Human Thymine DNA Glycosylase Binds to Apurinic Sites in DNA but Is Displaced by Human Apurinic Endonuclease 1*

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In vitro, following the removal of thymine from a G-T mismatch, thymine DNA glycosylase binds tightly to the apurinic site it has formed. It can also bind to an apurinic site opposite S^6-methylthioguananine (SMeG) or opposite any of the remaining natural DNA bases. It will therefore bind to apurinic sites formed by spontaneous depurination, chemical attack, or other glycosylases. In the absence of magnesium, the dissociation rate of the glycosylase from such complexes is so slow (k_{off} 1.8 \times 10^{-7} \text{s}^{-1}, i.e. half-life between 5 and 10 h) that each molecule of glycosylase removes essentially only one molecule of thymine. In the presence of magnesium, the dissociation rates of the complexes with GAP and SMeG:AP are increased more than 20-fold, allowing each thymine DNA glycosylase to remove more than one uracil or thymine from C-U and SMeG-T mismatches in DNA. In contrast, magnesium does not increase the dissociation of thymine DNA glycosylase from GAP sites sufficiently to allow it to remove more than one thymine from G-T mismatches. The bound thymine DNA glycosylase prevents human apurinic endonuclease 1 (HAP1) from cutting the apurinic site, so unless the glycosylase was displaced, the repair of apurinic sites would be very slow. However, HAP1 significantly increases the rate of dissociation of thymine DNA glycosylase from apurinic sites, presumably through direct interaction with the bound glycosylase. This effect is concentration-dependent and at the probable normal concentration of HAP1 in cells the dissociation would be fast. This interaction couples the first step in base excision repair, the glycosylase, to the second step, the apurinic endonuclease. The other proteins involved in base excision repair, polymerase β, XRCC1, and DNA ligase III, do not affect the dissociation of thymine DNA glycosylase from the apurinic site.

In mammalian cells, 2–7% of the total cytosine is methylated. Spontaneous deamination of 5-methylcytosine, which is somewhat faster than cytosine (1), generates G-T mispairs in DNA. The repair of these G-T mismatches is initiated by thymine DNA glycosylase which excises the mismatched thymine (2). 5-Methylcytosine occurs almost exclusively in the sequence 5^‘CpG, and in keeping with its proposed role in the repair of G-T mispairs resulting from the deamination of 5-methylcytosine, thymine DNA glycosylase shows a strong preference for removal of thymine from CpG-T sequences (4–7). The human enzyme has been cloned and overexpressed (8) and has been shown to belong to a family of uracil DNA glycosylases that remove uracil from G-U base pairs but that are distinct from the general uracil DNA glycosylase enzyme (9). Thymine DNA glycosylase removes uracil from G-U base pairs more rapidly than it removes thymine from G-T base pairs (10) and can also remove uracil from C-U, T-U, and A-U base pairs (7) and may therefore provide a backup function to the general uracil DNA glycosylase. The glycosylase also removes thymine from base pairs with S^6-methylthioguanine (SMeG) that are thought to occur in the DNA of cells treated with the drug 6-thioguanine (7, 11). Kinetic studies in our laboratory showed that each molecule of thymine DNA glycosylase can remove only one thymine molecule because the glycosylase remains bound to the apurinic site it produces (7). Since this strong binding may have physiological significance, it has been studied in more detail.

Removal of an incorrect base by a DNA glycosylase is the first step of base excision repair (reviewed in Refs. 12 and 13), and one possible role of the bound thymine DNA glycosylase might be to recruit the enzymes needed to complete the repair process. Removal of the base is followed by cleavage of the phosphodiester bond 5’ to the abasic sugar (Fig. 1). In humans, this reaction is most probably carried out by the apurinic endonuclease, HAP1 (also known as APE, APEX, and Ref-1), as this enzyme is responsible for around 95% of all incisions at apurinic sites in HeLa cell extracts (14) and can function satisfactorily in reconstituted in vitro base excision repair systems (15, 16). Following cleavage of the sugar-phosphate backbone, DNA polymerase β then removes the deoxyribose 5’-phosphate and fills the single base gap (17–19). Ligation of the nick completes repair and is believed to be carried out by DNA ligase III (15), which is present in cells as a heterodimer with XRCC1 (20). The XRCC1 may act to ensure that polymerase β adds only one nucleotide (15). The strong binding of thymine DNA glycosylase to apurinic sites suggested that it might recruit the other proteins involved in base excision repair. Consequently we investigated possible interactions between the thymine DNA glycosylase bound to the apurinic site and the other proteins involved in base excision repair. We found that repair cannot take place while the thymine DNA glycosylase is bound to the apurinic site, and so the extremely slow dissociation of thymine DNA glycosylase from these sites would...
greatly inhibit repair. However, the apurinic endonuclease HAP1, which catalyzes the second step in base excision repair, can displace the bound thymine DNA glycosylase. This effect, which couples the first and second steps in base excision repair, is concentration-dependent and would be expected to be significant at the concentrations of HAP1 reported in mammalian cells.

