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Rapid, large-scale purification and characterization of 'Ada protein' (O^6 methylguanine-DNA methyltransferase) of *E.coli*

D.Bhattacharyya[1], K.Tano[1], G.J.Bunick[3], E.C.Uberbacher[1], W.D.Behnke[4] and S.Mitra[2]*

Protein Engineering and Molecular Mutagenesis Program and [1]University of Tennessee Graduate School of Biomedical Sciences, [2]Biology Division and [3]Solid State Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831 and [4]Department of Biochemistry and Molecular Biology, University of Cincinnati College of Medicine, Cincinnati, OH 45267, USA

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ABSTRACT

The *E. coli* Ada protein (O^6-methylguanine-DNA methyltransferase) has been purified using a high-level expression vector with a yield of about 3 mg per liter of *E. coli* culture. The 39-kDa protein has an extinction coefficient ($E^{280nm}_{1\%}$) of 5.3. Its isoelectric point of 7.1 is lower than that predicted from the amino acid content. The homogeneous Ada protein is fully active as a methyl acceptor from O^6-methylguanine in DNA. Its reaction with O^6-methylguanine in a synthetic DNA has a second-order rate constant of $1.1 \times 10^9\ M^{-1}\ min^{-1}$ at 0^oC. Both the native form and the protein methylated at Cys-69 are monomeric. The CD spectrum suggests a low α-helical content and the radius of gyration of 23 Å indicates a compact, globular shape. The middle region of the protein is sensitive to a variety of proteases, including an endogenous activity in *E. coli*, suggesting that the protein is composed of N-terminal and C-terminal domains connected by a hinge region. *E. coli* B has a higher level of this protease than does K12.

INTRODUCTION

Simple alkylating mutagens and carcinogens alkylate a number of nucleophilic sites in DNA, including the ring nitrogens (N-3 and N-7) of purines and the exocyclic oxygens of bases and phosphate residues (1). The biological consequences of all the alkyl adducts are not established. However, Loveless proposed that O^6-alkylguanine is the critical promutagenic and procarcinogenic lesion because of its potential for mispairing with thymine during DNA replication (2). This prediction has subsequently been proven correct (3-5). Other alkyl adducts, e.g. 3-alkylpurines, are toxic in *E. coli* (6,7). Samson and Cairns observed the phenomenon of *the adaptive response* in *E. coli* when the bacteria treated with a low level of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) became resistant to both the toxic and the mutagenic effects of the alkylating agents (8). The *in vivo* evidence showing that this phenomenon involves induction of a repair system for toxic and mutagenic alkylated lesions in DNA, including O^6-methylguanine (9), is supported by the identification a unique repair protein in adapted *E. coli*

that demethylates O^6-methylguanine in DNA *in situ* (10-12). It adds the methyl group to one of its own cysteine residues in a "suicide-inactivation" reaction (13). The repair protein was initially purified as a 19-kDa species and named O^6-methylguanine-DNA methyltransferase (MGMT) (12). Subsequently, MGMTs that undergo a similar stoichiometric reaction have been identified in mammalian cells (7,14,15). However, *E. coli* methyltransferase is more complex than the mammalian methyltransferases.The *E. coli ada* gene, responsible for adaptive response, has been cloned (16-19) and its regulation has been extensively studied (20-22). The primary product of the *ada* gene, a 39-kDa "Ada protein", has pleiotropic functions. It has two methyl-accepting cysteine residues. Cys-69 accepts a methyl group from S-diastereomers of methylphosphotriesters (7). The resulting methylated protein acts as an activator for transcription of the *ada* regulon. This includes not only the *ada* gene itself but also *alkA*, (which codes for an *N*-glycosylase that repairs *N*-alkylpurines and O^2-alkylpyrimidines) and other genes involved in alkylation damage repair (21,22). Cys-321 of the Ada protein accepts an alkyl group from O^6-alkylguanine and O^4-alkylthymine in DNA (20,22). The 19-kDa MGMT characterized by Demple *et al.* (12) is the C-terminal fragment of the Ada protein which results from *in vivo* cleavage by an uncharacterized proteolytic activity in *E. coli* (19).

