Biphasic Kinetics of the Human DNA Repair Protein MED1 (MBD4), a Mismatch-specific DNA N-Glycosylase*

The human protein MED1 (also known as MBD4) was previously isolated in a two-hybrid screening using the mismatch repair protein MLH1 as a bait, and shown to have homology to bacterial base excision repair DNA N-glycosylases/lyases. To define the mechanisms of action of MED1, we implemented a sensitive glycosylase assay amenable to kinetic analysis. We show that MED1 functions as a mismatch-specific DNA N-glycosylase active on thymine, uracil, and 5-fluorouracil when these bases are opposite to guanine. MED1 lacks uracil glycosylase activity on single-strand DNA and abasic site lyase activity. The glycosylase activity of MED1 prefers substrates containing a G:T mismatch within methylated or unmethylated CpG sites; since G:T mismatches can originate via deamination of 5-methylcytosine to thymine, MED1 may act as a caretaker of genomic fidelity at CpG sites. A kinetic analysis revealed that MED1 displays a fast first cleavage reaction followed by slower subsequent reactions, resulting in biphasic time course; this is due to the tight binding of MED1 to the abasic site reaction product rather than a consequence of enzyme inactivation. Comparison of kinetic profiles revealed that the MED1 5-methylcytosine binding domain and methylation of the mismatched CpG site are not required for efficient catalysis.

The integrity of genetic information is constantly challenged by a variety of endogenous and exogenous DNA damaging agents (1–3). Cellular DNA transactions occur in aqueous solution containing reactive oxygen species, and, as such, DNA is prone to both hydrolytic and oxidative damage. Hydrolysis of the N-glycosyl bond yields apurinic and, less frequently, apyrimidinic sites that are highly mutagenic. Hydrolytic deamination of cytosine and 5-methylcytosine (M) generates G:U and G:T mismatches, respectively. Oxidative lesions include 8-oxoguanine, thymine glycol, and formamidopyrimidine derivatives of adenine and guanine (1, 2). In addition to endogenous damaging processes, DNA is exposed to the attack of exogenous reactive species, including alkylating agents and the carcinogens vinyl chloride and ethyl carbamate. Alkylating agents primarily alkylate the N3 position of purines and the N7 and O6 positions of guanine (1, 2), whereas metabolites of vinyl chloride and ethyl carbamate generate cyclic (etheno) DNA adducts, such as 3,N4-ethenocytosine, 1,N6-ethenoadenine, 1,N2-ethenoguanine and N2,3-ethenoguanine (4, 5).

Efficient correction of these DNA lesions relies on the action of several enzymes belonging to the base excision repair system (2, 6–9). Unlike nucleotide excision repair or long-patch mismatch repair (MMR), base excision repair enzymes usually act in a lesion-specific fashion on a single damaged or mismatched nucleotide. Given the mutagenic potential of DNA lesions, continuing elucidation of the biochemical activities, damage spectrum and specificity of base excision repair enzymes has direct implications on cancer and aging (2).

In an effort to isolate new human proteins involved in DNA repair, we recently conducted a yeast two-hybrid screening with the MMR protein MLH1 as a bait and identified a novel human DNA repair protein, named MED1 (methyl-CpG-binding endonuclease 1) (10). The 580-amino acid MED1 protein, also known as MBD4 (11), has a tripartite structure with an N-terminal 5-methylcytosine binding domain (MBD), a central region with five putative nuclear localization signals, and a C-terminal catalytic domain with homology to bacterial DNA repair proteins (11). MED1 may act as a caretaker of genomic fidelity in a lesion-specific fashion on a single damaged or mismatched nucleotide. Given the mutagenic potential of DNA lesions, continuing elucidation of the biochemical activities, damage spectrum and specificity of base excision repair enzymes has direct implications on cancer and aging (2).

As to the function of MED1 in DNA repair, we had previously suggested three possibilities. (i) MED1 may function in long-patch MMR; this would explain the interaction with MLH1 and, possibly, the binding to hemi- and fully methylated DNA, in analogy to the E. coli MutSLH system. (ii) MED1 may work in a short-patch MMR pathway, similar to the E. coli Vsr performance liquid chromatography; Bis'Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane.
endonuclease; this would also explain the interaction with MLH1. (iii) MED1 may act as a glycosylase/lyase in a pathway of base excision repair (10). In the present paper, we show that MED1 acts as a mismatch-specific glycosylase active on thymine, uracil, and 5-fluorouracil (5-FU) paired with guanine. Due to its high affinity for the abasic (apurinic/apyrimidinic) (AP) site reaction product, MED1 displays biphasic kinetics with a rapid initial burst of product formation followed by much slower reactions.

