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Methyl phosphotriesters in alkylated DNA are repaired by the Ada regulatory protein of *E. coli*

Tommie V.McCarthy and Tomas Lindahl

Imperial Cancer Research Fund, Mill Hill Laboratories, London NW7 1AD, UK

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ABSTRACT

The E.coli ada^+ gene product that controls the adaptive response to alkylating agents has been purified to apparent homogeneity using an overproducing expression vector system. This 39 kDa protein repairs O^6-methylguanine and O^4-methylthymine residues in alkylated DNA by transfer of the methyl group from the base to a cysteine residue in the protein itself. The Ada protein also corrects one of the stereoisomers of methyl phosphotriesters in DNA by the same mechanism, while the other isomer is left unrepaired. Different cysteine residues in the Ada protein are used as acceptors in the repair of methyl groups derived from phosphotriesters and base residues.

INTRODUCTION

Alkylating agents such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and N-methyl-N-nitrosourea (MNU) react with cellular DNA to generate a number of products, which may be corrected by DNA repair. For example, the most abundant potential cell killing lesion, 3-methyladenine, is removed efficiently by a constitutively expressed DNA glycosylase in all cells investigated (1-3). In E.coli, repair of several other alkylation lesions in DNA is inducible by treatment with methylating agents, and the repair pathway has been termed the "adaptive response" to alkylating agents (4). Thus, the methylated bases, 3-methylguanine, O^2-methylcytosine, and O^2-methylthymine are excised by an inducible DNA glycosylase (5). The important mutagenic lesion O^6-methylguanine is repaired by an entirely different mechanism, involving transfer of the methyl group to a cysteine residue within the inducible transferase itself (6-8). The adaptive response to alkylating agents is under the positive control of the regulatory gene ada^+ (9,10). The gene product, a 39 kDa protein, undergoes rapid cleavage in cell extracts at the

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single Lys-Gln bond in the protein to generate a 19 kDa fragment. This domain (representing the C-terminal half of the Ada protein) is identical with the previously isolated, active O^6-methylguanine-DNA methyltransferase (11,12).

The unusual mode of correction of O^6-methylguanine residues in DNA has stimulated a search for analogous repair functions that would recognize other alkylation lesions. Three groups have recently described an E.coli activity that in a similar fashion transfers a methyl group from an O^4-methylthymine residue in DNA to a protein cysteine residue (5,13,14). The enzyme responsible was shown to be the same methyltransferase as the one acting on O^6-methylguanine, rather than a separate protein (5). Schendel and co-workers have discovered a methyltransferase with an apparent molecular weight of 39,000 which acts on methyl phosphotriesters in DNA (13). Our preliminary experiments (reported in ref. 11) indicated that this activity was yet another function of the Ada protein. This reaction has now been investigated in greater detail, and the repair of one of the two stereoisomers of methyl phosphotriesters in alkylated DNA by the homogeneous Ada protein is described.

MATERIALS AND METHODS

Purification of the Ada protein

The plasmid pBAR2, which contains the entire E.coli B ada^+ gene cloned into the pEMBL8 vector, was obtained from Dr. B. Sedgwick. The insert was located at the HindIII site of the vector, under control of the lac promoter (12). E.coli 1157 nalA harbouring pBAR2 were grown at 30°C to 2 x 10^8 cells $\cdot$ ml^{-1} in 2 l of L-Broth containing 50 µg $\cdot$ ml^{-1} ampicillin. The incubation temperature was then shifted rapidly to 42°C and isopropyl β-D-thiogalactoside (IPTG) added to a final concentration of 1 mM. After 10 hr at 42°C the cells were harvested by centrifugation, washed and resuspended in 25 ml of buffer A (50 mM Tris $\cdot$ HCl, pH 7.9, 20 mM 2-mercaptoethanol, 3 mM EDTA, 5% glycerol) containing 0.3 M NaCl. All operations were performed at 0°C to 4°C. After disruption of the bacteria by ultrasonic treatment, the cell debris was removed by centrifugation at 12 000 x g for 20 min and the supernatant was recovered (Fraction I, 23 ml). Nucleic acids were

