Effect of Metal Ions on Transcription of the *ada* Gene Which Encodes O^6-Methylguanine-DNA Methyltransferase of *Escherichia coli*

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The effect of metal ions on transcription of the *ada* gene of *Escherichia coli* which is promoted by Ada protein in the presence of methylated deoxyribonucleic acid (DNA) was examined in a reconstituted system. Their effect on the O^6-methylguanine-DNA methyltransferase (MGTase) activity of Ada protein was also examined. Ag^+, Cd^{2+}, Cu^{2+} and Hg^{2+} severely inhibited transcription of the *ada* gene at a dose which inhibited neither transcription of the *lacUV5* gene nor MGTase activity. Zn^{2+} inhibited both transcription and MGTase activity in the same dose range. Al^{3+} and Fe^{3+} inhibited transcription of both *ada* and *lacUV5* genes without affecting the MGTase activity of Ada protein. Inhibitory mechanisms are discussed.

Keywords DNA repair; adaptive response; *ada* transcription; O^6-methylguanine-DNA methyltransferase; metal ions; cadminum ion; mercuric ion

Introduction

In *Escherichia coli*, there are deoxyribonucleic acid (DNA) repair networks induced by exposure of cells to mutagenic methylating agents such as *N*-methyl-*N*-nitrosourea (MNU), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and methyl methanesulfonate.[1-5] One of these repair networks, termed the adaptive response, is crucial for resistance to the mutagenic and cytotoxic effects of methylating agents. Cells incapable of inducing the adaptive response are extremely sensitive to the mutagenic and toxic effects of a methylating agent.[6,7] This repair network is regulated by the *ada* gene product.[8,9] Ada protein, O^6-methylguanine-DNA methyltransferase (MGTase), repairs mutagenic lesions of methylated bases, *i.e.*, O^6-methylguanine, O^4-methylthymine and methylphosphotriester, by transferring a methyl group to acceptor sites (cysteine moieties) on Ada protein. Ada protein possesses two methyl-acceptor sites; one (Cys-321) accepts a methyl group from methylated bases and the other (Cys-69) accepts a methyl group from methylphosphotriesters.[10-13] The latter type of methylated Ada protein then functions as a transcriptional activator of the *ada* gene.[13,14] Once Ada protein is overproduced in this manner, it takes part in activating transcription of its own and other genes belonging to the *ada* regulon, thereby generating large amounts of enzymes that can repair various types of methylated lesions in DNA.

We recently reported that Cd^{2+} and Hg^{2+} inhibit induction of the adaptive response in *E. coli* leading to potentiation of mutagenesis by methylating agents such as MNU and methyl methanesulfonate.[15,16] Scicchitano and Pegg reported that several metal ions, including Cd^{2+} and Hg^{2+}, inhibit MGTase activity in preparations partially purified from rat liver and *E. coli*, and that this inhibition is likely caused by the reversible inhibitory interaction of the metal ions with SH groups of methyl-acceptor sites on Ada protein.[17] However, we considered that *ada* gene expression promoted by methylated Ada protein might be inhibited by these metal ions in *E. coli* prior to inhibition of cellular MGTase.[16] Our assumption was confirmed using a reconstituted system by means of which Cd^{2+} and Hg^{2+} were shown to inhibit the transcriptional promoting activity of methylated Ada protein at a dose which did not inhibit MGTase activity.[18] In the present study, so-called soft cations able to bind to SH groups were assayed for inhibition of gene promotion and MGTase activity. In addition to Cd^{2+} and Hg^{2+}, the metal ions examined were Ag^+, Al^{3+}, Cu^{2+}, Fe^{3+}, Pb^{2+} and Zn^{2+}, all of which are known to inhibit MGTase in *in vitro* systems.[17,19]

