION

The results presented here demonstrate that Drosophila ribosomal protein S3 is able to remove precised AP sites that exist either 3' or 5' to the initial incision event. Similar to the Fpg protein (7) but not to other dRpase-like activities in E. coli (5, 6), S3 is shown to remove incised 5' AP sites by a β-elimination reaction. Interestingly, Fpg is the only other purified activity that also shares with S3 the ability of S3 to act on 8-oxoguanine residues in DNA that is subsequently followed in vitro by the formation of a β, δ-elimination product. Unlike Fpg, however, is the ability of S3 to also remove 3'-incised AP sites through a Mg2+-dependent hydrolytic mechanism. Whereas Fpg lacks this activity, 2 the removal of 3' AP sites nevertheless has been observed for another dRpase that exists in E. coli (5) that has a K_m value (0.06 µM) for the release of this product very close to that determined for S3 (0.075 µM). The in vitro ability of S3 to liberate an AP site existing at a 3' terminus is also shared by 5'-acting AP endonucleases present in both prokaryotes and eukaryotes (17, 18). That S3 is able to carry this reaction out in vivo as well as in vitro comes from studies examining the rescue of the methyl methane sulfonate sensitivity of a bacterial strain lacking the major 5'-acting AP endonucleases (RPC501), where Drosophila S3 was able to significantly protect RPC501 from cell killing (11). As an explanation of this result, it was noted that once S3 catalyzed a β-elimination reaction at an AP site formed by methyl methane sulfonate damage to DNA, the ribosomal protein dissociated from the DNA and on a second encounter cleaved 5' to the abasic site by δ-elimination, thus leaving a 3' phosphoryl group that was hypothesized to be less deleterious to the cell than a 3'-modified deoxyribose produced by δ-elimination. However, based on the results presented here, it is conceivable that in the presence of endogenous Mg2+, on the second encounter S3 completely released the existing 4-hydroxy-2-pentenal-5-phosphate, thus leaving a one-nucleotide gap and an efficient 3' terminus for DNA polymerase I to fill.

In eukaryotic organisms, recent results indicate that rat DNA polymerase β can catalyze the release of 5'-incised AP sites (9). It is unknown whether the same is true for the Drosophila β polymerase, but if so, it would suggest that S3 would only act in a back-up role for the removal of 5' deoxyribose-phosphate, or it is possible that S3 has a more important role in the proliferating cell nuclear antigen-dependent abasic site repair using DNA polymerase δ (19).

We have been examining another Drosophila ribosomal protein, PO, that also contains DNase activity, but unlike S3, possesses only modest AP endonuclease activity. However, similar to S3 is the ability of PO to significantly reverse the sensitivity of E. coli RPC501 to MMS (20). Thus, both ribosomal proteins are capable of acting as DNA repair proteins in vivo although it remains to be seen whether, in fact, this is the case in Drosophila. It should be noted that both PO (20) and S3 (21) are associated with the nuclear matrix in Drosophila cells, therefore suggesting roles that are distinct from protein translation.

2 M. Sandigursky and W. A. Franklin, unpublished observations.
In conclusion, it seems that *Drosophila* S3 has a broader substrate specificity toward the removal of an AP site than any eukaryotic protein thus far characterized. Additionally, *Drosophila* S3 is capable of acting on DNA containing formamidopyrimidine (FapyGua) and 8-oxoguanine residues. Taken together, the combination of these activities could result in a one-nucleotide gap by processing oxidatively damaged bases by a concerted N-glycosylase/AP lyase activity, followed by S3 dissociating from the substrate, and, in the presence of Mg$^{2+}$, subsequently removing the remaining 4-hydroxy-2-pentenal-5-phosphate. The combination of these activities would suggest a more efficient means for preparing the DNA for gap filling, as opposed to recruiting a 5' acting AP endonuclease to remove the blocked 3' termini in preparation for polymerase $\beta$.

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