precipitated by the addition of neutralized Polymin-P to a final concentration of 0.5 %. After 20 min the mixture was centrifuged at 12 000 x g for 30 min and the supernatant recovered (Fraction II, 25 ml). Crystalline ammonium sulfate was added to 28 % saturation, and the small precipitate was removed by centrifugation. Additional ammonium sulfate was then added to the supernatant to a final concentration of 52 % saturation, and the resulting precipitate was collected by centrifugation and suspended in 2 ml of buffer A containing 0.5 M NaCl (Fraction III, 4 ml). Gel filtration was carried out by applying the sample directly to a column (2.6 x 150 cm) of Ultrogel AcA54 (LKB Products) equilibrated with the same buffer. When the eluted protein fractions were assayed for O^6-methylguanine-DNA methyltransferase activity, a major symmetrical peak of activity was observed, corresponding to an apparent molecular weight of about 39 000. The most active fractions were pooled and dialysed overnight against buffer B (30 mM potassium phosphate, pH 7.9, 20 mM 2-mercaptoethanol, 3 mM EDTA, 10 % glycerol) containing 0.2 M NaCl (Fraction IV, 21ml). One volume of buffer B (without NaCl) was added to fraction IV, and the solution was immediately loaded onto a single-stranded DNA cellulose column (1.5 x 10 cm) equilibrated with buffer B containing 0.1 M NaCl. After washing with the same buffer and buffer B containing 0.18 M NaCl, the enzyme was eluted with buffer B containing 0.3 M NaCl. Active fractions were pooled (Fraction V, 12 ml), dialysed against the elution buffer supplemented with glycerol to a final concentration of 50 %, and stored at -20°C. This Ada protein fraction was 35-fold purified, in 36 % yield. Fraction V was stable as a 39 kDa protein at -20°C for several weeks with little or no decrease in methyltransferase activity. A summary of the purification procedure is given in Table 1. Protein concentrations were measured by the Coomassie Blue method (15).

Alkylated DNA

A double stranded alkylated DNA substrate containing 75 % of its total radioactivity as O^6-methylguanine residues (spec. act. 920 cpm $\cdot \mu g^{-1}$ DNA) was prepared by methylation of *Micrococcus luteus* DNA with [^{3}H]MNU (declared spec. act. 1.0 Ci $\cdot$ mmol^{-1}, NEN Inc.), followed by heating at 80°C for 16 h to remove N-alkylated

TABLE I Purification of E.coli Ada protein

Fraction		Total Protein (mg)	Total Activity (units)	Specific Activity (units • mg^{-1})
I.	Crude Extract	322	157,000	490
II.	Polymin-P	218	-	-
III.	Ammonium Sulfate	130	119,000	920
IV.	Gel Filtration	7.2	74,800	10,400
V.	DNA-Cellulose	3.45	56,700	17,300

purines by selective depurination (16). An aliquot of this DNA substrate (220 µg) containing 460 pmol O^6-methylguanine was incubated with a two-fold excess of the previously purified homogeneous 19 kDa form (8) of O^6-methylguanine-DNA methyltransferase (920 units) for 30 min at 37°C. The protein was then removed from the DNA by extractions with phenol and chloroform/isoamyl alcohol. After ethanol precipitation, the DNA was redissolved in 10 mM Tris • HCl, pH 7.5, 1 mM EDTA; it contained less than 1% of its total radioactivity as O^6-methylguanine residues (as determined by HPLC analysis of hydrolysates), with > 80% of the ^{3}H-labelled material in the form of methyl phosphotriesters (spec. act.220 cpm • μg^{-1} DNA). Poly(dT) and poly(dA) were prepared with terminal deoxynucleotidyltransferase. For alkylation of poly(dT) 1 mCi of [^{3}H]MNU (spec. act. 1.0 Ci • $mmol^{-1}$,) in ethanol was lyophilised to remove the solvent and then employed to treat 83 µg of the polymer in 0.2 ml of 10 mM Tris • HCl, pH 7.5, 1 mM EDTA for 1 h at 37°C. After extensive dialysis, the methylated poly(dT) (spec. act. 3180 cpm • μg^{-1}) was annealed with an equimolar amount of poly(dA). From HPLC analyses of hydrolysates, it was estimated that the phosphotriester Tp(Me)T accounted for 90% of the total alkylation products.