Materials and Methods

Materials Ada protein was prepared from *E. coli* YN3 cells harboring pYN3059, as described.[20] A 176-bp *Hin*dIII–*Eco*RI fragment containing the *ada* promoter region and a 205-bp *Eco*RI fragment containing the *lacUV5* promoter region were prepared from plasmids pYN3066 and pYN3077, respectively.[8] All metal salts, $AlCl_3$, $CuCl_2$, $CdCl_2$, $FeCl_3$, $HgCl_2$, $PbCl_2$, $Pb(CH_3COO)_2$, and $ZnCl_2$, were purchased from Wako Pure Chemical Ind., Ltd. (Osaka), except for $AgNO_3$ which was purchased from Nakarai Tesque, Inc. (Kyoto). *E. coli* ribonucleic acid (RNA) polymerase and bovine serum albumin (BSA) (nuclease-free) were purchased from Pharmacia LKB Biotechnology Inc. (Uppsala). Methylated DNA used as a methyl donor in the transcription assay was prepared by treatment of 2.76 mg *Micrococcus luteus* DNA (Sigma, St. Louis) with 90 mM MNU in 5 ml of 20 mM Ammediol buffer (pH 10), as described in our previous paper.[21] [^{3}H]Methylated DNA used as a substrate in the methyltransferase assay was prepared by treatment of 10 mg calf thymus DNA (Sigma) with 1.25 μmol [^{3}H]MNU (Amersham Corp., Buckinghamshire; 0.8 Ci/mmol) in 6 ml of 20 mM Ammediol buffer (pH 10). The specific activity of [^{3}H]methylated DNA was 9200 dpm/μg DNA.

Assay for *ada* and *lacUV5* Transcripts To transfer methyl groups from methylated DNA to Ada protein, a mixture containing 2 pmol Ada protein, DNA fragments containing the *ada* promoter region (0.2 pmol) or *lacUV5* promoter region (0.1 pmol) and 100 ng methylated *M. luteus* DNA was incubated in the presence of metal ion at 37 °C for 15 min in 33 μl of buffer consisting of 50 mM Tris–HCl (pH 7.8), 3 mM magnesium acetate, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM dithiothreitol, 50 mM NaCl, and 25 μg/ml BSA. After the addition of 2 pmol *E. coli* RNA polymerase, this mixture was further incubated at 37 °C for 30 min to form the open complex required for initiation of transcription. Fifteen μl of a prewarmed solution containing 200 μg/ml heparin, 50 μM [α-^{32}P]uridine triphosphate ([α-^{32}P]UTP, 2 μCi/tube) and 160 μM each of adenosine triphosphate (ATP), guanosine triphosphate (GTP), and cytidine triphosphate (CTP) were added, and the mixture was incubated at 37 °C for 10 min. The reaction was terminated by addition of 50 μl of a solution containing 100 μg/ml *E. coli* ribosomal RNA (Sigma) and 40 mM EDTA. The transcripts were precipitated with ethanol, dried under vacuum, and then dissolved in formamide–dye solution. Electrophoresis was then carried out at 1500 V for about 1.5 h on a 10% polyacrylamide gel containing 8.3 M urea. The transcripts separated into a 98 base *ada* and a 63 base *lacUV5*. Transcription levels were quantified by direct measurement of radioactivity on the gel using a Betascope B603 Blot Analyzer (Betagence Co., Waltham, MA) or by scanning autoradiographic

spots of the transcription products using a densitometer (Shimadzu, Kyoto).

Assay for Methyltransferase Activity The reaction mixture which contained 2 pmol Ada protein and 8 μg [^{3}H]MNU-treated calf thymus DNA in 33 μl of the buffer described above was incubated at 37 °C for 15 min. After addition of 0.1 mg BSA dissolved in 167 μl of H_2O and 400 μl of trichloroacetic acid (TCA), the mixture was heated at 90 °C for 15 min to hydrolyze DNA, and then chilled in ice-water. The protein was collected by centrifugation and rinsed with 400 μl of 5% TCA. The pellet was then dissolved in 200 μl of 0.1 N NaOH. After heating at 90 °C for 2 min, 200 μl of 0.1 N HCl were added to neutralize the pH, and radioactivity was determined on a liquid scintillation counter using a Scintisol EX-H (Dojin Lab, Kumamoto).