**EXPERIMENTAL PROCEDURES**

**Enzymes**—Thymine DNA glycosylase was expressed in *Escherichia coli* as described previously (8) and was purified in four chromatographic steps (7). HAP1 was a gift from Dr. I. Hickson and Dr. D. Rothwell; DNA polymerase β, XRCC1, and DNA ligase III were gifts from Dr. G. Daly and Dr. T. Lindahl; and *E. coli* Endonuclease IV was provided by Dr. T. Barrett and Dr. L. Pearl.

**Synthesis and Purification of Oligodeoxynucleotides**—34-Base pair DNA duplexes of the general sequence AGC TTG GCT GCA GGC were synthesized and purified as described previously (21). Oligodeoxynucleotides with a uracil opposite position of bound and free 32P-labeled DNA was located by autoradiography. The amount of bound 32P-labeled DNA was quantified by scintillation counting and was plotted against the time of removal of thymine by thymine DNA glycosylase, and the extent of scission of the resultant apurinic site by HAP1.

**Measurement of the Rate of Dissociation of the Glycosylase from an Apurinic Site in DNA**—Complexes of thymine DNA glycosylase and DNA containing an apurinic site opposite guanine (GAP) were preincubated with 1 mM ATP for 10 min at room temperature. When used, competitor oligodeoxynucleotides were pre-mixed with the radiolabeled DNA at a 40-fold excess, before adding the thymine DNA glycosylase. After incubation, samples were loaded onto a non-denaturing 6% polyacrylamide gel, and electrophoresis was carried out for 2 h (8 V/cm at 10–15 °C). The gel was then dried and the position of the DNA visualized by autoradiography.

The band shift experiment in Fig. 6 was carried out as above except that DNA containing a G-T mismatch was incubated with thymine DNA glycosylase and HAP1 (amount as shown in the legend) for 2.5 h at room temperature before loading onto the gel. Also, part of each sample was assayed by chromatography, as described above, to measure the extent of removal of thymine by thymine DNA glycosylase, and the extent of scission of the resultant apurinic site by HAP1.

**RESULTS**

**Thymine DNA Glycosylase Binds to DNA Containing an Apurinic Site Opposite All Four DNA Bases**—In a previous report (7) we showed that thymine DNA glycosylase forms a complex with DNA containing an apurinic site opposite guanine (Fig. 2A). Tight binding of the glycosylase requires the apurinic site because 40-fold excess unlabeled DNA containing a G-Ap site virtually eliminates formation of the radioactive complex, whereas excess perfectly matched DNA only reduces the complex by about 60%. Formation of the complex does not depend upon the apurinic site being opposite a guanine, since...
of thymine DNA glycosylase from an $\text{SMeG-AP}$ site. In the absence of magnesium thymine DNA glycosylase dissociated only slightly more rapidly from an $\text{SMeG-AP}$ site than from a G-AP site, but magnesium increased the dissociation of the glycosylase from an $\text{SMeG-AP}$ site by about 20-fold (Fig. 3B and Table I). This affects the glycosylase activity of the enzyme on an $\text{SMeG-T}$ mismatch. In the absence of magnesium, thymine DNA glycosylase could only remove a stoichiometric amount of thymine from an $\text{SMeG-T}$ mismatch because it bound to the $\text{SMeG-AP}$ site that it produced, but in the presence of magnesium its more rapid dissociation from the $\text{SMeG-AP}$ site allowed turnover of $\text{SMeG-T}$ mismatches (Fig. 3F).

Magnesium has an even greater effect on the dissociation of thymine DNA glycosylase from C-AP sites (Fig. 3C and Table I). As one would expect, this faster dissociation from the C-AP site affected the glycosylase action of thymine DNA glycosylase on C-U mismatches to an even greater extent than on $\text{SMeG-T}$ mismatches (Fig. 3G).

In the experiments shown in Fig. 3, $E$–$G$, the concentration of DNA was 10 nM, and the concentration of thymine DNA glycosylase was 3 nM, whereas in Fig. 3D the concentration of G-T DNA was 100 nM and glycosylase 30 nM. These higher concentrations were used because when 10 nM G-T DNA was incubated with 3 nM enzyme, it was found that the initial reaction rate was lower in 2 mM magnesium than in EDTA (Fig. 4A). Although magnesium increased the amount of thymine finally removed from $\text{SMeG-T}$ DNA by thymine DNA glycosylase, it did not affect the initial rate of removal of the thymine (Fig. 3F). The reactions with G-U DNA (Fig. 3E) and C-U DNA (Fig. 3G) were too fast to be able to see whether the initial rates were affected by magnesium.