We and others have previously shown that the MED1 (MBD4) gene is mutated in human colorectal, endometrial, and pancreatic carcinomas exhibiting a defect in the MMR genes MLH1 and MSH2 (12, 13). The mutations target polyadenine microsatellites in the central region and cause frameshifts; this predicts the synthesis of truncated proteins lacking the C-terminal catalytic domain (12, 13). Thus, the present findings suggest that a fraction of human carcinomas are defective not only in long-patch MMR but also in MED1 thymine, uracil, and 5-FU glycosylase activity. The defect in MED1 activity may facilitate accumulation of DNA damage, thereby contributing to tumor development, and, in addition, may have implications in cancer treatments based on chemotherapy with 5-FU.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Recombinant Proteins**—Expression constructs of wild type MED1 protein and deletion mutants were prepared in the vector pET28(b) (Novagen), which provides a 6-histidine tag, and were propagated in E. coli strain XL-1 Blue, as described previously (10). BL21(DE3)(pLysS) cells transformed with the expression vectors were grown to an A600 of 0.4 and induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside at 37 °C for 2 h. Cells collected by centrifugation were lysed in Buffer A (10 mM Tris-HCl, pH 8, 500 mM NaCl, 0.1% Nonidet P-40, 10% glycerol, and “Complete” protease inhibitors (Roche Molecular Biochemicals)). After clarification by centrifugation at 12,000 × g, the soluble protein fraction was diluted to 150 mM NaCl.

**β-Mercaptoethanol** (7 mM) was added, and the sample was applied to a 5-ml SP Sepharose cation exchange column (Amersham Pharmacia Biotech) connected in series to a 5-ml Q Sepharose anion exchange column (Amersham Pharmacia Biotech). After disconnecting the Q Sepharose column, the SP Sepharose column was washed with 30 ml of Buffer B (10 mM Tris-HCl, pH 8, 10% glycerol, and 7 mM β-mercaptoethanol) supplemented with 100 mM NaCl. Elution was performed with 10 ml of Buffer B supplemented with 1.5 mM NaCl. The SP Sepharose eluate, supplemented with 20 mM imidazole, was applied to a 1.5-ml nickel-chelating Sepharose column (Amersham Pharmacia Biotech). The nickel affinity column was washed with 10 ml of Buffer C (10 mM Tris-HCl, pH 8, 300 mM NaCl, 10% glycerol, 2 mM β-mercaptoethanol) supplemented with 20 mM imidazole. Recombinant MED1 proteins were eluted with 5 ml of Buffer C, supplemented with 150 mM imidazole. Proteins were further purified by size exclusion chromatography using a Superdex 200 PC 5.2/30 gel filtration column (Amersham Pharmacia Biotech), equilibrated with Buffer D (20 mM HEPES, pH 7.5, 150 mM NaCl, and 1 mM EDTA), pumped at a rate of 40 μl/min using a SMART chromatography system (Amersham Pharmacia Biotech). UV absorbance was monitored at 214, 256, and 280 nm, and 40-μl fractions were collected every 1 min. Throughout the purification, fractions were followed by SDS-polyacrylamide gel electrophoresis (PAGE) and assayed for glycosylase activity (see below). The final MED1 preparation was estimated to be >95% pure by SDS-PAGE. Upon the addition of 10% glycerol, purified MED1 fractions were frozen in liquid nitrogen and stored at –70 °C. A slow decay of glycosylase activity was observed upon prolonged storage at –70 °C. Protein concentration was determined with the Bradford assay kit (Bio-Rad), using bovine γ-globulin as a standard.

**Synthesis of Oligonucleotides, Preparation of Substrates, and Sequencing Reactions**—Oligonucleotides (37- and 64-mers) were synthesized on an automated DNA synthesizer (Applied Biosystems) and purified by denaturing 8.3M urea, 15–20% PAGE. Oligonucleotide bands were visualized by ultraviolet shadowing and excised. DNA was eluted from gel slices using an Amicon 5005 electroleuter, as described previously (14). Oligonucleotides containing 5-FU were purchased from Genset (Paris, France) and gel-purified as above. The AP site-containing oligonucleotide was prepared by incubating an oligonucleotide containing uracil with E. coli uracil DNA glycosylase (Udg) (PerkinElmer Life Sciences). The AP site reaction product was purified by ion exchange chromatography at pH 12.

**Nucleotide-strand oligonucleotide substrates** were prepared by annealing complementary, gel-purified single-strand oligonucleotides, as summarized in Table I. Duplex substrates were labeled at the 3′ end of the bottom strand by a fill-in reaction with exounucleotide-deficient DNA polymerase I Klenow fragment (Prime-It, Stratagene) and [α-32P]dATP (PerkinElmer Life Sciences). Unincorporated label was removed with G25 spin columns (Amersham Pharmacia Biotech).