While the regulatory role of the Ada protein has recently been elucidated, many of its biophysical and biochemical properties have not been determined. We have been able to overproduce the Ada protein using a cloned *ada* gene from which the regulatory sequence has been deleted (23). This system has provided an opportunity for a rapid and large-scale purification of the Ada protein to homogeneity. The purification and some physico-chemical properties of the protein are described in this paper.

MATERIALS AND METHODS

Materials

[^3H-CH_3]*N*-methyl-*N*-nitrosourea (MNU) (4 Ci/mmol) was purchased from Moravek Biochemicals, Brea, CA. Calf thymus DNA, proteinase K, dithiothreitol, bovine serum albumin were purchased from Sigma. Ultrogel AcA 44 and AcA 54 were from LKB. Polyethyleneimine (Polymin P) and sodium dodecyl sulfate (SDS) were obtained from BDH and isopropyl-β-thiogalactoside (IPTG) from IBI.

[^3H-CH_3]O^6-methylguanine-containing DNA was prepared by the reaction

of calf thymus DNA with [^{3}H]MNU as described previously (11,15). The methylated DNA, after removal of N-methylpurines by heating, contained about half of the radioactivity as O^6-methylguanine and most of the remainder as methylphosphotriesters. The use of poly(dG,dC,[8-^{3}H]m^{6}dG), as a substrate for O^6-methylguanine-DNA methyltransferase assay, has been discussed in detail elsewhere (10). Human MGMT was purified from placenta as described earlier (15).

Plasmid Coded Expression and Purification of Ada Protein

The construction and characterization of high-level *ada* expression vector, pSM41, has been described earlier (23). *E. coli* JM107 containing *lacI*^{q} and transformed with pSM41 were induced for *ada* gene by IPTG, which was added at 0.5 mM to log-phase cultures (A_{550nm} = 0.2) grown under vigorous aeration. After 4 hrs of induction at 37°C, the cells were harvested by centrifugation and stored at -70°C.

In a typical experiment, the frozen cells from a 2-liter culture were suspended in (30-40) ml of 20 mM Tris-HCl (pH 8.0), 10 mM DTT, 1 mM EDTA and 10% glycerol (buffer A) containing 0.4 M NaCl and were disrupted by sonication. After removal of cell debris by centrifugation at 35,000 rpm for 15 min in a Beckman Ti50 rotor at 4°C, 5% Polymin P (neutralized to pH 8.0 with HCl) was added to the supernatant at the rate of 18 μg per A_{260} of the extract. The precipitated nucleic acids were removed by centrifugation (10,000 x g, 20 min). The supernatant (Fraction II) was made to 45% saturation with ammonium sulfate and the precipitate containing Ada protein was collected by centrifugation (10,000 x g, 20 min). The precipitate was dissolved in 1.0 to 1.5 ml buffer A (Fraction III), centrifuged and placed on an Ultrogel AcA 54 column (1.8 x 47 cm) which was eluted with buffer A containing 50 mM NaCl. The elution of Ada protein was monitored by A_{280} measurement and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of eluted fractions. The second major A_{280} peak (Fraction IV) contained homogeneous Ada protein. The fractions containing Ada protein were stored in buffer A containing 50% glycerol and 0.25 M NaCl at -20°C.

Assay of O^6-methylguanine-DNA Methyltransferase

The stoichiometric reaction of Ada protein with O^6-methylguanine in DNA was assayed by conversion of [8-^{3}H]O^6-methylguanine to [8-^{3}H]guanine in poly(dC,dG,[8-^{3}H]m^{6}dG). The details of the assay and separation of guanine and O^6-methylguanine have been described elsewhere (10,24). One unit of Ada protein is defined as the amount that demethylates 1 pmol of O^6-methylguanine. The kinetics of the reaction of the Ada protein with

poly(dC,dG,[8-^{3}H]m^{6}dG) was determined at 0°C in individual 2-ml reactions for each time point. The reaction was started with the addition of the protein, stopped by adding SDS to 1% and the products were then analyzed as before.