Enzyme assays

For the purification of the Ada protein, a standard assay for O^6-methylguanine-DNA methyltransferase activity measuring the disappearance of O^6-methylguanine residues from alkylated DNA was employed (8), but incubations were performed for 1 h at 37°C. The kinetics of removal of O^6-methylguanine from [^{3}H]MNU-treated

DNA by the purified Ada protein were determined as described (19). For methyl phosphotriester repair assays, the transfer of radioactive methyl groups from alkylated DNA to a protease-sensitive form was measured (6,13). Reaction mixtures (100 µl) contained 70 mM Hepes • KOH, pH 7.8, 1 mM EDTA, 10 mM dithiothreitol, 5% glycerol, 8 µg of the ^{3}H-alkylated DNA substrate containing largely methyl phosphotriesters or 3 µg of [^{3}H]MNU-treated poly(dT) • poly(dA), and Ada protein. After 60 min at 37°C (unless indicated otherwise), the reactions were stopped by rapid chilling to 0°C followed by addition of an equal volume of 0.8 M trichloroacetic acid. The acid-precipitated material was collected by centrifugation and redissolved in 0.1 M Tris • HCl, pH 8.0, 1 mM EDTA (100 µl), and proteinase K (Merck) was added to a final concentration of 250 µg • ml^{-1}. The reaction mixtures were incubated at 37°C for 1 h and re-precipitated with trichloroacetic acid. The supernatants containing protease-digested material were recovered after centrifugation and their radioactivity determined. In order to assess the amount of methylated residues remaining in the DNA substrate, the acid precipitated material was resolubilized by boiling in 0.1 M Tris• HCl, pH 8.0, 2 % SDS and its radioactivity determined. When [^{3}H]MNU-treated poly(dT) • poly(dA) was employed as substrate, the acid-precipitable material remaining after proteinase K digestion was washed in 80 % ethanol and redissolved in 100 µl of 50 mM Tris • HCl, pH 7.5, 0.1 mM EDTA, 8 mM $MgCl_2$. The polymer was then hydrolysed to deoxyribonucleosides by incubation with DNase I, snake venom phosphodiesterase and bacterial alkaline phosphatase (5). Non-radioactive Tp(Me)T was added as an authentic marker to each sample before analysis by HPLC.

HPLC analysis of methyl phosphotriesters

The methyl phosphotriester Tp(Me)T was a generous gift from Dr. P.D. Lawley. The compound had been synthesized according to Swenson et al. (17) and comprised approximately equal amounts of the R and S stereoisomers. Separation of the two isomers of Tp(Me)T was achieved by reverse phase HPLC on a Varian MHC-10 column according to an elution procedure developed by Dr. M. Weinfeld (pers. commun.). A flow rate of 2 ml • min^{-1} with 10 % methanol in water was employed from time 0 to 20 min, followed by

a linear gradient to 40 % methanol from 20 min to 80 min. Material appearing between 53 min and 60 min was collected as 0.2 min fractions and contained the stereoisomers of Tp(Me)T, eluting as two separate peaks at 55.5 and 57 min.

RESULTS

Isolation of the Ada protein

The plasmid pBAR2 was constructed and used for determining the DNA sequence of the ada^+ gene (12). A cloned HindIII DNA fragment of E.coli B that encodes the entire sequence for the Ada protein and its promoter was inserted into the structural gene for a β-galactosidase fragment, in the vector pEMBL8 (18). In pBAR2 the ada^+ gene is under control of the lac promoter, and consequently the Ada protein should be overproduced in bacteria transformed with pBAR2 when induced for lac expression. Fusion of the Ada protein to the β-galactosidase fragment was not likely to occur since the cloned HindIII fragment encoding the ada^+ gene has translational stop codons in the 5' and 3' flanking regions of the structural sequence (12). When E.coli AB1157 nalA harbouring the plasmid pBAR2 were grown at 42°C in the presence of the lac operon inducer IPTG, and crude cell extracts were subsequently analysed by gel electrophoresis, a 39 kDa protein was observed to be produced in large amounts accounting for 2-3 % of the total protein present (Fig. 1, lane b). This protein was identified as the Ada protein by immunoblotting (data not shown) with antibodies directed against the 19 kDa form of O^6-methylguanine-DNA methyltransferase (11). No such protein was detected in cells harbouring only the vector plasmid pEMBL8 (data not shown).

