Rare DNA lesions that are chemically stable and refractory to repair may add disproportionately to the accumulation of mutations in long-lived cells. 3-Methylthymine is a minor lesion that is induced by DNA-methylating agents and for which no repair process has been described previously. Here we demonstrate that this lesion can be directly demethylated in vitro by bacterial and human DNA dioxygenases. The Escherichia coli AlkB and human ABH3 proteins repaired 3-methylthymine in both single-stranded and double-stranded polydeoxynucleotides, whereas the human ABH2 protein preferred a duplex substrate. Thus, the known substrates of these enzymes now include 3-methylthymine in DNA, as well as 1-methyladenine and 3-methylcytosine, which all have structurally similar sites of alkylation. Repair of 3-methylthymine by AlkB and ABH3 was optimal at pH 6, but inefficient. At physiological pH, 3-methylthymine, which is a minor methylated lesion, was optimal at pH 6, but inefficient. Major damaged DNA bases that have the potential to directly miscode, such as 8-hydroxyguanine and O6-methylguanine (O6-meG), are rapidly repaired; therefore, minor DNA lesions that are chemically stable and refractory to repair may contribute disproportionately to the accumulation of mutations (3). Such lesions that block the progression of DNA replication may persist and result in mutagenesis if bypassed by error-prone translesion DNA polymerases (4, 5). Furthermore, accumulation of DNA lesions in non-dividing cells may generate mutant proteins (6). Two methylated bases, 3-methylthymine (3-meT) and 3-methylcytosine (3-meC), for which no DNA repair process was known, were proposed previously as possible candidates for persistent lesions (3). However, 3-meC is now known to be corrected by a new mechanism of oxidative demethylation catalyzed by DNA dioxygenases (7–10). In this work, we examine whether the minor stable lesion 3-meT is a substrate of such enzymes or whether this lesion is refractory to DNA repair.

Methylating agents can generate base adducts at ring nitrogens and exocyclic oxygens (11). The mechanisms by which most of these lesions are repaired are now well characterized (12). The major harmful lesions formed in double-stranded DNA are O6-meG and 3-methyladenine (3-meA). O6-meG, a directly miscoding lesion, is rapidly demethylated by O6-meG-DNA methyltransferases, which are conserved from bacteria to man. These proteins transfer the methyl group onto one of their own active cysteine residues resulting in their self-inactivation (13, 14). 3-meA in DNA blocks DNA replication and is a cytotoxic lesion, but it may occasionally generate mutations when bypassed by translesion DNA polymerases that have flexible base-pairing properties. To prevent the harmful effects of 3-meA, it is excised from DNA by ubiquitous 3-methyladenine-DNA glycosylases in the initial step of the base excision-repair pathway (15). 7-meG is an abundant lesion that is relatively innocuous; it is slowly lost from DNA by spontaneous hydrolysis and also by very slow excision by 3-methyladenine-DNA glycosylases (15). Several minor adducts induced in double-stranded DNA, including 3-methylguanine, O2-methylthymine, and O4-methylcytosine, are excised by the AlkA 3-meA-DNA glycosylase in Escherichia coli, and O2-methylthymine is demethylated by O2-meG-DNA methyltransferases in E. coli and human cells (for review, see Ref. 12).

1-Methyladenine (1-meA) and 3-meC are induced in single-stranded regions of DNA, where they are the main alkylation lesions formed (11, 16). As a consequence, these non-coding lesions probably arise in vivo at DNA replication forks and transcription bubbles, where they will block the progression of DNA and RNA polymerases (17, 18). 1-meA and 3-meC are chemically stable lesions that do not appear to be liberated by a DNA glycosylase (19); instead, a novel DNA repair mechanism is used to destabilize the methyl group (7, 8, 19–21). α-Ketoglutarate/Fe(II)-dependent dioxygenases are a superfamily of enzymes that couple oxidative decomposition of α-ketoglutarate to hydroxylation of their target substrates (22). The E. coli AlkB and human ABH2 and ABH3 DNA dioxygenases are members of this family and use iron-oxo intermediates to hydroxylate the methyl groups of 1-meA and 3-meC in DNA. The resulting hydroxymethyl groups are unstable and are released catalytically as formaldehyde, with the damaged bases reverting to the unmodified form (7–10, 23). AlkB and ABH3 can also slowly repair these lesions in methylated RNA, but the biological significance of this activity is unclear (10, 24).
A minor methylated base for which no DNA repair process has been described yet is 3-meT (3, 25). The 3-meT lesion is of particular interest because of its great chemical stability (11). The 3-position of thymine is normally involved in base pairing; thus 3-meT (like 3-meC and 1-meA) is predicted to be a non-coding lesion. Indeed, both 3-meT and 3-ethylthymine in synthetic polymers completely block DNA synthesis by *E. coli* DNA polymerase I or the Klenow fragment of this enzyme (26–28). Consequently, unrepaired 3-meT in DNA could be a threat to cell viability or may result in mutagenesis if bypassed by translesion DNA polymerases. It was therefore of interest to determine whether this lesion can be actively repaired by DNA dioxygenases. At present the only known methylated substrates of these enzymes are 1-meA and 3-meC in DNA (7–9, 19), so their substrate range appears to be restricted. They do not demethylate 3-meA, 7-meA, 7-meG (7–9), 5-methylcytosine, or thymine (5-methyluracil) in DNA (see below). The 3-meC and 1-meA bases differ from 3-meT in DNA, and such structural features could be essential for substrate recognition by DNA dioxygenases (Fig. 1). To further analyze the specificity of these enzymes, we have investigated whether the *E. coli* AlkB and human ABH2 and ABH3 dioxygenases can demethylate 3-meT residues in DNA.