The slower initial rate in the presence of magnesium for the removal of thymine from a G-T mismatch suggested that magnesium might lower the affinity of the glycosylase for the G-T mismatch. To investigate the effect of magnesium on the initial rate of thymine removal from G-T DNA, rates were determined at four different concentrations of DNA and enzyme (Fig. 4B). The results are plotted as moles of thymine removed per mol of enzyme to facilitate comparison between the different concentrations. As the concentration increased, so did the relative rate of reaction, implying that at these concentrations not all of the substrate G-T DNA was bound to the enzyme (i.e. $K_d$ in Scheme I must be greater than 2 nM). This is in contrast to the reaction in EDTA in which the $K_d$ has been reported to be considerably less than 2.5 nM (7, 24). The data in Fig. 4B could not be used to calculate a $K_d$ because the rate of reaction at the lowest concentrations was less than one would expect from a single reaction with a single $K_d$ and because the reaction appeared to be biphasic (see Fig. 4A). Taken together, these suggest that at the lowest concentrations some of the enzyme was inactivated or bound in a non-productive complex.

\[
K_d \quad E + \text{DNA(G-T)} \quad k_{\text{cat}} \quad \text{DNA(G-AP)} + T \quad E + \text{DNA(G-AP)}
\]

**Scheme I**

The Action of the Apurinic Endonuclease HAP1 on Apurinic Sites Complexed with Thymine DNA Glycosylase—The accessibility of the apurinic site in the thymine DNA glycosylase-apurinic DNA complex was measured. Naked apurinic sites in DNA are cleaved rapidly by the human type II apurinic endonuclease, HAP1. The reported $K_d$ for the binding of HAP1 to an apurinic site in a 49-base pair oligodeoxynucleotide is 0.8 nM and the $k_{\text{cat}}$ 10 s$^{-1}$ (25). As would be expected from these

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**Fig. 2.** Thymine DNA glycosylase can bind to an apurinic site opposite any of the four natural DNA bases. 1.3 nM thymine DNA glycosylase was incubated in EDTA binding buffer with 0.5 nM $^{32}$P-labeled DNA containing an apurinic site opposite either guanine (A), thymine (B), cytosine (C), or adenine (D) for 30 min, and then the samples were run on a non-denaturing gel as described under “Experimental Procedures.” Competitor oligodeoxynucleotides, as indicated above the lanes, were mixed with the $^{32}$P-DNA at a 40-fold excess before addition of the glycosylase. The positions of bound and free DNA are shown by arrows.
values, we found that when 20 nM DNA containing a G\textsubscript{z}T mismatch was treated with 6 nM HAP1 the DNA was entirely cleaved in less than 1 min. The effect of two unrelated type II apurinic endonucleases, HAP1 from human cells and Endonuclease IV from \textit{E. coli}, on the apurinic site in the complex with thymine DNA glycosylase is shown in Fig. 5. In this experiment thymine DNA glycosylase and DNA containing a G\textsubscript{z}T mismatch were incubated together with one of the apurinic endonucleases. The effect of adding Endonuclease IV was very different from the effect of adding HAP1. Endonuclease IV had no discernable effect on the progress of the reaction (Fig. 5A), even when it was present in a considerable excess (60 nM) over the amount of thymine DNA glycosylase (6 nM) or DNA (20 nM). By contrast in the presence of HAP1 the reaction of thymine DNA glycosylase with DNA containing a G-T mismatch (Fig. 5B) continued after reaching the stoichiometric limit seen in Figs. 3D and 5A. Although this second phase was slow, it was much faster than in the absence of HAP1. The samples from the incubation were analyzed to measure the approximate amount of intact DNA still containing the G\textsubscript{z}T mismatch, DNA containing an apurinic site, and DNA containing a strand break where the apurinic site had been cleaved by HAP1. The amount of DNA cleaved by HAP1 was measured by adding an equal amount of saturated