Using E.coli carrying plasmid pBAR2 as a source of material, we have purified the Ada protein. In order to minimize proteolytic cleavage of this labile protein in crude enzyme fractions, the initial purification steps were performed at NaCl concentrations > 0.3 M. After ammonium sulfate fractionation and gel filtration, two major protein bands were visible on SDS-polyacrylamide gels with apparent molecular weights of 39 000 and 41 000 (Fig. 1, lane e). In the final purification step, the Ada protein bound to single stranded DNA-cellulose at low ionic

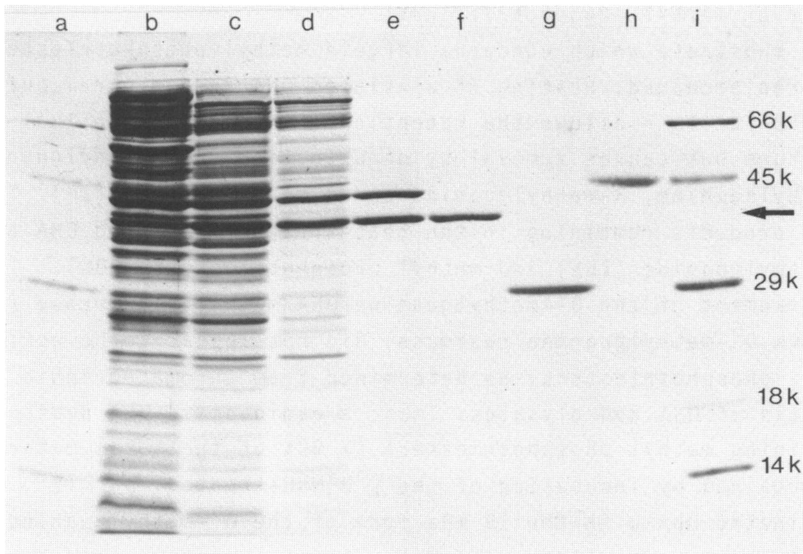

Fig. 1. SDS-polyacrylamide gel electrophoresis of the Ada protein during purification. Aliquots of fractions I-V containing 60 units of transferase activity, and reference proteins (2 µg per lane) with apparent molecular weights of 66 000, 45 000, 29 000, 18 400, and 14 300 were denatured by heating at 100°C for 2 min in a mixture of 50 mM Tris•HCl, pH 6.8, 2 mM EDTA, 2 % SDS, 5 % 2-mercaptoethanol, 10 % glycerol and electrophoresed on a 15 % polyacrylamide gel containing 0.1 % SDS in a running buffer of 50 mM Tris base, 0.384 M glycine, 0.1 % SDS for 2 h at 25 mA (32). Protein bands were visualized by staining with Coomassie Brilliant Blue. The 39 kDa protein band is indicated by an arrow.
Lanes a,g,h,i: Reference proteins. Lanes b-f: Ada protein, fractions I-V respectively.

strength while the major protein contaminant was not adsorbed. The 39 kDa Ada protein was then eluted in apparently physically pure form at a higher salt concentration (Fig. 1, lane f). The purified Ada protein is stable as a 39 kDa protein in 50 mM Tris•HCl, pH 7.9, for at least 4 h at 37°C, in the presence or absence of alkylated DNA. Thus, in contrast to the Ada protein in crude cell lysates which is cleaved rapidly (11), the purified protein does not undergo proteolysis. These data indicate that the processing activity is due to another protein which is removed during purification of the Ada protein. Similar results have been obtained by Y. Nakabeppu and M. Sekiguchi (pers. commun.).