Maxam-Gilbert sequencing reactions of the substrate oligonucleotides were conducted as described previously (15), with the exception that the DNA was labeled at the 3′ end.

**Glycosylase Assays**—Substrate DNA, typically 5 nm, was preincubated at 37 °C in Buffer E (20 mM HEPES, pH 7.5, 1 mM diethiothreitol, 1 mM EDTA, 1 mg/ml bovine serum albumin). Reaction was started by the addition of an equimolar amount, typically 5 nm, of purified MED1. At different time points (ranging from 15 s to 10 h), 15-μl aliquots of the reaction were taken, quenched with 3.8 μl of 500 mM NaOH, and immediately transferred to 90 °C for 30 min. An equal volume (18.8 μl) of denaturing loading dye (95% formamide, 0.04% bromophenol blue, 0.04% xylene cyanol) was added, and 7.5 μl of the heat-denatured samples were separated by 8.3 M urea, 15% PAGE. Gels were exposed to a PhosphorImager (Fuji) screen for 1 h, which yielded autoradiograms within the dynamic range of the instrument. The substrate and product bands were quantified to evaluate the percentage of substrate converted into product. Reaction time courses were then analyzed using the kinetic simulation program KINSIM (16–18). KINSIM uses numerical integration to predict the time dependence of a reaction from a given mechanism and initial concentrations without relying upon the assumptions of steady state kinetic analysis.

When the incubation with alkali was omitted, the AP site was sta-
The catalytic domain of MED1 bears distant homology to several bacterial DNA repair glycosylases/lyases (10), including MutY (20) and endonuclease III from *E. coli* (21), Mig.*M. thermoautotrophicum* (22), and UV endonuclease from *E. coli* (26–28). Mig.*M. thermoautotrophicum* is a thymine glycosylase active on G:T mismatches; the enzyme is equally active on G:U and a less degree on G:G, A:G, T:C, and U:C mismatches (22).

Based on the homologies with these enzymes, MED1 was assayed for glycosylase activity on mismatched bases. Purified recombinant MED1 protein was incubated with 32P-labeled oligonucleotide substrates carrying all the eight possible mismatches of the normal DNA bases. The products of the reaction were treated with strong alkali to cleave at AP sites and then separated by electrophoresis on denaturing polyacrylamide gels. As shown in Fig. 1A, a cleavage product was detected only on the 32P-labeled, thymine-containing strand of a G:T substrate. A sequencing ladder indicated that the migration of the cleavage product corresponds to the site of the mismatched thymine (Fig. 1B). In addition, no cleavage product was detected on C:T or T:T mismatches (Fig. 1A), or when MED1 was incubated with matched unmethylated, hemimethylated, or fully methylated oligonucleotide substrates, or with substrates containing 1–5 extrahelical bases (Fig. 1A and data not shown). These results suggest that MED1 has thymine glycosylase activity specific for G:T mismatches. We next conducted a MALDI-TOF mass spectrometry analysis of the MED1 glycosylase reaction with a G:T substrate. As shown in Fig. 1C, in addition to the peaks corresponding to the parental mismatched top (G) and bottom (T) single-strand oligonucleotides, a third peak is detected upon MED1 incubation, with a mass/charge ratio consistent with that of the reaction product, i.e., the bottom oligonucleotide lacking thymine. This experiment provides conclusive evidence on the thymine glycosylase activity of MED1.

**FIG. 1.** G:T mismatch-specific thymine glycosylase activity of MED1. **A,** the indicated 64-mer double-stranded oligonucleotides (Table I), bearing all possible mismatches and 32P-labeled at the 3′ end on the bottom strand (marked by the *asterisk*), were treated with purified recombinant MED1 protein at 37°C for 30 min, in order to cleave the sugar-phosphate backbone at the AP site. A band representing a cleavage product was detected for the G:T-containing oligonucleotide substrate labeled on the thymine-containing strand (*lane 4*). Arrows mark the expected migration of the substrate and product bands. **B,** G+A- and T-specific sequencing reactions of the 38-mer G:T duplex oligonucleotide substrate (Table I), labeled at the 3′ end of the bottom strand, were run as a standard next to a MED1 glycosylase reaction. The sequence of the bottom strand of the substrate is reported on the left. The product band comigrates with the mismatched T (indicated in *bold*). **C,** upper panel, MALDI-TOF mass spectrum of annealed starting G:T mismatched duplex oligonucleotide untreated with MED1 (37-mer, see Table I). The separate G and T oligonucleotide strands were used as internal standards for mass calibration; their neutral masses are 11,358.5 and 11,395.5 Da, respectively. Lower panel, MALDI-TOF mass spectrum of annealed G:T mismatched duplex oligonucleotide treated with MED1 and reduced with sodium borohydride. The new oligonucleotide ion (marked −T) is 105.7 Da less than the T-containing oligonucleotide, consistent with the expected delta mass of 106.1 Da from the hydrolysis of the N-glycosidic bond of the thymine base and subsequent reduction of the AP site.
MED1 is a monofunctional glycosylase lacking lyase activity. A, the indicated fractions from a gel-filtration purification of recombinant MED1 were incubated with the 38-mer G:T-mismatch substrate. An aliquot of the reaction was processed with NaOH before electrophoresis (upper panel), whereas the remaining directly underwent electrophoresis (lower panel). A product band was detected for fractions 14–18 after incubation with alkali; no cleavage product was detected for these fractions when this incubation was omitted. B, reaction of Udg with a 37-mer G:U oligonucleotide substrate generates an AP site that can be cleaved by treatment with NaOH (lane 3), but not by incubation with MED1 for 30 min (lane 4).