Isoelectric Focusing - Polyacrylamide Gel Electrophoresis

Isoelectric focusing was carried out in nondenaturing slab gels of 12.5% polyacrylamide containing ampholines (Ampholyte pH 3.0 - 10.0, Pierce Chemical Co.) (25). The pH gradient after electrophoresis was calibrated by the use of isoelectric-point marker proteins (Sigma) placed in the same gel. At the end of electrophoresis (3 hr, 900 V), the gels were treated with a fixative solution containing 30% methanol, 3.5% sulfosalicylic acid and 12% trichloroacetic acid (TCA) before staining for protein bands with Coomassie Brilliant Blue and subsequent destaining.

The integrity and size of the Ada protein were determined by denaturing gel electrophoresis in 15% polyacrylamide containing 0.1% SDS according to Laemmli (26). After electrophoresis, the protein bands were visualized by staining with Coomassie Brilliant Blue dissolved in 50% TCA.

X-ray Scattering Studies

Small-angle scattering data were collected using the 10-meter X-ray scattering instrument of the National Center for Small-Angle Scattering Research (located at Oak Ridge National Laboratory). For this experiment, the sample to detector distance was configured at 2 meters providing a usable k-range of 0.030 to 0.197 $\AA^{-1}$ using graphite monochromated CU Kα radiation (λ = 1.5418 Å). The samples consisted of Ada protein at 3.5 mg/ml and the buffer (buffer A containing 0.2 M NaCl) used in preparing the protein. These were measured repeatedly in one hour segments, the protein for a total of sixteen hours and the buffer for ten hours. The individual runs for each sample were combined prior to processing. The two dimensional data were corrected for instrumental effects, detector sensitivity, sample transmission, and normalized by the beam-monitor count. The scattering by the buffer was subtracted from the protein scattering data and the radially averaged scattering curve was calculated.

Other Methods

Protein concentrations were determined by the bicinchoninic acid procedure (27) with bovine serum albumin as the standard. Radioactivity in liquid samples was quantitated in a Beckman liquid scintillation spectrometer after addition of ACS scintillation solvent. Circular dichroism (CD) spectra were obtained in a JASCO 500 spectropolarimeter at 22°C.

RESULTS

Purification of Ada Protein

As shown in Table 1, the high level expression of the Ada protein from the recombinant plasmid pSM41, induced under the control of *lac* promoter, yields more than 10% of the total soluble protein in crude *E. coli* extracts as Ada protein. This allows a rapid and large-scale purification of the protein to near homogeneity in essentially three steps with an overall recovery of about 50%. The purification can be achieved in 8-10 hrs with a yield of 3 mg protein per liter of *E. coli* culture. The critical step in the purification is precipitation of nucleic acids with Polymin P. We have established the maximum amount of the polymer that could be added to remove some proteins along with most of the nucleic acids without precipitation of the Ada protein.

The purity of Ada protein preparations was tested by SDS-PAGE and isoelectric focusing (Fig. 1). The observed trace contaminants, which included Ada degradation products (see later) did not account for more than 2-5% of the total protein. The size of 39-kDa for the protein is in agreement with earlier results and prediction from the DNA sequence of the cloned gene (19,20,28).

The isoelectric point of Ada protein was observed to be 7.1 ± 0.1 (Fig. 1B). This was surprising because of the excess of basic over acidic amino acids by ten residues in the Ada protein sequence (19). In fact, it was predicted earlier that the protein would be significantly basic (19).

Table 1
Purification of E. coli Ada protein[a]

Fraction		Total protein (mg)[b]	Total activity (Units) x 10^3	Specific activity (Units/mg)
I.	Crude extract	74	321	4,330
II.	Polymin P supernatant	53	319	6,010
III.	Ammonium sulfate supernatant	14	276	19,710
IV.	Gel filtration (Pooled peak fractions)	5.8	171	29,430

[a] *E. coli* cells from 2 liter culture was used as the starting material.
[b] Protein was determined after precipitation with TCA.