Repair of methyl phosphotriesters

A DNA substrate which contains largely methyl phosphotriesters has been prepared. Heating of alkylated DNA in a neutral buffer at 80°C for 16 h allows the retention of its double helical structure but causes removal by depurination of 7-methylguanine, 7-methyladenine, 3-methylguanine and 3-methyladenine (16). The major products remaining in the heat-treated alkylated DNA are O^6-methylguanine (75%) and methyl phosphotriesters (20%). The 19 kDa fragment of the O^6-methylguanine-DNA methyltransferase (which repairs O^6-methylguanine residues) did not appear to be active on methyl phosphotriesters, as determined from chromatographic analysis of DNA hydrolysates. Thus, a radioactive DNA substrate containing methyl phosphotriesters (> 80% of the total material) was obtained by incubation of the [^{3}H]MNU-treated, partly depurinated DNA with the 19 kDa form of the O^6-methylguanine-DNA methyltransferase. (In addition to phosphotriesters, minor lesions such as 1-methyladenine persist in this substrate and account for 10-20% of the radioactive material). Incubation of a two-fold excess of the purified 39 kDa Ada protein with the DNA substrate containing largely [^{3}H]methyl phosphotriesters resulted in rapid disappearance of 35% of the methyl groups from the DNA, with a concomitant rapid appearance of an equal amount of radioactive material in protease sensitive form (Fig. 2). These data indicate that methyl residues were transferred enzymatically from approximately one-half of the methyl phosphotriesters in the DNA to the Ada protein. Chromatographic analysis of amino acid hydrolysates of the self-methylated Ada protein showed that > 90% of the radioactive material was present as S-methylcysteine. When limiting amounts of Ada protein were employed in reaction mixtures, the extent of methyl phosphotriester repair was proportional to the protein concentration but independent of time between 5 and 50 min (results not shown), reflecting suicidal enzyme inactivation as a consequence of the reaction of the Ada protein with the alkylated DNA. From the specific radioactivity of the alkylated DNA that contained largely methyl phosphotriesters and the amounts of purified Ada protein employed, it could be estimated that about 0.8 methyl groups were removed from the DNA per protein molecule. This probably reflects

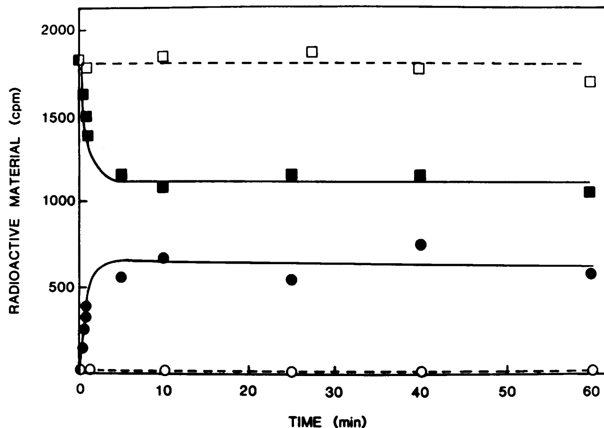

Fig. 2. Reaction kinetics of enzymatic repair of methyl phosphotriesters in [^{3}H]MNU alkylated DNA by purified Ada protein at 37°C. Alkylated DNA containing > 80 % of its radioactivity as methyl phosphotriesters was incubated with a 3-fold excess of the enzyme. Each reaction mixture contained 4 pmol methyl phosphotriesters in 8 µg alkylated DNA and 12 pmol of purified 39 kDa Ada protein (closed symbols) or 12 pmol of the purified 19 kDa protein fragment with O^6-methylguanine-DNA methyltransferase activity (open symbols). The transfer of radioactive material to a proteinase K-sensitive form (circles) and the radioactive material remaining in the DNA substrate (squares) were determined.

a 1:1 stoichiometric relationship.