**EXPERIMENTAL PROCEDURES**

*Non-radiolabeled Heavily Methylated Poly(dT)—* 0.25 mg of poly(dT) (Amersham Biosciences) in 0.5 ml of 200 mM NaH2PO4, pH 10.8, was exposed to 5 μl of dimethylsulfoxide (DMS) at 20 °C for 6 h. During this reaction the pH falls; therefore, 50 μl of 1 M NaOH and a further 5 μl of DMS were added, and the incubation continued for a further 16 h. The methylated poly(dT) was ethanol-precipitated twice. HPLC analysis of acid hydrolysates showed that 70% of the thymine bases had been converted to 3-meT.

*i4C* MePoly(dT)—0.75 mg of poly(dT) in 1 ml of 200 mM NaH2PO4, pH 10.8, was added to 1 mCi of ice-cold *i4C* MeI (54 mCi/mmol, Amersham Biosciences) and incubated at 20 °C for 6 h. To remove the unreacted *i4C* MeI, the methylated polymer was ethanol-precipitated three times and dialyzed against water. HPLC analysis showed that ~3% of the thymine bases were methylated to generate *i4C* 3-meT. The specific activity of the *i4C*-methylated poly(dT) substrate was 1580 cpm/μg of poly(dT) and 118 cpm/pmol of 3-meT. To prepare a double-stranded substrate, the *i4C*-methylated poly(dT) was annealed to poly(dA) at 20 °C for 1 h. *i4C*-Methylated poly(dC) was prepared as described previously by treatment of poly(dC) with *i4C* MeI at pH 7 for 6 h (7). The specific activity of this substrate was 880 cpm/μg of polymer and 125 cpm/pmol of 3-meT.

**HPLC Analysis**—Thymine residues were released from methylated poly(dT) by hydrolysis in 90% formic acid in sealed glass vials at 180 °C for 40 min. The samples were evaporated to dryness, resuspended in 100 μl of water, and filtered through an Amicon Ultrafree-MC filtration unit (10,000 nominal molecular weight limit). After re-evaporation, the samples were resuspended in 100 μl of water, and 10 μl was analyzed by HPLC on a Phenomenex Synergi Hydro reverse phase column (dimensions, 4.6 × 250 mm) in 50 mM ammonium acetate, pH 3.5, at a flow rate of 0.6 ml/min. After 5 min, a linear gradient of 0–50% methanol was applied over 20 min. The retention times of thymine and 3-meT were 19.5 and 26.2 min, respectively. When analyzing radioradiolabeled substrates, fractions were collected at 0.5-min intervals, and radioactive methylated bases were detected by scintillation counting. When using non-radiolabeled substrates, methylated and unmethylated bases were detected by *A* 100 measurements. The absorption maxima at pH 4 of thymine and 3-meT are 264 and 260 nm, respectively (29). The 3-meT marker was prepared by deamination of 3-methylthymidine (obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repository) in 90% formic acid at 180 °C for 40 min. After evaporation to dryness, the hydrolysate was resuspended in water. HPLC analysis revealed a single UV light-absorbing peak of 3-meT.