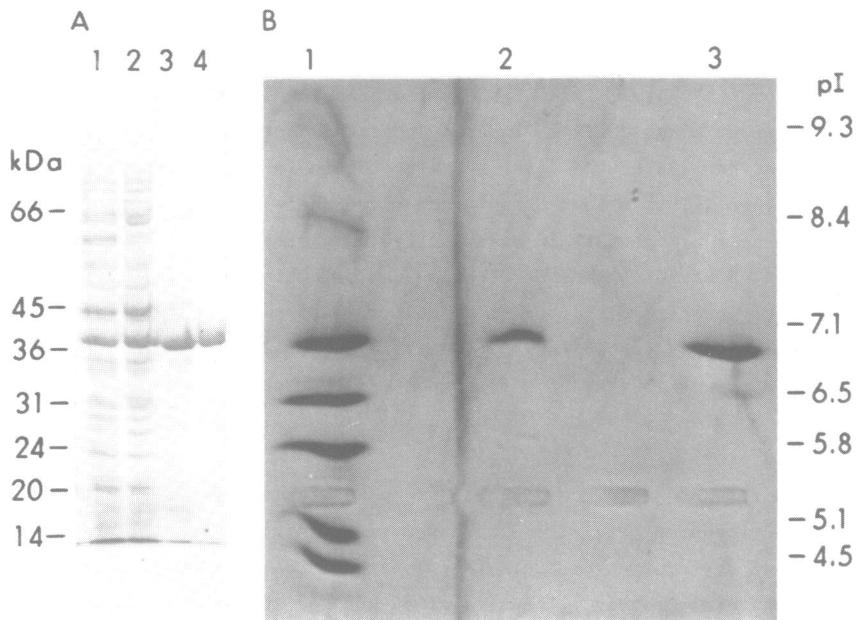

FIGURE 1: A. SDS-PAGE of Ada protein. Aliquots (14 to 8 μg of protein) of Fractions I through IV (Table 1), in lanes 1 through 4 respectively, were electrophoresed in 15% polyacrylamide with 4.5% stacking gel according to Laemmli (26). The positions of molecular weight markers are indicated (bovine serum albumin, 66-kDa; ovalbumin, 45-kDa; glyceraldehyde-3-phosphate dehydrogenase, 36-kDa; carbonic anhydrase, 31-kDa; trypsinogen, 24-kDa; soybean trypsin inhibitor, 20-kDa and α-lactalbumin, 14-kDa).
B. Isoelectric focusing of Ada protein (Fraction IV) (lane 2). The positions of marker proteins in lane 1 are indicated (trypsinogen, pI 9.3; lactate dehydrogenase, pI 8.4; horse heart myoglobin, pI 7.1; human carbonic anhydrase B, pI 6.5; bovine carbonic anhydrase B, pI 5.85; β-lactoglobulin, pI 5.1 and soybean trypsin inhibitor, pI 4.5). Lane 3 contains only horse heart myoglobin.

Clearly, the neutral pI of the protein is due to the lowering of pKa's of the basic amino acids in the folded molecule.

Extinction Coefficient of Ada Protein

The extinction coefficient of the Ada protein was determined after extensively dialyzing Fraction IV against 20 mM sodium-phosphate (pH 7.0) containing 0.4 M NaCl. The absorbance at 280 nm and the protein content of the dialyzed sample were determined. An aliquot was hydrolyzed for amino acid analysis in a Beckman 121 M amino acid analyzer. Based on the quantitation of several amino acids, whose relative amounts agreed with the predicted values, the extinction coefficient ($E_{1\%}^{280\ nm}$) of Ada protein was

calculated to be 5.3. Using bovine serum albumin (BSA) as the standard and estimating the protein by the BCA reagent (27), the $E^{280}_{1\%}$ was calculated to be 5.0.