Selective methyl group transfer from one stereoisomer

The two isomeric DNA methyl phosphotriesters (isomer I and II), in the R and S configuration, were found to account for 90% of the total alkylation products in poly(dT) treated with MNU, as determined by HPLC analyses of enzymic hydrolysates. Thus, the minor alkylation lesions O^4-methylthymine, O^2-methylthymine, and 3-methylthymine accounted for less than 10% of the total radioactivity. Schendel and coworkers (13) found that extracts from E.coli cells induced for the adaptive response contain a methyltransferase activity, apparently distinct from the 19 kDa O^6-methylguanine-DNA methyltransferase, which removes 50 % of the methyl phosphotriesters present in [^{3}H]MNU-treated poly(dT)·poly(dA). We have obtained similar results with regard to phosphotriester removal using the purified Ada protein and an alkylated DNA substrate (Fig. 2). The two most likely

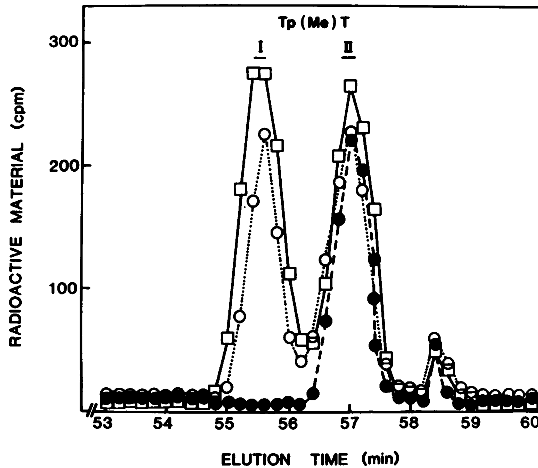

Fig. 3 Repair of one of the two stereoisomers of the methyl phosphotriester Tp(Me)T in alkylated poly(dT) by purified Ada protein. Three µg of [^{3}H]MNU-treated poly(dT) • poly(dA), containing 11 pmol of [^{3}H]methyl phosphotriester in the (dT) strand, were enzymatically hydrolysed to deoxynucleosides and methylated dinucleoside monophosphates and analysed by reverse phase HPLC. Authentic, chemically synthesized Tp(Me)T eluted after the radioactive deoxynucleosides as two distinct peaks at the positions designated I and II, representing the two different isomers. (□) Control, no enzyme added. (●) Reaction mixtures containing 22 pmol of Ada protein. (○) Reaction mixtures containing 22 pmol of the purified 19 kDa form of O^6-methylguanine-DNA methyltransferase.

explanations for this observation are that the Ada protein either repairs about 50 % of both stereoisomers of methyl phosphotriesters in an alkylated DNA substrate, or that it is active only on one of the isomers. To distinguish between these possibilities a two-fold excess amount of Ada protein was incubated with [^{3}H]MNU-treated poly(dT) • poly(dA) for 60 min at 37°C. After the incubation, the [^{3}H]poly(dT) • poly(dA) was hydrolysed enzymatically to a mixture of deoxyribonucleosides and methylated dinucleoside monophosphates and analysed by HPLC in a system which separated the two stereoisomers of Tp(Me)T. One of them (isomer I) was found to be removed completely by the Ada protein while the other remained unchanged (Fig. 3). The 19 kDa form of O^6-methylguanine-DNA methyltransferase had no effect on either stereoisomer of Tp(Me)T (Fig. 3).

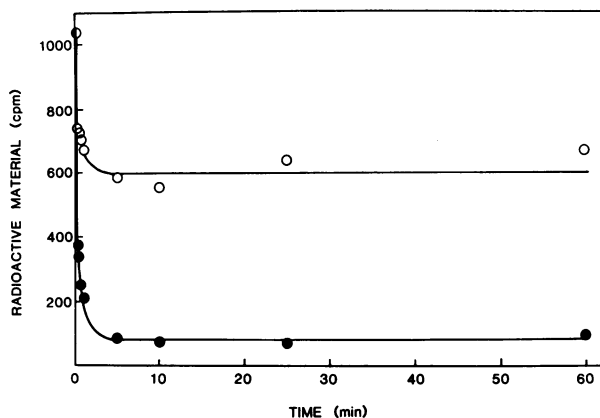

Fig. 4. Reaction kinetics for the removal of O^6-methylguanine from [^{3}H]MNU-treated DNA by the 39 kDa Ada protein. Each reaction mixture contained 5 pmol of O^6-methylguanine residues in 2.2 µg alkylated DNA. The DNA was incubated with 2 pmol (O) or 10 pmol (●) of purified Ada protein.