**Protein Purification**—*His*₆-tagged AlkB protein was overexpressed in *E. coli* and purified as described previously by nickel-agarose affinity chromatography (7). *His*₆-tagged ABH2 and *His*₆-tagged ABH3 were overexpressed in recombinant baculovirus-infected S. frugiperda (Spain) cells as described previously (23). The method was slightly modified; after loading the cell extracts onto nickel-nitriilotriacetic acid agarose (Qiagen), the column was washed with buffer containing 40 mM imidazole, and the proteins were eluted in buffer containing 250 mM imidazole. Purity of both the recombinant enzyme and ABH2 and ABH3 proteins was ~98% as observed by SDS-PAGE.

**Assays of AlkB, ABH2, and ABH3**—AlkB, ABH2, or ABH3 (10 or 20 pmol) was added to the *i4C* MeI poly(dT) substrate (800–3000 cpm) in a reaction mixture containing 50 mM HEPES-KOH or MES-HCl, 75 μM Fe(NH₄)₂(SO₄)₂, 1 mM α-ketoglutarate, 5 mM ascorbate, 50 μg/ml bovine serum albumin, and incubated at 37 °C. The reactions were stopped by adding EDTA to 10 mM, and the polynucleotide substrate was ethanol-precipitated in the presence of carrier calf thymus DNA. Two-thirds of the ethanol-soluble radioactive material was monitored by scintillation counting. When determining the optimum pH for activity on methylated poly(dT), 50 mM MES-HCl or 50 mM HEPES-KOH buffer was used with pH values between 5 and 6.5 or between 7 and 8.5, respectively.

**RESULTS**

**Methylation of Poly(dT)—** 3-meT is a minor product when single- or double-stranded DNA is treated with methyl methanesulfonate, DMS (S₉₂ agents), or methyl nitrosourea (S₉₁ agent) at neutral pH, constituting up to 0.8% of the total alkylated residues (for review, see Ref. 11). The yield of 3-meT is increased when DNA is treated with methyl nitrosourea in alkaline conditions (30) or when free thymidine is exposed to DMS at high pH (31). Thus, to prepare a DNA substrate containing 3-meT, poly(dT) was treated with DMS at pH 10.8. The methylated polymer was hydrolyzed with formic acid, and the released pyrimidine bases were analyzed by HPLC. After treatment of poly(dT) with 100 mM DMS at pH 10.8, ~70% of the thymine bases were converted to 3-meT (Fig. 2). Other possible
minor methylation products of thymine are O2-methylthymine and O4-methylthymine. However, O-alkyl base derivatives are dealkylated by acid hydrolysis (32) and would not be detected by the procedures used here. In any case, O-methylated bases would be very minor products because S,2 agents such as MeI and DMS rarely alkylate oxygens in DNA (11). To prepare a substrate containing radiolabeled 3-meT, poly(dT) was treated with [14C]MeI at pH 10.8 to convert an estimated 3% of the thymine residues to a methylated form.

**Repair of 3-Methylthymine in Poly(dT) by AlkB—**Repair of 1-meA in DNA by *E. coli* AlkB protein was assayed previously by the release of [14C]formaldehyde from [14C]MeI-treated poly(dA) as ethanol-soluble radiolabeled material (7). The ability of AlkB to repair 3-meT in 14C-methylated poly(dT) was therefore initially examined using the same assay. In conditions that are optimal for AlkB to catalyze repair of 1-meA in DNA (7), no detectable release of ethanol-soluble radiolabeled material from the 14C-methylated poly(dT) was observed. AlkB repairs 1-meA in DNA with a sharp pH optimum of 8 (7, 33). The possibility that AlkB may repair 3-meT with a different pH optimum was tested, and activity was observed initially at pH 6.5. The [14C]3-meT bases remaining in the substrate were quantitated by HPLC and showed that 3-meT was actively removed by AlkB (Fig. 3). AlkB directly reverses 1-meA and 3-meC in DNA to adenine and cytosine. To determine whether AlkB reverts 3-meT to thymine, AlkB was incubated with the heavily methylated non-radiolabeled poly(dT) under standard assay conditions at pH 6.5. HPLC analysis of the substrate and integration of the peak areas showed that AlkB catalyzed the stoichiometric reversion of 3-meT lesions directly back to thymine (Fig. 4). Because the peak of thymine increased in the presence of AlkB, we can also conclude that AlkB does not demethylate thymine (5-methyluracil) itself.