FIG. 3. A–C, dissociation rates of complexes between thymine DNA glycosylase and DNA containing an apurinic site and the effect of magnesium. Complexes between 1.4 nM thymine DNA glycosylase and 1 nM \textsuperscript{32}P-labeled apurinic DNA containing an apurinic site opposite guanine (A), \textit{S\textsuperscript{m}eG\textsubscript{z}}AP and cytosine (C) were preformed in binding buffer containing either EDTA (●) or 2 mM magnesium (○). The dissociation rates were determined using the band shift assay described under “Experimental Procedures.” The dissociation rates determined from the exponential curves are given in Table I. D–G, effect of magnesium on the action of thymine DNA glycosylase. In buffer containing EDTA (●), thymine DNA glycosylase could only remove a stoichiometric amount of thymine or uracil from mismatches. In the presence of 2 mM magnesium (○), the increased dissociation rates of complexes between thymine DNA glycosylase and \textit{S\textsuperscript{m}eG\textsubscript{z}}AP or C\textsubscript{z}AP allowed the glycosylase to remove more than an equimolar amount of mismatched base from \textit{S\textsuperscript{m}eG\textsubscript{z}}T or C\textsubscript{z}U substrates. Magnesium failed to increase the amount of mismatched base removed from G-T or G-U mismatches. D, removal of thymine by thymine DNA glycosylase (30 nM) from DNA containing a G-T mismatch (100 nM); E–G, removal by thymine DNA glycosylase (3 nM) of uracil or thymine from DNA (10 nM) containing either a G-T mismatch (E), an \textit{S\textsuperscript{m}eG\textsubscript{z}}T mismatch (F), or a C-U mismatch (G). The amount of mismatched uracil or thymine removed was monitored by chromatographic assay.

| Bound DNA | Dissociation constant, $k_{off}$ (s\textsuperscript{-1}) |
|-----------|------------------|
|           | EDTA buffer | Magnesium buffer |
| G-AP      | $1.8 \times 10^{-5}$ | $2.8 \times 10^{-5}$ |
| \textit{S\textsuperscript{m}eG\textsubscript{z}}-AP | $3.5 \times 10^{-5}$ | $7.3 \times 10^{-5}$ |
| C-AP      | $3.6 \times 10^{-5}$ | $1.0 \times 10^{-3}$ |

FIG. 4. The effect of magnesium upon the initial rate of reaction of thymine DNA glycosylase with G-T DNA. A, reaction of thymine DNA glycosylase (3 nM) with \textsuperscript{32}P-labeled DNA (10 nM) containing a G-T mismatch in buffer containing either EDTA (●) or 2 mM magnesium (○). The amount of thymine removed was measured by chromatographic assay. B, reaction between equal amounts of thymine DNA glycosylase and DNA containing a G-T mismatch in 2 mM magnesium. The concentrations of DNA and enzyme were 20 nM (●), 10 nM (○), 5 nM (□), and 2 nM (▲). For easier comparison of the relative rates, the reactions have been plotted as moles of mismatched thymine removed per mol of glycosylase.
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Fig. 5. The effect of the apurinic endonucleases HAP1 and Endonuclease IV upon the reaction of thymine DNA glycosylase. A, E. coli. Endonuclease IV does not cut the apurinic site in the glycosylase-GAP complex and does not affect the progress of the glycosylase reaction with a G-T mismatch. 20 nM GT DNA was incubated with 6 nM thymine DNA glycosylase in the absence (□) or the presence of 6 nM (●) and 60 nM (×) Endonuclease IV. B, the apurinic site in the complex of thymine DNA glycosylase and apurinic DNA is also inaccessible to HAP1, but HAP1 increases the rate of reaction by increasing the dissociation of thymine DNA glycosylase from the apurinic site. DNA containing a G-T mismatch (20 nM) was incubated with 6 nM thymine DNA glycosylase and 6 nM HAP1 in 2 mM magnesium buffer. At intervals samples were taken and were either analyzed by chromatography to measure the amount of DNA cleaved by HAP1 (●) or treated with NaOH to cleave any remaining apurinic DNA and then analyzed by chromatography to measure the total amount of thymine removed (□). C, concentration dependence of the effect of HAP1 on the glycosylase reaction with a G-T mismatch. GT DNA (20 nM) was incubated with thymine DNA glycosylase (6 nM) in 2 mM magnesium in the absence of (□) or the presence of 6 nM (●), 12 nM (×), 24 nM (▲), and 600 nM (■) HAP1. D, effect of HAP1 on the glycosylase reaction with a C-U mismatch. CU DNA (10 nM) was incubated in 2 mM magnesium with thymine DNA glycosylase (3 nM) in the presence of 10 nM HAP1 (●). The first four data points from Fig. 3G for the reaction in magnesium buffer have been plotted to show the rate of reaction in the absence of HAP1 (□).