In order to determine whether MED1 has AP lyase activity, fractions from the last step of purification of recombinant MED1 (gel filtration) were incubated with the [32P]-labeled G:T substrate. Following incubation with MED1, an aliquot of the reaction was processed with NaOH before electrophoresis, whereas the remaining directly underwent electrophoresis. As shown in Fig. 2A, no cleavage was detected when the incubation of the MED1 reaction products with alkali was omitted. Similarly, MED1 did not further process an AP site generated by incubation of a G:U oligonucleotide substrate with the uracil glycosylase Udg (Fig. 2B). These data indicate that MED1 is a monofunctional glycosylase that lacks a detectable lyase activity.

**MED1 Thymine Glycosylase Activity in the Context of a Methylated or Unmethylated CpG Site**—In its G:T mismatch-specific glycosylase activity, MED1 is similar to the above mentioned Mig.Mth (22) and the human mismatch-specific thymine glycosylase TDG (29, 30) in that all three enzymes may counteract mutagenesis caused by spontaneous deamination of 5-methylcytosine to thymine, which would give rise to a G:T mismatch (22, 29, 30). Since in mammalian cells genome-wide cytosine methylation occurs exclusively at CpG sites, we investigated whether a cytosine or 5-methylcytosine preceding the mismatched guanine is preferred for MED1 thymine glycosylase activity. MED1 was incubated with oligonucleotide substrates in which the mismatched G is immediately 3’ to A, C, G, T, or M. As shown in Fig. 3, thymine glycosylase activity was high with CpG/TpT and MpG/TpT substrates and low with ApG/TpT, GpG/TpC, and TpG/TpA substrates. Thus, the fact that CpG/TpT and MpG/TpT are the optimal substrates for MED1 thymine glycosylase activity confirms that MED1 may preferentially counteract mutagenic consequences of deamination of 5-methylcytosine to thymine at CpG sites.

**MED1 Is Also Active on Uracil and 5-Fluorouracil Paired with Guanine**—Both Mig.Mth and TDG have a mismatch-specific uracil glycosylase activity (22, 29). Based on the similarities with these enzymes, we tested the uracil glycosylase activity of MED1 on oligonucleotide substrates in which uracil was paired with A, C, G, or T. As expected, MED1 uracil glycosylase activity is specific for G:U mismatches (Fig. 4A). MED1 did not exhibit uracil glycosylase activity on single-stranded DNA (Fig. 4B). MED1 efficiently removed the uracil analog 5-FU opposite a guanine (Fig. 4C).

**Biphasic Kinetics of MED1 Glycosylase Activity**—A kinetic analysis was performed on MED1 glycosylase reaction in order to elucidate the mechanism of this important DNA repair enzyme. Kinetic studies were preceded by experiments in which we assayed the glycosylase activity of MED1 under various reaction conditions. The results indicated that MED1 is active over a wide range of temperature and pH, whereas it has a more restricted optimum of ionic strength (48). All

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**Fig. 2.** MED1 is a monofunctional glycosylase lacking lyase activity. A, the indicated fractions from a gel-filtration purification of recombinant MED1 were incubated with the 38-mer G:T-mismatch substrate. An aliquot of the reaction was processed with NaOH before electrophoresis (upper panel), whereas the remaining directly underwent electrophoresis (lower panel). A product band was detected for fractions 14–18 after incubation with alkali; no cleavage product was detected for these fractions when this incubation was omitted. B, reaction of Udg with a 37-mer G:U oligonucleotide substrate generates an AP site that can be cleaved by treatment with NaOH (lane 3), but not by incubation with MED1 for 30 min (lane 4).