Repair of O^6-methylguanine residues by the Ada protein

The 19 kDa fragment of the Ada protein acts very rapidly *in vitro* to repair O^6-methylguanine in alkylated DNA, the reaction being completed in <2 sec at 37°C (19). Since the 19 kDa domain is derived by cleavage of the 39 kDa Ada protein, it was of interest to investigate whether the entire Ada protein had similar properties. The rate of disappearance of O^6-methylguanine from an alkylated DNA substrate was measured after addition of a limiting amount, and a two-fold excess, of purified Ada protein to a standard reaction mixture. Representative reaction kinetics were similar to those previously obtained with the 19 kDa protein fragment (Fig. 4). For unknown reasons, different preparations of the Ada protein showed variable initial reaction rates, but suicide kinetics (19) were always observed. We estimate that approximately 0.7 methyl groups derived from O^6-methylguanine were removed from DNA per 39 kDa Ada protein molecule.

It has been shown previously that the 19 kDa form of O^6-methylguanine-DNA methyltransferase is active in the demethylation of O^4-methylthymine residues in alkylated DNA (5). Under similar experimental conditions, the 39 kDa Ada protein is

also active in the repair of this minor alkylation lesion (data not shown).

Two active sites in the Ada protein

Incubation of the purified Ada protein with alkylated DNA substrates containing either largely O^6-methylguanine, or methyl phosphotriesters, indicated that the Ada protein can accept 1 methyl group per molecule from each type of substrate (Figs. 2 and 4). In contrast, the 19 kDa C-terminal domain of the Ada protein only accepts methyl groups from O^6-methylguanine. These data indicate that the active sites for methyl phosphotriester repair and O^6-methylguanine repair are distinct, and that the former may be a function of the N-terminal half of the Ada protein. In support of this model, it was observed that when the purified Ada protein was incubated with an excess of a mixture of the two different DNA substrates, containing O^6-methylguanine and methyl phosphotriesters, respectively, the Ada protein was found to accept 1.5 to 2 methyl groups per protein molecule. Moreover, preincubation of the Ada protein with a two-fold excess amount of the alkylated DNA substrate containing largely O^6-methylguanine completely saturated its ability to repair O^6-methylguanine, while the protein still retained most of its methyl phosphotriester repair activity.

DISCUSSION

DNA methyl phosphotriesters are formed by modification of phosphate residues of the DNA backbone. The reaction with an alkylating agent causes covalent linkage of a methyl group to either of the two oxygen atoms not involved in the 5'-3' phosphodiester bond. A pair of stereoisomers, in the R or S configuration, is generated (Fig.5). Holy and Scheit (20) demonstrated that methyl phosphotriesters are stable in oligodeoxyribonucleotides at neutral pH, while phosphate alkylation in RNA under the same conditions is followed by spontaneous hydrolysis and chain breakage. Various alkylating agents react with DNA to generate different amounts of phosphotriesters. Thus, alkylation at phosphate residues represents 1 % *vs*. 15 % of the total modification when DNA is treated with methyl methanesulfonate or ethyl methanesulfonate,

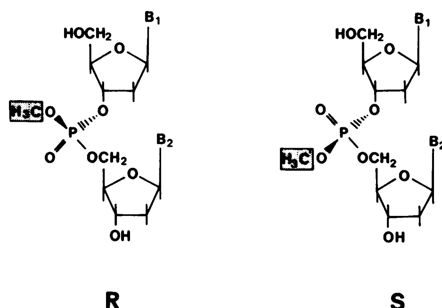

Fig. 5. Schematic drawing of the R and S stereoisomers of methyl phosphotriesters produced by alkylation of DNA phosphate residues.

respectively (21). Agents such as MNU and ethyl nitrosourea are much more reactive with oxygen atoms in nucleic acids and yield 3 % and 60 %, respectively, of the total alkylation products in DNA as phosphotriesters (22,23). Lawley (22) has shown that formation of methyl phosphotriesters in MNU-treated DNA is apparently random and independent of base sequence. The observed resistance of dinucleotide phosphotriesters to the activities of DNA degrading enzymes such as snake venom and spleen phosphodiesterases (24,25) facilitates their quantitation in DNA.