urea containing 10 mM EDTA and 0.5% Triton X-100 before chromatography to separate cleaved from intact DNA. The total amount of DNA from which thymine had been removed (i.e., the combined amount of DNA cleaved by HAP1 and DNA still containing an apurinic site) was measured by treating the samples with NaOH to cleave the apurinic sites before chromatography. Fig. 5B shows the rate of thymine removal and the rate of production of DNA cleaved by HAP1. It can be seen that thymine DNA glycosylase rapidly produced an approximately stoichiometric amount of apurinic DNA. After this initial burst, the glycosylase removed thymine at a much slower rate. The amount of DNA cleaved by HAP1 accumulated at approximately the same rate as the slower phase of the thymine DNA glycosylase reaction so that there was always an approximately constant amount of intact apurinic DNA complexed with the glycosylase. The amount of this intact apurinic DNA, estimated by subtracting the amount of DNA cleaved by HAP1 from the total cleaved by HAP1 and by NaOH, was, on average, only about 4 nM (i.e., two-thirds of the glycosylase present). If thymine DNA glycosylase bound only to DNA containing an apurinic site one would expect that there would be 6 nM bound apurinic DNA. This discrepancy is probably an artifact arising from the method of analysis. In this analysis the reaction was stopped, the DNA and protein dissociated, and the DNA strands separated, by addition of urea and EDTA. It is probable that this treatment exposed some of the apurinic sites in the thymine DNA glycosylase-apurinic DNA complex before all the HAP1 activity had been lost. This explanation is supported by the observation (Fig. 7, discussed below) that an exactly stoichiometric amount of apurinic DNA and glycosylase is not repaired by a complete base excision repair system and also by analysis of the products of reaction with Endonuclease IV using the same method as used for the study of HAP1. Endonuclease IV does not cut the apurinic sites in the thymine DNA glycosylase-apurinic DNA complex, and no cleaved DNA should have been obtained, but when the reaction mixture at the end of the experiments shown in Fig. 5A was analyzed by the same method as used for the HAP1 experiment shown in Fig. 5B, it was found that 25–39% of the apurinic sites had been cleaved.

The results in Fig. 5B suggest that HAP1 can displace thymine DNA glycosylase from an apurinic site in DNA. The dependence of the rate of this displacement on the concentration of HAP1 is shown in Fig. 5C. From 6 to 24 nM HAP1 the rate of removal of thymine after the stoichiometric limit had been reached was roughly proportional to the amount of HAP1 present. In the presence of 600 nM HAP1, almost all of the 20 nM GT DNA had reacted after 1 h. HAP1 had a similar, but greater, effect on the removal of uracil from a C-U mismatch. Despite the fact that thymine DNA glycosylase dissociates more rapidly from a C-A mismatch than a G-A site (Table I), the removal of uracil from C-U DNA by thymine DNA glycosylase is still limited by slow product release (Fig. 3G). Addition of 10 nM HAP1 increased the rate of turnover of C-U DNA by more than 150-fold so that the reaction was complete in around 15 min (Fig. 5D). The increased rate of uracil removal seen when HAP1 was added was not due to contamination of the HAP1 preparation by uracil DNA glycosylase because addition of the uracil glycosylase inhibitor did not affect the rate. In addition, no uracil was removed when C-U DNA was incubated with HAP1 alone.

The results in Fig. 5 suggest that HAP1 and the glycosylase interact. This interaction is probably with the complex of glycosylase and the apurinic site and not with the free glycosylase, because essentially the same results were obtained when HAP1 was mixed with the DNA before the glycosylase was added, as were obtained when thymine DNA glycosylase was preincubated with HAP1 before addition of the DNA. The possibility that this interaction might involve the formation of a ternary complex of apurinic DNA, glycosylase, and HAP1 was investigated by a band shift experiment (Fig. 6). 32P-Labeled DNA containing a G-T mismatch was incubated for 2.5 h with thymine DNA glycosylase and various concentrations of HAP1 and then subjected to electrophoresis on a non-denaturing polyacrylamide gel. When only thymine DNA glycosylase was present, a band corresponding to the complex between the glycosylase and DNA containing a GAP site was observed. The mobility of this complex of thymine DNA glycosylase with GAP DNA was not altered in the presence of up to 24 nM HAP1, indicating that HAP1 does not bind tightly to the glycosylase-apurinic DNA complex. However, a new band appeared that became more intense as the concentration of HAP1 increased. This complex had the same mobility as a complex seen when GAP DNA was incubated with HAP1 (Fig. 6B, +HAP1). Chro-
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**Fig. 6.** Non-denaturing gel of the reaction shown in Fig. 5C. A. 32P-labeled DNA containing a G-T mismatch was incubated with thymine DNA glycosylase in the presence of four different concentrations of HAP1 for 2.5 h under the same conditions as Fig. 5C and then run on a non-denaturing gel as described under "Experimental Procedures." B, 10 nM 32P-labeled DNA containing a G-AP site was incubated with either 5 nM thymine DNA glycosylase (+TDG) or 5 nM HAP1 (+HAP1) for 30 min at room temperature and then analyzed on a non-denaturing gel. The positions of free DNA, HAP1-DNA complexes, and thymine DNA glycosylase-DNA complexes are shown by arrows. Note that the free DNA migrates more slowly in the presence of HAP1 than in the presence of thymine DNA glycosylase. This is presumably because the nicked DNA produced by HAP1 migrates differently to the intact G-AP DNA in the 1st lane (+TDG).