**Fig. 3.** Sequence context of MED1 G:T mismatch-specific thymine glycosylase activity: preference for CpG sites. MED1 was incubated at 37 °C for 60 min with the indicated G:T-containing oligonucleotide substrates, in which the mismatched G followed A, C, G, T, or M (Table I). Highest thymine glycosylase activity was detected with CpG/TpG and MpG/TpG substrates, which contained a G:T mismatch in the context of a methylated or unmethylated CpG site. Only low amounts of products were generated with ApG/TpT, GpG/TpC, and TpG/TpA substrates.

**Fig. 4.** MED1 has a G:U and G:5-FU mismatch-specific uracil and 5-fluorouracil glycosylase activity. A, the indicated double-stranded oligonucleotides containing uracil (U) paired with A, C, G, and T, were 32P-labeled at the 3’ end on the bottom strand (marked by the asterisk) and treated with purified recombinant MED1 protein at 37 °C for 60 min. The reactions were then treated with 100 mM NaOH at 90 °C for 30 min, in order to cleave the AP site. A cleavage product was detected for the G:U-containing oligonucleotide substrate labeled on the uracil-containing strand. Arrows mark the expected migration of the substrate and product bands. B, a 37-mer single-stranded uracil-containing (U) oligonucleotide was 32P-labeled at the 5’ end and treated at 37 °C for 60 min with purified recombinant MED1 protein or Udg, as a positive control. The reactions were then treated with 100 mM NaOH at 90 °C for 30 min, in order to cleave the AP site. A cleavage product was detected for the Udg-treated (lane 2) but not for the MED1-treated reaction (lane 1). Arrows mark the expected migration of the substrate and product bands. C, recombinant MED1 protein was incubated at 37 °C for 15 min with the indicated oligonucleotide substrates 32P-labeled on the bottom strand and containing 5-fluorouracil (FU) paired with A, C, G, and T. MED1 displayed 5-FU glycosylase activity specific for G:5-FU mismatches. The G:U oligonucleotide substrate constitutes a positive control. Arrows mark the expected migration of the substrate and product bands.
the kinetic studies were then conducted at the following optimal conditions: temperature = 37 °C, pH = 7.5, and [NaCl] = 0 mM, in Buffer E (see “Experimental Procedures”).

For the kinetic experiments, the 32P-labeled G:T oligonucleotide substrate was incubated with MED1 and aliquots of the reaction were removed at time points ranging from 15 s to 10 h. Reactions were stopped by incubation with NaOH at high temperature, which also cleaves at the AP site. Reactions were run in denaturing gels to separate the substrate (38-mer) from the product (16-mer), and the intensities of the substrate and product bands were quantified with a PhosphorImager. The reaction curve is biphasic with an initial burst followed by a slower phase (Fig. 5, A and B). Near-maximum product generation takes place after approximately 2 h; for the subsequent 8 h, there is almost no additional formation of product and the reaction proceeds at a very slow rate (Fig. 5, A and B). This and subsequent experiments were conducted at equimolar or near-equimolar concentrations of substrate and enzyme (as determined by protein assay with the Bradford method, under “Experimental Procedures”). Under these conditions, 30–50% of the substrate was typically converted into product in 10 h. When the assay was performed with different enzyme concentrations, all lower than substrate concentration, an estimate of the active enzyme concentration could be derived (32). Since the turnover rate is slow, the amplitude of the burst directly reflects active site concentration (32). We concluded that the activity of the various preparations of MED1 ranges from 30% to 50% (data not shown). (An additional source of interassay variability, i.e. the efficiency of annealing of the mismatched oligonucleotides, was deemed negligible because only annealed molecules act as templates for the DNA polymerase to incorporate the 3′-label.) Thus, since the concentration of reaction product is limited by the concentration of active MED1 enzyme, there is a stoichiometric or near-stoichiometric relationship between enzyme and substrate/product. This indicates that each molecule of active MED1 enzyme efficiently removes only approximately one mismatched thymine. To further explore this near-stoichiometric relationship and rule out the artificial possibility that a fraction of substrate could not be converted into product, we added additional aliquots of enzyme after the reaction reached its slow phase. This resulted in additional conversion of substrate into product (Fig. 5C), showing that the remaining substrate was competent to partake in the reaction.