By assaying ethanol-soluble material released from 14C-methylated poly(dT) at various pH values, AlkB was shown to repair 3-meT in DNA with a pH optimum of 6 (Fig. 5). Even at this optimal pH, AlkB repaired 3-meT inefficiently, with 10 pmol of the protein removing only 1.4 pmol of 3-meT in 30 min. To verify that AlkB was acting as an α-ketoglutarate/Fe(II)-dependent dioxygenase and therefore repairing 3-meT by oxidative demethylation, the activity was shown to be completely dependent on α-ketoglutarate and inhibited by 0.2 mM EDTA. In the presence of EDTA, the addition of 0.7 mM Fe(II) regenerated the activity (data not shown).

**Repair of 3-Methylthymine in Poly(dT) by Human ABH2 and ABH3—**Like AlkB, ABH3 has a sharp pH optimum of 8 for repair of 1-meA in DNA, whereas ABH2 has a broader optimum of pH 7–7.5 (9, 33). The ability of these human proteins to demethylate 3-meT in DNA was examined over a range of pH values using the 14C-methylated poly(dT) substrate. Similarly to AlkB, ABH3 repaired 3-meT in the single-stranded polymer with an acidic pH optimum of 5.5–6.5 (Fig. 5). Both these enzymes repair 1-meA and 3-meC in single- and double-stranded DNA but prefer the single-stranded substrate (7–10). This is also the case for 3-meT residues, and both AlkB and ABH3 repaired 3-meT in a double-stranded poly(dT)poly(dA) substrate at 20–30% of the rate observed with single-stranded poly(dT) (data not shown). ABH2 activity was not detectable on a single-stranded polynucleotide substrate (Fig. 5). ABH2 repairs 1-meA and 3-meC more efficiently in double-stranded than in single-stranded DNA (10). The possibility that ABH2 might preferentially repair 3-meT in a double-stranded substrate was therefore examined. ABH2 slowly released [14C]ethanol-soluble material from 14C-methylated poly(dT) annealed to poly(dA) (Fig. 6a), and the repair of 3-meT in this
double-stranded polydeoxynucleotide also had a low pH optimum of 6.5 (Fig. 6B).

Repair of 3-meT and 3-meC at Physiological pH—The optimal pH for in vitro repair of 3-meT by E. coli AlkB, human ABH2, and human ABH3 is pH 6–6.5, whereas 3-meC is repaired optimally by these proteins at pH 7.5–8 (7, 33). To assess the relative significance of these activities at physiological pH, the abilities of AlkB and ABH3 to repair 3-meT and 3-meC were compared at pH 7.4 (Fig. 7). At this pH, both AlkB and ABH3 repaired 3-meC more efficiently than 3-meT. Repair of 3-meT at pH 7.4 occurred but was extremely inefficient, requiring ~30 pmol of either protein to repair 1 pmol of 3-meT during a 10-min incubation.

DISCUSSION

Prior to this work, known substrates of E. coli AlkB and human ABH2 and ABH3 were 3-meA, 1-meA, and 1-ethyladenine in DNA, and within this limited set of substrates the larger 1-ethyladenine lesion was inefficiently repaired by the human enzymes (9). E. coli AlkB may be more versatile as in vivo observations suggest that it also dealkylates hydroxethyl, propyl, and hydroxypropyl lesions (23). In this paper, we have extended the substrate range of all three enzymes to include the minor methylation lesion 3-meT. Comparable with the repair of 1-meA and 3-meC, ABH2 primarily repaired 3-meT in double-stranded DNA, whereas AlkB and ABH3 demethylated this lesion more efficiently in single-stranded than in double-stranded DNA.

During the course of this work, we also tested whether two other stable methylated bases, N2-ethylguanine and 5-methylcytosine, were substrates of these dioxygenases. N2-Ethylguanine is generated in DNA upon exposure to acetaldehyde, the primary metabolite of ethanol and a possible initiating event in alcohol-induced cancer (34). It is a stable lesion for which no specific DNA repair process is known. Using oligonucleotides containing a single lesion, we have observed that N2-ethylguanine is not detectably dealkylated by E. coli AlkB.2 Because human ABH2 and ABH3 only inefficiently repair 1-ethyladenine residues (9), it seems unlikely that N2-ethylguanine would be a substrate of these activities. Possibly, the bulky N2-ethylguanine can be excised by an alternative process such as nucleotide excision-repair. The naturally occurring methylated base 5-methylcytosine may have a reversible role in the epigenetic control of gene expression in mammalian cells (35), but the mechanism by which 5-methylcytosine is actively demethylated is uncertain. The possibility that this could occur by oxidative demethylation catalyzed by a dioxygenase has been suggested (7). However, neither of the human DNA dioxygenases ABH2 and ABH3 nor AlkB detectably demethylated radiolabeled 5-methylcytosine in DNA in in vitro assays under a variety of conditions (data not shown). Other related but as yet uncharacterized dioxygenases could possibly be involved in 5-methylcytosine demethylation.