matographic analysis of the DNA in Fig. 6B showed that in the presence of thymine DNA glycosylase alone (+TDG) the G-AP DNA was intact, whereas in the presence of HAP1 only (+HAP1) the DNA was entirely cleaved, and thus HAP1 was bound to DNA that it had already cleaved. Complexes between HAP1 and DNA containing an uncleaved tetrahydrofuran analogue of an apurinic site (26) to intact apurinic DNA that could not be cleaved because of the absence of magnesium (25) and to a DNA fragment with a 3'-phosphoribose produced by heat-induced cleavage of an apurinic DNA (25) have been detected previously in band shift experiments, but this seems to be the first report of a complex between HAP1 and apurinic DNA that it had itself cleaved.

**Complete In Vitro Repair of G-T Mismatches**—The experiments reported above show that thymine DNA glycosylase binds particularly strongly to the apurinic sites produced from its supposed physiological substrate, a G-T mismatch in the sequence CpG-T, but that the thymine DNA glycosylase can be displaced from this complex by HAP1, the enzyme that catalyzes the second step in base excision repair. This suggested the possibility that the physiological function of the bound thymine DNA glycosylase might be to recruit proteins involved in base excision repair, i.e., HAP1, DNA polymerase β, XRCC1, and DNA ligase III (15), to the site of DNA damage. The influence of these proteins on the repair of a G-T mismatch is shown in Fig. 7. The DNA containing the G-T mismatch was incubated with either (i) thymine DNA glycosylase; (ii) thymine DNA glycosylase and HAP1; (iii) thymine DNA glycosylase, HAP1, DNA polymerase β, XRCC1, and DNA ligase III without ATP; or (iv) with thymine DNA glycosylase, HAP1, DNA polymerase β, XRCC1, and DNA ligase III and ATP. At intervals samples were removed, treated with hydroxide, and the amount of cleaved DNA measured. With thymine DNA glycosylase alone the cleavable DNA that is measured represents the total DNA containing an apurinic site, either as free apurinic DNA or as the thymine DNA glycosylase-apurinic site complex. When both thymine DNA glycosylase and HAP1 are present in the incubation, the cleaved DNA that is measured represents the sum of the apurinic DNA cleaved by HAP1 and the apurinic DNA in the thymine DNA glycosylase-apurinic site complex that has been cleaved by the hydroxide. When all the proteins are present, but without ATP (i.e. in the absence of ligase action), the cleaved DNA measured represents the sum of three different species: (i) apurinic DNA that has been cut by HAP1; (ii) apurinic DNA that has been cut by HAP1 and a deoxyxycytidine nucleotide added to the 3'-end by polymerase β; and (iii) the apurinic DNA in the thymine DNA glycosylase-apurinic site complex that has been cleaved by the hydroxide. The final mixture in which there are all the proteins and ATP represents a positive control because if all the proteins are active, and present in the required amounts, then all the apurinic DNA, except that bound in the thymine DNA glycosylase complex, will be repaired and the two fragments religated. The results showed that, in the presence of all the proteins, but in the absence of ATP, the cleaved DNA accumulated at a similar rate to that seen with thymine DNA glycosylase and HAP1 alone. This shows that, unlike HAP1, these other repair enzymes do not enhance the rate of dissociation of thymine DNA glycosylase from the apurinic site. When all these proteins and ATP were present, the amount of cleavable DNA remained at a level similar to the amount of thymine DNA glycosylase. This is most likely because any apurinic DNA that was released from the glycosylase-G-AP complex was immediately repaired and ligated to form G-C DNA. This repair of apurinic DNA shows that the enzymes used were active and present in sufficient quantity to carry out complete repair. The observation that the glycosylase-G-AP complex persisted even in the presence of all these proteins confirms the conclusion drawn from the experiments shown in Fig. 5B that the apurinic site in the complex cannot be repaired until the glycosylase is displaced from it. Although the glycosylase-apurinic DNA complex slows down the process, the DNA in the complex was repairable since overnight incubation of the reaction shown in Fig. 7, in which all of the repair components were present, resulted in more than 90% repair.