**Kinetic Analysis of MED1 Glycosylase Reaction**—The single-turnover of MED1 can be explained by two alternative mechanisms: (i) MED1 is inactivated during the reaction, or (ii) MED1 does not efficiently release its product. In order to discriminate between the two possibilities, we conducted an analysis of the kinetic data. Because the kinetic studies were conducted under conditions of similar concentrations of substrate and enzyme, and within a 10-h incubation, the assumptions required for steady state kinetic analysis are not applicable. In particular, in our experiments, the concentration of enzyme is not much smaller than the concentrations of substrate/product, and there is only a slow (but measurable) enzyme turnover within the reaction time course. Applying standard steady state kinetic analyses, under the required experimental conditions of enzyme concentration much less than substrate concentration, would thus require impractically long reaction times (32). We therefore analyzed the data using the kinetic simulation program KINSIM. KINSIM utilizes numerical integration to predict the time course of a reaction as governed by a kinetic mechanism and the reactant concentrations (16–18). For MED1 and a particular substrate, the time courses at different protein and DNA concentrations could all be fit using the same rate constants, supporting the validity of the kinetic mechanism. We then derived kinetic parameters (Table II) for MED1 glycosylase reaction according to Scheme I.

\[
E + \text{DNA(G:T)} \stackrel{k_{in}}{\longrightarrow} E \cdot \text{DNA(G:T)} \stackrel{k_{cat}}{\longrightarrow} E + \text{DNA(G:AP)}
\]

**Scheme I**

The lower limit for the substrate binding rate (200 μM⁻¹ s⁻¹)
is estimated from the value required to fit the data at the lowest substrate concentrations. This value is near the diffusion limit, showing that the enzyme is quite efficient in substrate recognition. The $k_{\text{cat}}$ for the G:T mismatch is comparable to that reported for TDG, 0.015 s$^{-1}$ (33). Finally, analysis of the data parameters revealed that indeed the rate of release of the AP site product ($k_{\text{off}}$) is very small (approximately $8 \times 10^{-6}$ s$^{-1}$) (Table II).

### Table II

Comparison of MED1 glycosylase activity on G:T and G:U mismatches

| Substrate | $k_{\text{on}}$ | $k_{\text{cat}}$ | $k_{\text{off}}$ |
|-----------|----------------|----------------|----------------|
| G:T       | $\geq 200$     | 0.012          | $8 \times 10^{-6}$ |
| G:U       | $\geq 200$     | 0.05           | $8 \times 10^{-6}$ |

- The lower limit for $k_{\text{on}}$ is obtained from the minimal value required to reproduce the initial phase of the reaction at the lowest reactant concentrations.
- The uncertainty in these values is ±20%. Given the non-steady state kinetics, the term $k_{\text{on}}$ is used here loosely to indicate the constant rate of the glycosylase step, i.e. in place of $k_c$.
- The uncertainty in these values is ±50%, as a result of the very slow rates.

Preincubation with the Reaction Product (AP Site) Inhibits MED1 Glycosylase Activity—The small value of $k_{\text{on}}$ predicts that incubation of MED1 with DNA containing an AP site prior to reaction with the G:T substrate would reduce the concentration of free enzyme and, therefore, lead to a decrease in thymine release from the substrate. This has been shown previously for TDG (33). We then preincubated MED1 with a double-stranded oligonucleotide containing an AP site opposite a G; as controls, MED1 was preincubated with a double-stranded oligonucleotide containing a G:C match or with reaction buffer lacking DNA. The preincubation was followed by incubation with a radioactive G:T substrate. As shown in Fig. 6, in comparison to the buffer and G:C oligonucleotide controls, the rate of thymine removal from the G:T substrate is reduced by preincubation with the AP site oligonucleotide. Thus, this confirms that the biphasic kinetics of MED1 glycosylase reaction is a consequence of high product affinity with slow release rather than enzyme inactivation.

Comparison of MED1 Thymine and Uracil Glycosylase Reactions; Substrate Binding Is Not Limiting for Glycosylase Activity—We next conducted a kinetic analysis of the MED1 uracil glycosylase activity on a G:U substrate. Assuming that the $k_{\text{cat}}$ of the AP site is the same as determined for the G:T substrate and that the rate of release of the free uracil is similar to that of free thymine, the KINSIM simulation should fit the kinetic data simply by changing the chemical step. Indeed, the $k_{\text{on}}$ is the major difference between the G:T and G:U substrate (Table II): uracil is removed by MED1 from G:U mismatches faster than thymine is removed from G:T mismatches. In order to test the alternate possibility that the differences in glycosylase activity result from different binding of MED1 to the two DNA substrates, we conducted kinetic analysis at increasing equimolar concentrations of MED1 and G:T or G:U substrates. The relative reaction rate did not change at 2.5, 5, and 10 nM concentrations (49), indicating that at these concentrations binding of the enzyme to the two substrates is essentially saturated (33). This also confirms the KINSIM prediction that the different reaction rate on G:U and G:T substrates is not due to differences in substrate binding but rather reflects intrinsic differences in $k_{\text{on}}$ (see Table II). If the association and dissociation rates of the enzyme-substrate and enzyme-product complexes are comparable, the values of $k_{\text{on}}$ and $k_{\text{off}}$ in Table II provide an estimate of the affinity of the complexes of approximately $10^{-14}$ M. Enzyme-substrate dissociation constants in the subnanomolar range have also been reported for MutY and TDG (32–34), indicating that each of these DNA repair enzymes will efficiently recognize a low density of mismatches in DNA.