Stability of Ada Protein

Earlier studies showed that the 39-kDa Ada protein was susceptible to degradation by an endogenous protease in *E. coli*. We investigated the possibility of contamination of the purified Ada protein with the protease by measuring its stability on the basis of both size and activity. Because Ada protein reacts stoichiometrically, the activity was measured by the extent of methyl transfer from an excess of O^6-methylguanine in DNA. The results can be summarized as follows.

The 39-kDa Ada protein can be stored in concentrated solution in 20 mM Tris-HCl (pH 8.5), 1 mM EDTA, 5 mM DTT, 0.2 M NaCl and containing 10% glycerol at -80° or in the same buffer containing 50% glycerol at -20° for several weeks without loss of activity or degradation of size. Also, several cycles of freezing and thawing did not destroy the activity or damage the integrity of the protein to any significant extent. In fact, the protein is quite stable at room temperature. Incubation of the protein in the presence of BSA (100 μg/ml) up to 6 hrs at 37°C did not result in any extensive degrdation or inactivation (see later). On the other hand, the methyltransferase activity was rapidly lost without loss of the 39-kDa species when the Ada protein was incubated in a dilute solution in the absence of BSA (data not shown).

Specific Activity and Kinetics of Methyltransferase Reaction

The specific activity of Ada protein in Fraction IV was calculated to be about 1.1 mole of methyl acceptor (from O^6-methylguanine) per mole of protein (Table 1). Thus the pure Ada protein did not lose any activity during the purification steps.

We have shown that the methyl transfer reaction of human MGMT follows second-order kinetics (15). Lindahl *et al.* (13) showed that the reaction of *E. coli* 19-kDa MGMT was extremely fast at 37°C and the kinetics was measurable only at 0°C. We have measured the kinetics of reaction of pure Ada protein with O^6-methylguanine in a synthetic DNA substrate at 0°C and calculated the second-order rate constant to be 1.1×10^9 M^{-1} min^{-1} (Fig. 2)

Monomeric State of Native and Methylated Ada Proteins

Earlier gel filtration studies indicated that the native Ada protein is monomeric (28). We prepared [^{3}H-CH_3] Ada protein methylated at Cys-69 after incubation of the active protein with [^{3}H-CH_3]-labeled DNA depleted of

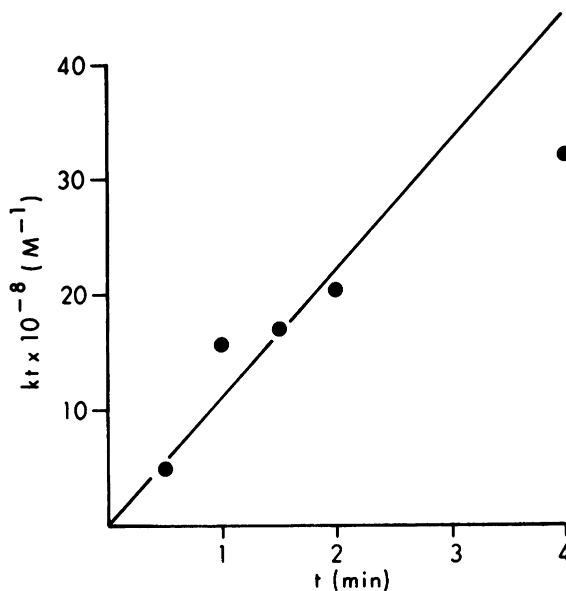

FIGURE 2: Kinetics of reaction of Ada protein (6.4×10^{-11} M) with poly(dG,dC,[8-^{3}H]m^{6}dG) (4.35×10^{-10} M m^{6}dG) at 0°C plotted according to the second-order rate equation (36).

its [^{3}H-CH_3] O^6-methylguanine. This selective removal of O^6-methylguanine was accomplished by treating the methylated DNA with an excess human MGMT which, unlike *E. coli* Ada protein, has no methylphosphotriester-methyltransferase activity (7,14). The methylated Ada protein was purified from the DNA by chromatography on DEAE-cellulose and eluted from an LKB Ultrogel AcA 44 column in buffer A containing 0.2 M NaCl. The unmethylated and methylated protein eluted at nearly identical positions corresponding to a native molecular mass of about 40-kDa (data not shown). This suggests that the Ada protein methylated at Cys-69 is monomeric in its native state.