Results

The inhibitory effect of 8 metal ions on transcriptional promoting activity was quantified in a reconstituted system by analysis of the transcript from the DNA fragment containing the *ada*-regulatory region in the presence of Ada protein and methylated *M. luteus* DNA. The transcript from the DNA fragment containing the *lacUV5*-regulatory region was also measured as a positive control for the RNA synthetic machinery. The transcription levels of *ada* and *lacUV5* genes observed in the presence of metal ion were plotted in Fig. 1. The values were expressed as a percentage of the controls in the absence of metal ion. Of the 8 metal ions examined, Ag^+, Cd^{2+}, Cu^{2+}, Hg^{2+} and Zn^{2+} inhibited *ada* transcription at a dose which did not significantly inhibit *lacUV5* transcription. Al^{3+} and Fe^{3+} inhibited both *ada* and *lacUV5* transcription in the same dose range. Pb^{2+} did not affect either of the transcriptions within the dose range used in this experiment.

Next, the effect of metal ions on the MGTase activity of Ada protein was examined. The MGTase assay was carried out in the same buffer used for the transcription assay. MGTase activity was determined by measuring radioactivity in the protein fraction after incubation of Ada protein with [^{3}H]methylated calf thymus DNA. The MGTase activity observed in the presence of metal ion were plotted in Fig. 1, together with the transcription levels. The MGTase activity were also expressed as a percentage of the controls in the absence of metal ion. Ag^+, Al^{3+}, Cd^{2+}, Cu^{2+}, Hg^{2+} and Zn^{2+} inhibited MGTase activity. On the other hand, $FeCl_3$ and $PbCl_2$ did not significantly affect MGTase