The MED1 5-Methylcytosine Binding Domain and Methylation of the Mismatched Substrate Are Not Required for Efficient Catalysis—The preferential activity of MED1 on substrates presenting a G:T mismatch within the context of a CpG site (Fig. 3) raises the possibility that recognition of methylated DNA by the MBD domain is important for MED1 glycosylase activity. To test this possibility, we compared the thymine glycosylase activity of wild type MED1 and a recombinant deletion mutant lacking the MBD and encompassing only the catalytic domain (amino acids 455–580) (10). The two polypeptides processed an Mpg/TpG substrate with very similar kinetics (Fig. 7A). In a parallel experiment, a deletion mutant (amino acids 1–454) (10) lacking the catalytic domain was completely inactive (data not shown). Thus, the catalytic domain of MED1 is necessary and sufficient for glycosylase activity, whereas the MBD is dispensable in this assay.

Since the MBD is dispensable, then methylation of the mismatched CpG site may also be nonessential. We then determined the kinetic profile of the MED1 thymine glycosylase reaction on methylated and unmethylated mismatched CpG substrates. Mpg/TpG and Cpg/TpG substrates were processed by MED1 with very similar kinetics (Fig. 7B). Thus, methylation of the mismatched substrate is not required for efficient catalysis by MED1.

### DISCUSSION

In the present study, we sought to define the biochemical and enzymatic properties of the DNA repair protein MED1, also known as MBD4, which we identified as an interactor of MLH1. We show here that MED1 acts as a mismatch-specific thymine and uracil DNA N-glycosylase. Conclusive demonstration of MED1 thymine glycosylase activity is provided by the direct mass spectrometric detection of the reaction product, i.e. the thymine-less oligonucleotide generated by incubation of MED1 with a G:T-containing oligonucleotide substrate. The uracil glycosylase activity of MED1 is limited to G:U mismatches; MED1 does not have glycosylase activity on uracil paired with adenine, cytosine, or thymine in double-stranded DNA substrates, or on uracil present in single-strand DNA. The enzyme lacks AP lyase activity; MED1 fails to cleave the AP site generated by its glycosylase activity or by an heterologous glycosylase, such as UDG.

These observations are in agreement with an initial description of the mismatch-specific thymine and uracil glycosylase activity of this enzyme, which was reported during the course of our investigation (35). Importantly, our current findings further address central questions concerning the function of MED1, namely its detailed mechanism of action, substrate spectrum, and relative roles of the catalytic and 5-methylcytosine binding domains.

In its thymine and uracil glycosylase activity on G:T and G:U mismatches and its preference for substrates containing these mismatches in the context of CpG sites, MED1 is similar to TDG (29–31). Both enzymes, despite the lack of sequence similarity, appear to counteract the mutagenic potential of the deamination of 5-methylcytosine and cytosine by removing the mismatched thymine and uracil. These deamination reactions occur spontaneously at a remarkable rate of 2–300 events/genome/day and, if not repaired, lead in the next round of DNA replication to C → T and G → A transitions on complementary strands (1, 2, 36–38). These transitions at CpG sites are a frequent source of interspecies genetic divergence on an evolutionary scale (39), contribute to human genetic variation (40),
and, most importantly, comprise the most frequent mutations in human cancer (36–38, 41). Thus, for their biochemical activity, MED1 and TDG seem to act as “caretakers” of genomic fidelity at CpG sites and raise the possibility that they may be relevant to the pathogenesis of human cancer. In keeping with this possibility, we and others demonstrated that the MED1 (MBD4) gene is frequently mutated in human colorectal and extracolonic carcinomas exhibiting microsatellite instability (12, 13). In colorectal cancer specimens, we also detected loss of heterozygosity at the MED1 (MBD4) locus, suggesting that this gene may act as a tumor suppressor (12). On the contrary, TDG mutations in human carcinomas have been elusive (42, 43).