**DISCUSSION**

Having recently found (7) that each molecule of thymine DNA glycosylase can only remove one thymine because the glycosylase binds so tightly to the G-AP product of the reaction that it is unable to react with another G-T mismatch, we investigated the interaction of thymine DNA glycosylase with
apurinic sites in DNA in more detail. We found that the glycosylase can bind to DNA containing an apurinic site opposite guanine, $S^6$-methylthioguanine (7), cytosine, thymine, and adenine (Fig. 2). Thus thymine DNA glycosylase shows no absolute preference for the base opposite the apurinic site but seems only to require the apurinic site itself. Furthermore, thymine DNA glycosylase binds to all apurinic sites with a relatively low $K_d$. These findings suggest that thymine DNA glycosylase may bind to apurinic sites in the cell produced by the following: (i) spontaneous deamination; (ii) the action of alkylating agents and other chemicals that react with the bases in DNA to produce adducts with unstable glycosidic bonds, such as 3-alkyladenine or 7-methylguanine (27); and, (iii) the action of other DNA glycosylases such as uracil DNA glycosylase and the glycosylases that remove modified bases (28).

We have measured the dissociation rates for glycosylase-apurinic DNA complexes using a band shift assay that has been used previously for measuring dissociation rates of protein-nucleic acid complexes (23, 29, 30). The absolute $k_{off}$ values obtained here need to be confirmed using other techniques and should be treated with caution. However, the dissociation rates are consistent with the results for the reaction of thymine DNA glycosylase (see Fig. 3 and discussion below). In EDTA buffer, the dissociation rates of complexes between thymine DNA glycosylase and all three apurinic containing DNA duplexes tested in Fig. 3 are extremely slow ($1.8\times10^{-5}$ s$^{-1}$; see Table I) and correspond to half-lives for the complexes of between 5 and 10 h. Assuming a typical second-order association rate ($k_{on}$) of $10^{7}$ M$^{-1}$ s$^{-1}$, these results imply that the binding constants for thymine DNA glycosylase to these apurinic sites in DNA are between 2 and 4 pm. These results confirm our previous finding that the thymine DNA glycosylase action is limited by extremely slow product release (7). The E. coli mismatch-specific uracil glycosylase, MUG, which is a homologue of thymine DNA glycosylase (9), also binds to apurinic sites in DNA (31), although the reported $K_d$ (6 nm) for the binding of MUG to an apurinic site opposite guanine is a thousand times larger than the value obtained here for human thymine DNA glycosylase.

Thymine DNA glycosylase does not require magnesium, and initially we followed the practice of previous authors (2, 4, 6, 8) and carried out the thymine DNA glycosylase experiments in buffer containing EDTA. As the following step in base excision repair, cutting of the apurinic site by the apurinic endonuclease HAP1 requires at least 0.1 mM magnesium for maximum activity (32), the dissociation rates were also measured in the presence of 2 mM magnesium. Magnesium had little effect upon the rate of dissociation of thymine DNA glycosylase from G-AP DNA (only a 60% increase in $k_{off}$), but it increased the rate of dissociation of thymine DNA glycosylase from $S^6$MeG-AP and C-AP sites in DNA by 20- and 28-fold, respectively (Table I). This increase in the dissociation rate produced by magnesium has a great influence on the reaction of thymine DNA glycosylase with DNA containing a $S^6$MeG-T or a C-U mismatch allowing it to remove more than a stoichiometric amount of thymine from $S^6$MeG-T and uracil from C-U mismatches (Fig. 3, F and G). In contrast, magnesium had little effect on the dissociation of thymine DNA glycosylase from a G-AP site in DNA, so even in the presence of magnesium the glycosylase could remove only a stoichiometric amount of the mismatched thymine or uracil from DNA containing a G-T or a G-U mismatch. These results suggest that in the presence of magnesium and with limiting thymine DNA glycosylase, both C-U mismatches and $S^6$MeG-T mismatches would be better substrates for the glycosylase than G-T DNA. This is probably irrelevant to the repair of C-U base pairs because they would occur very rarely in cells, and it is almost certain that in vivo uracil would be removed from C-U by the more efficient and abundant uracil DNA glycosylase. However, thymine DNA glycosylase is the only glycosylase known to be able to remove thymine from $S^6$MeG-T base pairs so the reasonably rapid attack of thymine DNA glycosylase on $S^6$Me-G-T mismatches may be important in regard to the cytotoxicity of 6-thioguanine (11).