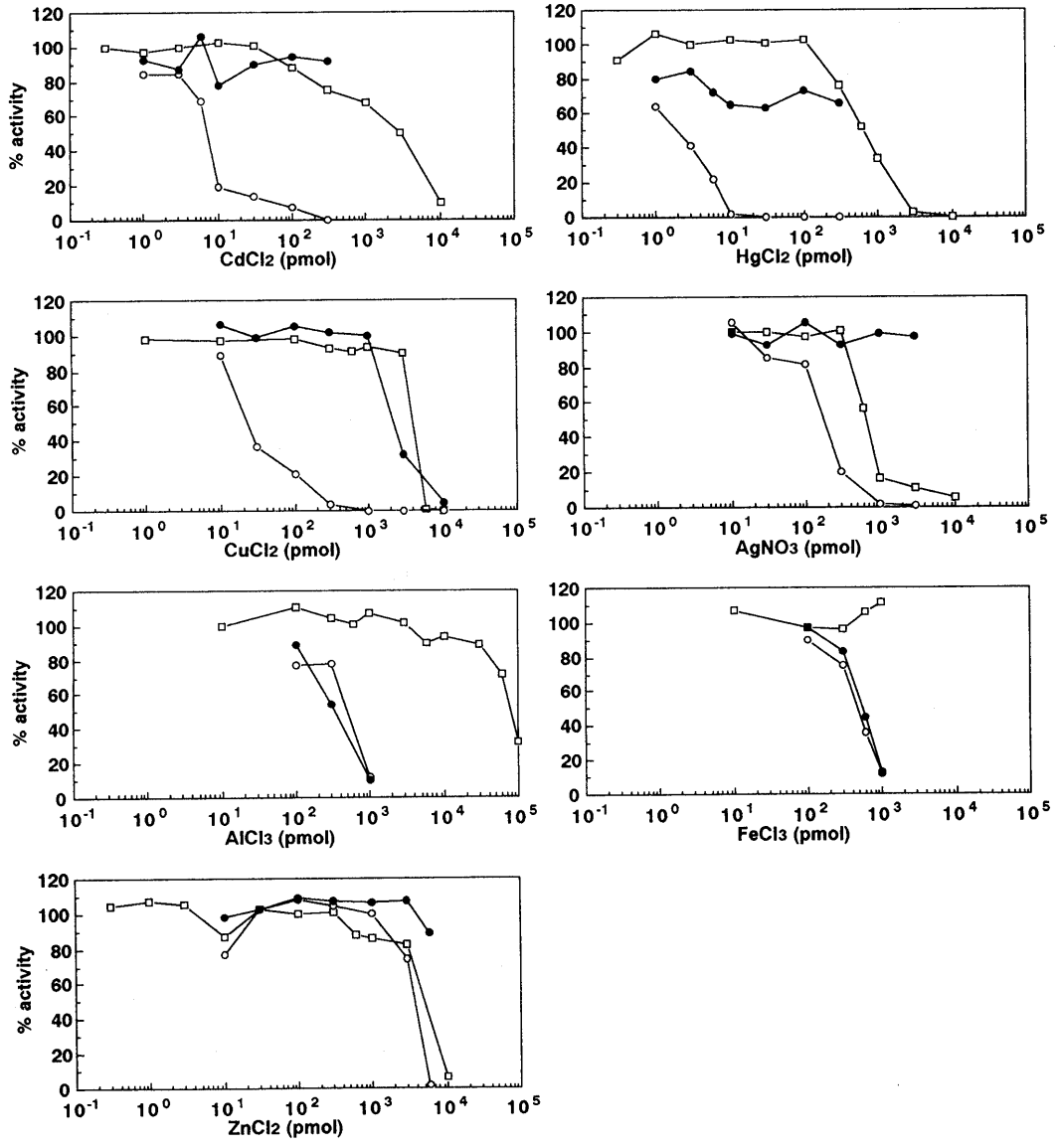

Fig. 1. Effects of Metal Ions on MGTase Activity and Transcription of *ada* and *lacUV5* Genes

The values are expressed as a percentage of the controls in the absence of metal ion. □, MGTase activity; ○, *ada* transcription; ●, *lacUV5* transcription.

activity at doses up to 1 nmol. Higher doses of the latter two metal salts were not available due to limited solubility. When $Pb(CH_3COO)_2$ was used instead of $PbCl_2$, no effect on MGTase was observed, even at a dose of 3 nmol (data not shown).

Effect of metal ions on the MGTase activity was compared with their effect on the transcriptions. Cd^{2+}, Cu^{2+} and Hg^{2+} inhibited *ada* transcription more effectively than MGTase activity, whereas Zn^{2+} inhibited *ada* transcription and MGTase activity equally. Ag^{+} had a marginal effect. Al^{3+} and Fe^{3+} inhibited both *ada* and *lacUV5* transcription in the same manner, even at a dose which did not affect MGTase activity. Pb^{2+} did not affect either the transcriptions or the MGTase activity.

Discussion

It has been reported that metal ions used in this study inhibit activity of MGTase partially purified from human placenta, rat-liver or *E. coli*.[17,19] The present study demonstrated that all metal ions used inhibit MGTase purified from *E. coli*, except for Pb^{2+} and Fe^{3+} which do not affect *E. coli* MGTase activity. Hg^{2+}, Ag^{+} and Cd^{2+} were most inhibitory to *E. coli* MGTase followed by Zn^{2+} and Cu^{2+}. However, Cd^{2+} and Cu^{2+} have been reported to be most inhibitory to rat-liver MGTase followed by Hg^{2+}, Zn^{2+} and lastly Ag^{+}.[17] It has been reported that Pb^{2+}, which does not inhibit *E. coli* MGTase, inhibits human MGTase but not rat-liver MGTase.[17,19]