Circular Dichroism Spectrum of Ada Protein

The CD spectrum of Ada protein in 50 mM Na-PO_4(pH 7.5), showed only one peak at 221 nm in the range of 210 to 300 nm. From the spectrum, the α-helix and β-sheet content of the protein was calculated to be 17% and 16% respectively (29).

Radius of Gyration of Ada Protein

In the low Q region, small-angle scattering data obey Guinier's law:

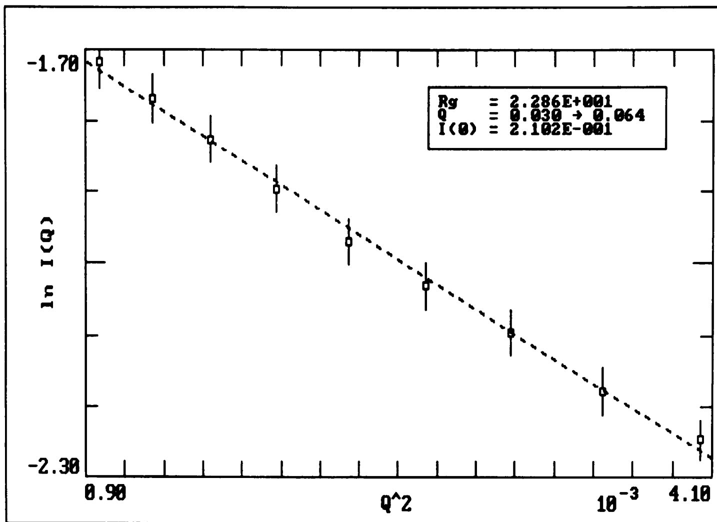

FIGURE 3: Guinier plot of X-ray scattering from Ada protein.

$I(Q) = I(0)e^{-Q^2 Rg^{2/3}}$, where I(0) is the forward scatter and Rg is the radius of gyration. The Rg and I(0) were determined by a weighted least-square it to a plot of ln I (Q) vs. Q^2. From the Guinior plot of the Ada protein (Fig 3), its Rg was calculated to be 22.9 Å (30). A number of geometric form factors were fit to the scattering curve of the protein [I(Q) vs. Q]. The geometric models consisted of a sphere, prolate and oblate ellipsoids, and a cylinder. The analysis indicated that the cylinder and ellipsoid models that best fit the MGMT scattering curve were consistent with a relatively compact molecule having an axial ratio less than 2:1. For a molecule with a molecular weight of 39-kDa the observed Rg of 22.9 Å is consistent with a compact globular molecule.

Proteolytic Cleavage of Ada Protein

Since the original isolation of *E. coli* MGMT as a 19-kDa protein (12) it has been shown that this is the C-terminal fragment of the 39-kDa Ada protein produced *in vivo* as a result of cleavage by an endogenous protease in *E. coli* (19,20). The Ada protein was shown to be cleaved *in vitro* by a number of proteases including one from *E. coli* into a 20-kDa N-terminal, and a 19-kDa C-terminal fragment followed by further cleavage of the N-terminal fragment into distinct fragments (7,31). We have confirmed similar distinct