The physiological effects of the presence of occasional phosphotriesters in DNA are unclear. It seems unlikely that they would be promutagenic lesions with effects similar to those of the mispairing base residues O^6-alkylguanine and O^4-alkylthymine. Moreover, the available evidence indicates that DNA alkyl phosphotriesters are not effective as cell-killing lesions. Miller and coworkers (26) employed a DNA template containing specific ethyl phosphotriester stereoisomers and observed that *E.coli* DNA polymerase I was inhibited only to 25 % and 50 % by the two different isomers. In contrast, it has been shown that the lethal lesion, 3-methyladenine, completely blocks DNA polymerase I in replication *in vitro* of MMS- and MNNG-treated DNA (27,28). The data indicate that DNA polymerase I reads through methyl phosphotriester lesions in DNA, albeit at a reduced rate. In summary, it seems likely that methyl phosphotriesters, akin to 7-methylguanine, represent an

apparently innocuous type of alkylation lesion. Several studies determining the persistence of alkyl phosphotriesters in mammalian cells *in vivo* have indicated that removal of such lesions does not occur at an appreciable rate (29,30). It is not known, however, if both stereoisomers of alkyl phosphotriesters, or only one, remain for long times in the DNA of mammalian cells. The most attractive explanation for the active reversal of methyl phosphotriesters in adapted *E.coli* cells is that a signal may be generated for positive control of the adaptive response. Preliminary experiments using the cloned *ada*$^+$ gene and purified Ada protein in an *in vitro* coupled transcription-translation system support this notion (I. Teo, B. Sedgwick, T.McC. and T.L., in preparation).

The Ada protein exhibits an apparently absolute stereospecificity in its removal of DNA methyl phosphotriesters. The stereoisomer of Tp(Me)T in alkylated poly(dT) • poly(dA) which is repaired has been tentatively identified by circular dichroism measurements as being in the S configuration; the methyl group of this isomer would protrude perpendicularly from the DNA double helix, while that of the R isomer may be accommodated within the major groove (M. Weinfeld, pers. commun.). The methyl group removed from a DNA phosphotriester by the Ada protein is transferred to one of the cysteine residues of the protein itself. O^6-Methylguanine is corrected by the Ada protein in a similar fashion, although a different cysteine residue is involved. G.P. Margison, D.P. Cooper and J. Brennand (pers. commun.) have also found that the Ada methyltransferase can repair methyl and ethyl phosphotriesters in DNA, employing an active site different from that involved in the repair of O^6-methylguanine residues.

In order to demonstrate the action of the Ada protein on methyl phosphotriesters in DNA, it was necessary to clone the *ada*$^+$ gene into an expression vector which would allow production of large amounts of this protein. The sequencing vector pEMBL8 with the *ada*$^+$ gene inserted under control of the *lac* promoter (12) proved useful in this respect (Fig. 1). The *ada*$^+$ gene was also cloned into expression vectors under control of the λ P_L promoter (31), however, cells containing these recombinant

plasmids only overproduced the Ada protein to 0.1% of the total cellular protein and were consequently less useful (our unpublished observations). Y. Nakabeppu and M. Sekiguchi (pers. commun.) have shown that after cloning of the ada^+ gene of E.coli K12 under lac promoter control in the vector pUC9, about 10% of the total cellular protein could be obtained as the ada^+ gene product. The availability of relatively large amounts of the intact Ada protein in purified form now allows further biochemical studies on its structure and mechanism of action, its specific processing, and its regulatory role in the adaptive response to alkylating agents.

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