Based on the effects of metal ions on MGTase activity of Ada protein and on transcription of *ada* and *lacUV5* genes, these ions can be classified into three groups. The first group includes Al^{3+} and Fe^{3+} which inhibit both *ada* and *lacUV5* transcriptions at a dose which does not inhibit MGTase activity. This suggests that these metal ions prefer to bind to RNA polymerase leading to nonspecific inhibition of transcription rather than to bind with SH groups of Ada protein which would normally accept methyl groups. These metal ions can be distinguished from other metal ions by the fact that their inhibitory activity is not reversible with the addition of dithiothreitol.[19] The second group includes Zn^{2+} which inhibits both *ada* transcription and MGTase activity to the same extent without inhibition of *lacUV5* transcription. It is thus probable that Zn^{2+} inhibits MGTase activity, and, as a result, sufficient amounts of the methylated MGTase are unavailable for potentiation of *ada* transcription. The third group includes Cd^{2+}, Cu^{2+} and Hg^{2+} which inhibit *ada* transcription even at a dose which does not significantly inhibit either MGTase activity or *lacUV5* transcription. Ag^{+} is also included in this category, although its effect is marginal. One possible mechanism which may explain the inhibitory effect on *ada* transcription is that these metal ions specifically block methyltransfer to Cys-69 which functions as the transcriptional activactor of the *ada* gene. However, this is not the case presented here. In this experiment, the MGTase activity is expressed as the sum of methyltransfers to both Cys-69 and Cys-321 when sufficient amounts of methylated DNA is present in the reaction mixture to methylate both acceptor sites of the Ada protein. If methyltransfer to Cys-69 was specifically blocked, the MGTase activity should have decreased to 50% within the dose range for complete inhibition of *ada* transcription. In addition, these metal ions inhibited *ada* transcription promoted by the methylated Ada protein prepared from Ada protein with methylated DNA in the absence of metal ion (data not shown). It is, therefore, safe to conclude that Cu^{2+}, Cd^{2+} and Hg^{2+} inhibit transcriptional promoting activity of the methylated Ada protein.

The present study indicates that Cd^{2+}, Cu^{2+} and Hg^{2+} bind preferentially to Ada protein at site(s) critical for activation of the *ada* transcription by binding with cysteine residues which are acceptor sites for methyl groups. The metal binding domain on methylated Ada protein responsible for inhibition of the *ada* transcription has not yet been identified. *In vitro* experiments have shown that methylation of Cys-69 renders Ada protein capable of binding to a specific sequence in the *ada* regulatory region, termed the "Ada box".[9,22,23] This binding facilitates further binding of RNA polymerase to the regulatory region which may promote open complex formation for initiation of transcription.[23] In this process, the N-terminal half of Ada protein is thought to be the main recognition site for binding to the *ada* regulatory sequence.[24] This is supported by the fact that Ada protein contains a potential helix-turn-helix motif spanning amino acids 100 to 121 on the N-terminal half, which is analogous to the amino acid sequence found in a typical DNA binding protein such as the lambda Cl repressor and MGTase from other microorganisms.[25-27] Moreover, it is reported that although the methylated N-terminal half of Ada protein binds to the *ada* promoter, it does not facilitate further binding of RNA polymerase. The C-terminal half of Ada protein may also be involved in binding RNA polymerase forming an active transcription initiation complex on the *ada* regulatory sequence.[24] It is probable that Cd^{2+}, Cu^{2+} and Hg^{2+} inhibit either binding of the methylated Ada protein to the *ada* regulatory sequence or binding of RNA polymerase to the sequence.

Among the metal ions which belong to the third group, only Cd^{2+} and Hg^{2+} inhibit *ada* gene expression in *E. coli*, as determined by measuring β-galactosidase expressed from the *ada'-'lacZ* fused gene, without inhibition of *umuDC* gene expression which is regulated by the *recA* and *lexA* genes.[15,16] In contrast, it was also previously reported that Ag^{+} and Cu^{2+} inhibit *ada* gene expression as well as *umuDC* gene expression in *E. coli*.[15] This is supported by the finding presented here that 50% inhibition of *ada* transcription was achieved by Cd^{2+} and Hg^{2+} in amounts of 4 and 1 molar eq of Ada protein, respectively, which are small as compared with the larger amounts of Ag^{+} and Cu^{2+} required. Alternatively, Cu^{2+} may inhibit both *ada* and *umuDC* gene expression by independent mechanisms.

Acknowledgements We are grateful to Dr. K. Sakumi for helpful advice. This work was supported by a grant from the Ministry of Education, Science and Culture of Japan.

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