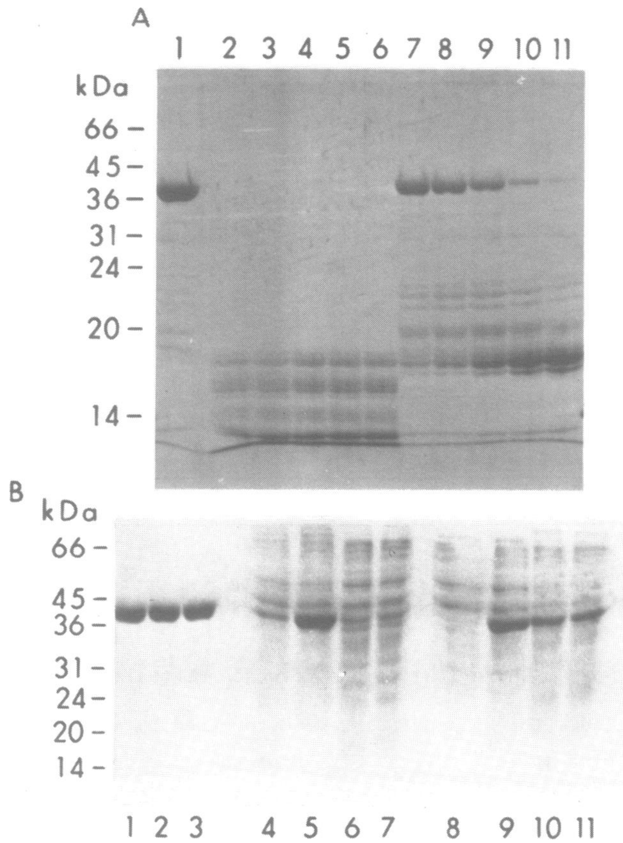

FIGURE 4: SDS-PAGE analysis of proteolytic cleavage products of Ada protein. A. Digestion of Ada protein with proteinase K. 100 μg/ml of protein was incubated with proteinase K at a weight ratio of 1:100 (lanes 2-6) and 1:1000 (lanes 7-11) for 1, 2, 5, 10, and 20 mins at room temperature. The reactions were arrested by freezing in liquid nitrogen. Each lane contains 5 μg of protein and the electrophoresis conditions were the same as described in Fig. 1. Lane 1 contains Ada protein and proteinase K (1:100) as the control at 0 min.
B. Digestion of Ada protein with *E. coli* protease. 4 μg Ada protein was incubated with extracts of *E. coli* B (lanes 4-7) or K12 (lanes 8-11) containing 8 μg total protein. Lanes 1-3: Ada protein without extract, lanes 4 and 8: extracts without Ada protein. Incubations were carried out at 37°C for 0 hr (lanes 1, 5, and 9) and 6 hrs (lanes 2, 3, 6, 7, 10, and 11). The incubation mixtures contained either buffer A (lanes 2, 6 and 10) or buffer A at pH 7.2 and including 0.5 M NaCl (lanes 3, 7, and 11).

cleavage sites of Ada protein by trypsin (data not shown) and for proteinase K. In particular, after limited digestion with proteinase K, a 19-kDa fragment resistant to further breakdown was observed by

electrophoresis in SDS/polyacrylamide gel (Fig. 4A). Because we have not observed a significant breakdown of plasmid-coded Ada protein in *E. coli* K-12, and because the 19-kDa MGMT was isolated from an *ada*-constitutive *E. coli* B mutant (BS21), we tested whether the levels of endogenous protease(s) responsible for site-specific cleavage of the Ada protein are different for the two strains. The incubations were carried out under conditions of both optimum protease activity (pH 7.2 and high ionic strength) and inhibition of this activity (pH 8.5 and low salt) (19). Fig. 4B shows the results of digestion of Ada protein with equal amounts of crude extracts of *E. coli* (BS23) and K12 (JM 107) strains. It is evident that the *E. coli* B extract caused more extensive proteolysis of the Ada protein.

DISCUSSION

The high level expression of *ada* gene in the recombinant plasmid pSM41, allows a rapid purification of the Ada protein to homogeneity involving a few simple steps. Furthermore, unlike some cases of overexpression of plasmid genes which result in accumulation of the inactive protein (32), the pSM41-coded Ada protein is fully active. Although the trace contaminants that are observed occasionally can be removed by additional steps (e.g. chromatography on hydroxyapatite, DNA-cellulose or phosphocellulose), these steps appear to be unnecessary for most purposes. Chromatography on phosphocellulose is very effective in purification and concentration of the protein. However, this step causes rapid inactivation of the protein, as was also observed earlier (19). The specific activity of our Ada protein preparations indicates that it is fully active compared to the 20% activity of the protein reported by Nakabeppu *et al.* (19). We suspect that this may be due to a partial inactivation of their protein during phosphocellulose chromatography.