In order to gain insight in the molecular mechanisms of MED1 action, we conducted a kinetic analysis of its glycosylase activity. We found that the MED1 glycosylase reaction follows pre-steady state burst kinetics. Each molecule of active enzyme efficiently removes approximately one mismatched thymine from G:T-containing substrates. Because of this stoichiometric or near-stoichiometric relationship between enzyme and substrate/product, the concentration of active MED1 enzyme limits the accumulation of reaction product. This results in a biphasic time course with an initial burst of activity followed by a slow phase. By using a preincubation experiment, we discriminated between the two possibilities that would result in biphasic kinetics, i.e. inactivation of the enzyme after a single reaction cycle or tight binding to the AP site reaction product. Indeed, incubation of MED1 with an AP site-containing oligonucleotide prior to incubation with the 32P-labeled G:T substrate inhibited MED1 thymine glycosylase activity. Binding of MED1 to an AP site-containing oligonucleotide in an electrophoretic mobility shift assay has also been reported (35). Here we show that this binding is very tight (estimated $k_{off}$ of approximately $8 \times 10^{-6} \text{s}^{-1}$) and is responsible for the biphasic kinetics of MED1.

Thus, MED1 is the third DNA glycosylase that exhibits biphasic kinetics, joining the adenine glycosylase MutY (32) and the thymine glycosylase TDG (33). All three enzymes exhibit single-turnover, pre-steady state kinetics due to the slow release of the AP site reaction product. This emerging feature of base excision repair glycosylases is likely to underscore an important biological function; the tight binding of the glycosylase allows protection of the AP site and prevents its nonspecific processing until subsequent repair activities are recruited at the lesion site (32, 33). Indeed, it has been shown that AP endonuclease could displace TDG from an AP site, thus increasing the turnover of the glycosylase (44). It would be interesting to determine whether MED1 is also displaced by the AP endonuclease. An additional and not necessarily alternative biological explanation of the slow $k_{off}$ of these glycosylases is that binding to the AP site provides a convenient way to signal a cell cycle checkpoint and thus minimize the possible mutagenic consequences of cell cycle progression in the presence of DNA damage. This possibility is particularly intriguing for MED1, since its mutations in human carcinomas would abrogate such a checkpoint. It would be interesting to evaluate whether this putative checkpoint is regulated via an association with other DNA repair and signaling molecules, including MLH1, a known interactor of MED1 (10).

The availability of kinetic information and the possibility of evaluating activity throughout the entire reaction timeline allowed us to make meaningful comparisons of different MED1 mutants and substrates. This provided the opportunity to explore the respective roles of MED1 catalytic and 5-methylcytosine binding domains. We found that MED1 processes with essentially identical kinetics oligonucleotide substrates containing a G:T mismatch in the context of a methylated or unmethylated CpG site (Fig. 7B). Moreover, an oligonucleotide substrate containing a G:T mismatch in the context of a methylated CpG site is processed with essentially identical kinetics by wild type MED1 or by a deletion mutant corresponding to the isolated catalytic domain (amino acids 455–580) and thus lacking the MBD domain (Fig. 7A). We concluded that the MBD domain and methylation of the substrate at the mismatched CpG site are not required for efficient catalysis. In an electrophoretic mobility shift assay, the MBD domain of MED1 was reported to bind to a 50-base pair oligonucleotide containing three Mpg/GpT substrate sequences within 27 base pairs (35). Based on this finding, it was suggested that the MBD domain might target mismatches for processing by the catalytic domain in a coordinate action (35). However, our comparison of the kinetic profile of MED1 mutants and methylated and unmethylated substrates does not support a role of the MBD and substrate methylation in the catalytic processing of oligonucleotide substrates. Thus, it appears that the isolated catalytic domain of MED1 can effectively recognize and process an unmethylated or methylated CpG/GpT substrate and that the MBD domain is dispensable for this function. Interestingly TDG, which, like MED1, has preferential activity toward G:T substrates in the
context of a CpG site regardless of its methylation status (34, 45–47), lacks an MBD domain altogether.

What is then the function of the MBD domain? It is possible that the MBD domain may facilitate the localization of MED1 to Mg-rich regions of the genome in vivo (11), where demethylation of 5-methylcytosine might be more frequent. This could reflect a specific role of MED1 in the repair of G/T mismatches, distinct from that of TDG, which lacks an MBD domain. Thus, MED1 and TDG, despite their biochemical similarities, may only be partially redundant in vivo and may display different roles in genomic fidelity and mutation avoidance in human cells.

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FIG. 7. The MBD domain and methylation of the mismatched substrate are not required for efficient catalysis by MED1. A, comparison of the kinetic profile of thymine glycosylase activity of wild type MED1 and a deletion mutant encompassing only the catalytic domain (cat. dom.), amino acids 455–580). The substrate was a duplex oligonucleotide with a Mpg/TpG mismatch. B, comparison of the kinetic profile of thymine glycosylase activity on methylated and unmethylated mismatched CpG substrates. The substrates were duplex oligonucleotides with a Mpg/TpG or CpG/TpG mismatch.