Glycosylases mediate the first step in the base excision repair pathway. The apurinic sites produced are then cut at the apurinic site by an apurinic endonuclease, probably HAP1 (reviewed in Ref. 33). We had expected that the bound thymine DNA glycosylase might recruit HAP1 and thus facilitate the cleavage of the apurinic site, but unexpectedly we found that neither HAP1 nor Endonuclease IV can cut at the apurinic site while thymine DNA glycosylase is bound to it (Fig. 5, A and B). Thus dissociation of the thymine DNA glycosylase from the apurinic site is a prerequisite for complete repair to take place (Fig. 7). HAP1 increases the rate of this dissociation. This increases the removal of thymine from G-T mismatches, because the displacement allows turnover of the glycosylase (Fig. 5) and accelerates the repair because it allows HAP1 to cut the apurinic site, which in turn allows the other enzymes involved in base excision repair to refill the gap in the DNA. The displacement of thymine DNA glycosylase from the apurinic site appears to be specific to HAP1 since Endonuclease IV, a type II endonuclease from E. coli, has no effect on the glycosylase reaction (Fig. 5A). No similar interaction between a DNA glycosylase and HAP1 has been reported before. The increase induced by 6 mM HAP1 in the rate of turnover of 6 mM thymine DNA glycosylase was quite small, but the increase is concentration-dependent and at 600 mM HAP1 a much larger increase in turnover was induced. From the purification of HAP1 from HeLa cells (14), we estimate that the concentration of HAP1 is about 0.1–1 mM (a similar figure is given in Ref. 34), and at this concentration HAP1 should produce a very substantial increase in the rate of dissociation of thymine DNA glycosylase from apurinic sites. The rate of removal of thymine from G-T mismatches by thymine DNA glycosylase in the presence of such a large concentration of HAP1 should be more than adequate to cope with the rate of deamination of 5-methylcytosine in cells (35). The high concentration of HAP1 in cells may, in part, reflect its role in maintaining the redox state of some transcription factors (33), but it may also reflect the necessity for a large concentration of HAP1 to dissociate the thymine DNA glycosylase, and conceivably other DNA glycosylases, from apurinic sites.

A multiprotein complex that fully repairs uracil sites in DNA has been purified suggesting that components of base excision repair may exist as a ‘repairosome’ that can carry out complete repair in a concerted manner (36). Kubota et al. (15) reconstituted $in vitro$ the repair of uracil in DNA using the human proteins uracil DNA glycosylase, HAP1, polymerase β, XRCC1, and DNA ligase III. In an analogous experiment, we reconstituted $in vitro$ repair of DNA containing a G-T mismatch using thymine DNA glycosylase, HAP1, polymerase β, XRCC1, and DNA ligase III. Our present data do not support the view that these proteins act as a repair complex analogous to that seen in nucleotide excision repair (reviewed in Ref. 37). Although this combination of proteins could completely repair the G-T mismatch, the rate of the glycosylase reaction was no greater than that seen with thymine DNA glycosylase and HAP1 alone (Fig. 7) showing that these other repair proteins are not able to cooperate with HAP1 in the displacement of the glycosylase from the apurinic site. Furthermore, there was no evidence from band shift assays that HAP1, which is the only one of these proteins that had any effect on the dissociation of the thymine DNA glycosylase from the apurinic site, formed a

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complex with the thymine DNA glycosylase bound to the apurinic site (Fig. 6).

The interaction between thymine DNA glycosylase and HAP1 would loosely coordinate the first and second step of base excision repair and appears similar to the recently reported interaction between HAP1 and DNA polymerase β (34) that would loosely coordinate the second and third step. An interaction between polymerase β and a complex of DNA ligase III and XRCC1 has also been described (15). The overall effect of these interactions might be to link all the steps in the pathway. However, this coordination does not entirely explain why the glycosylase binds so strongly to apurinic sites. If the site were not protected by the bound glycosylase it would be very rapidly cut by HAP1, which is a relatively fast enzyme (25), and so the binding of the glycosylase to apurinic sites slows, rather than accelerates, their repair. One possibility comes from the observation that in human tissue, the amount of HAP1 measured in some cell types is quite low, and in some cells HAP1 is predominantly cytoplasmic and thus may not be available for DNA repair (38). One could foresee that in cells where there was a lack of HAP1, the binding of thymine DNA glycosylase to apurinic sites might prevent oxidative damage to the apurinic site or might act as a physical block to DNA replication or transcription and prevent misincorporation opposite the apurinic site. Alternatively, it might act as a signal to halt the cell cycle until DNA repair can be completed or, in extreme cases, might act as the signal for apoptosis.

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