The Ada protein preparation has no detectable DNase activity as judged by the absence of nicking of supercoiled plamid DNA (M. Sikpi, unpublished results). Retention of both the activity and the structural integrity of the protein after its incubation for 6 hrs at 37°C, particularly under conditions of the optimum activity of *E. coli* protease, namely at pH 7.2 and in high salt (19), indicates a lack of contamination with proteases (Fig. 4B).

One of the many unusual properties of Ada protein and its 19-kDa fragment is their high rate of stoichiometric reaction with O^6-methylguanine in DNA (13). We have not as yet been able to measure the second-order rate

constant of this reaction for Ada protein with O^6-methylguanine in a duplex DNA substrate at 37°C. However, the rate constant with the synthetic substrate poly(dC,dG,m^6dG), with which the protein reacts much more slowly, was found to be 1.1×10^9 M^{-1} min^{-1} at 0°C. This value is some 500 times higher than that of the partially purified human MGMT with the same substrate at 37°C (D. Bhattacharyya, R. S. Foote, A. M. Boulden and S. Mitra, unpublished experiment). Assuming that the human protein preparation does not contain an inhibitor of the MGMT, this suggests a major difference in the affinity of the two proteins for the O^6-methylguanine in DNA which must be due to their structural differences. This possibility is further supported by the lack of cross reactivity of the human protein with the antibody to the 19-kDa MGMT (R. S. Foote and S. Mitra, unpublished experiment; D. Yarosh, personal communication).

Another unusual feature of the purified Ada protein is its stability after freezing and thawing in concentrated solution and yet its rapid inactivation in dilute solution, presumably due to surface denaturation in the absence of a carrier protein (BSA).

The elucidation of the detailed structure of the Ada protein will be possible only after its crystallization and X-ray diffraction analysis. However, we have investigated some physical properties of the protein in solution. First, our gel filtration data indicates that the protein is monomeric in 0.2 M NaCl, in agreement with the earlier data (28). More importantly, the protein methylated at Cys-69, that becomes a transcriptional activator of *ada* and *alkA* genes by binding to their regulatory sequences (7), is also monomeric. This is somewhat surprising because another transcriptional activator, the cAMP-CRP complex, as well as the *lac*, *gal* and lysogenic phage repressors, all of which recognize specific regulatory sequences containing regions of dyad symmetry, act as dimers or tetramers (33,34).

The Rg of 23 Å of Ada protein, calculated from small angle X-ray scattering data, predicts that it is globular in shape with an axial ratio of less than 2. The CD spectrum suggests that the protein has rather low α-helical and β-sheet contents. This is unexpected because of the potentially significant amount of α-helical regions predicted from the amino acid sequence on the basis of the Chou-Fasman procedure (35) (results not shown). Furthermore, α-helices have been shown to be critical in repressor proteins for their recognition of specific DNA sequences (34). At the same time, retention of methylphosphotriester methyltransferase and MGMT

activities by the N-terminal and C-terminal fragments respectively (7) suggests that the protein has two distinct and independently active domains. Thus, it appears that the two domains have significant tertiary structures and are in close proximity in order to have an overall globular conformation.

The unusually high sensitivity of the middle region of the Ada protein to a variety of proteases, including nonspecific proteinase K, strongly supports the argument that the protein has two protease-resistant domains connected by a hinge region (7). The first isolation of MGMT from an *ada*-constitutive *E. coli* B mutant (BS21) and subsequent cloning of the *ada* gene and isolation of its 39-kDa gene product (12, 19, 20) led to the identification of an *E. coli* protease responsible for degradation of the Ada protein to yield the 19-kDa C-terminal fragment (19,31). While the possible biological significance of this proteolysis of Ada protein is not clear, we observed no significant degradation of the Ada protein in *E. coli* K12. We suspected, therefore, that *E. coli* K12 has a much lower level of this protease, which was confirmed by comparing the *in vitro* activities of extracts of the two strains on Ada protein.

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*To whom correspondence should be addressed

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