Methylated bases that are not substrates of AlkB, ABH2, and ABH3 are now known to include 3-meA, 7-meG, 7-meA, thymine (5-methyluracil), 5-methylcytosine, and N2-ethylguanine. The sites alkylation on these bases are different, whereas those on 1-meA, 3-meC, and 3-meT are structurally equivalent, although numbered differently on the purine and pyrimidine rings (Fig. 1). Compared with 3-meC and 1-meA, 3-meT was a poor substrate of all three dioxygenases. Consequently, the exocyclic amino group adjacent to the methyl adduct or the positive charge on 1-meA and 3-meC (Fig. 1), although not essential features of the substrate, may be critical for efficient recognition or repair. It is also noteworthy that the different pH optima for repair of these lesions (pH 6–6.5 for 3-meT versus pH 7.5–8 for 3-meC and 1-meA) by AlkB, ABH2, and ABH3 may reflect a necessity for protonation of the substrate or of the metalloenzymes themselves and may influence the structure/3

2 P. J. Brooks, P. Koivisto, B. Sedgwick, and T. Lindahl, unpublished data.

FIG. 5. Optimal pH for repair of 3-meT in DNA by AlkB and ABH3. AlkB, ABH2, or ABH3 (10 pmol) was incubated with [14C]MeI-treated poly(dT) (total of 800 cpm) for 30 min in standard assay conditions at varying pH values. Release of ethanol-soluble radiolabeled material was monitored. ○, no protein; ●, AlkB; ▲, ABH2; ◆, ABH3.

FIG. 6. Repair of 3-meT in a duplex polymer substrate by ABH2. A, ABH2 (20 pmol) was incubated in standard assay conditions at pH 6.5 with the [14C]MeI-treated poly(dT) substrate or with this substrate annealed to poly(dA) (500 cpm/assay). Release of ethanol-soluble radiolabeled material was monitored after various incubation times. ○, single-stranded substrate; ●, double-stranded substrate. B, activity of ABH2 (10 pmol) on the duplex substrate assayed after incubation for 30 min at various pH values.

FIG. 7. Repair of 3-meT and 3-meC by AlkB and ABH3 at physiological pH. Different amounts of AlkB or ABH3 were incubated for 10 min in standard assay conditions at pH 6 or pH 7.4 with [14C]MeI-treated poly(dT) (2000 cpm) or poly(dC) (1000 cpm). Release of ethanol-soluble radiolabeled material was monitored. The substrates were as follows: ●, poly(dT) at pH 6; ●, poly(dT) at pH 7.4; ▲, poly(dC) at pH 7.4. A, AlkB; B, ABH3.
activity relationship of the proteins (36). Future determination of the three-dimensional structure of these proteins together with their substrates should clarify this issue. The restricted substrate range of AlkB, ABH2, and ABH3 can be contrasted with the diverse substrates of the 3-mea-DNA glycosylases that excise not only structurally unrelated alkylated bases such as 3-mea, 7-mea, and O5-meC but also non-alkylated base derivatives including hypoxanthine and ethenoadenine. The methyl group on the substrates of these enzymes apparently makes little contribution to substrate recognition (15).

The inefficient repair of 3-meT by DNA dioxygenases observed here is not surprising, as repair enzymes that act on several different DNA lesions are usually most efficient with their major substrates. For comparison, the major mutagenic DNA lesion O6-meG induced by S1-methylating agents is much more efficiently repaired by O6-meG-DNA methyltransferases than the minor lesion O4-methylthymine (37). The important point with regard to 3-meT is that this chemically highly stable lesion is actively repaired at all. Thus, it would not be expected to slowly accumulate to high levels in the DNA of long lived human cells over a period of many years and decades. In view of the sluggish repair of the lesion, it could still be present at a relevant but low steady-state level in DNA in vivo, interfering with transcription and replication or being bypassed by an error-prone DNA polymerase in translesion synthesis. This argument can also be made for other minor but chemically stable DNA lesions that are actively but slow repaired, such as the 8-diastereoisomer of 5,8-cyclocycloxyadenosine generated by oxygen free-radicals; this bulky endogenous DNA lesion is an exceptionally poor substrate for human nucleotide excision-repair and is not removed by any other known repair process (38). The inefficient repair of 3-meT and other stable DNA lesions therefore might lead to a gradual deterioration of the integrity of cellular DNA and conceivably affect cellular aging.

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