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Cadmium inhibits mismatch repair by blocking the ATPase activity of the MSH2–MSH6 complex

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ABSTRACT

Cadmium (Cd^{2+}) is a known carcinogen that inactivates the DNA mismatch repair (MMR) pathway. In this study, we have tested the effect of Cd^{2+} exposure on the enzymatic activity of the mismatch binding complex MSH2–MSH6. Our results indicate that Cd^{2+} is highly inhibitory to the ATP binding and hydrolysis activities of MSH2–MSH6, and less inhibitory to its DNA mismatch binding activity. The inhibition of the ATPase activity appears to be dose and exposure time dependent. However, the inhibition of the ATPase activity by Cd^{2+} is prevented by cysteine and histidine, suggesting that these residues are essential for the ATPase activity and are targeted by Cd^{2+}. A comparison of the mechanism of inhibition with *N*-ethyl maleimide, a sulfhydryl group inhibitor, indicates that this inhibition does not occur through direct inactivation of sulfhydryl groups. Zinc (Zn^{2+}) does not overcome the direct inhibitory effect of Cd^{2+} on the MSH2–MSH6 ATPase activity *in vitro*. However, the increase in the mutator phenotype of yeast cells exposed to Cd^{2+} was prevented by excess Zn^{2+}, probably by blocking the entry of Cd^{2+} into the cell. We conclude that the inhibition of MMR by Cd^{2+} is through the inactivation of the ATPase activity of the MSH2–MSH6 heterodimer, resulting in a dominant negative effect and causing a mutator phenotype.

INTRODUCTION

Mispaired bases result from incorporation errors during DNA biosynthesis that have escaped the proofreading activity of DNA polymerases as well as from the formation of heteroduplex DNA during recombination of divergent sequences. DNA mismatch repair (MMR) plays a major role in the recognition and correction of the mispaired bases, increasing replication fidelity and maintaining genome integrity. Defects in MMR are the underlying cause of a cancer susceptibility syndrome called HNPCC and account for 20% of sporadic cancers (1).

MMR is a complex reaction that involves multiple proteins, which recognize the mismatch, excise the DNA containing the error and resynthesize the correct DNA sequence. In eukaryotes, six functional MMR genes have been identified, *MSH2*, *MSH3* and *MSH6*, which are homologs of *MutS* in *Escherichia coli*, while *MLH1*, *MLH3* and *PMS2* (*PMS1* in yeast) are homologous to bacterial *MutL* (2–4). These genes are involved in the recognition of the mismatch, a critical step in the pathway, as illustrated by the fact that defects in these genes account for two-thirds of HNPCC cases. The initial recognition of mispairs is carried out by two protein complexes: the MSH2–MSH6 heterodimer, also known as MutSα, which recognizes base–base mismatches and frameshift mispairs (±1 bp), while the MSH2–MSH3 heterodimer, also known as MutSβ, recognizes frameshifts and larger insertion deletion mispairs (2–4 bp). The MutL homologs MLH1, PMS2 (PMS1 in yeast) and MLH3 form heterodimers MLH1–PMS1 and MLH1–MLH3, which participate in downstream events subsequent to the recognition of mismatches by the MSH complexes. Because of their requirement in the repair of both types of mispairs, MSH2 and MLH1 are regarded as the key factors in MMR as defects in the genes encoding these proteins result in a complete loss of repair.

ATP binding and hydrolysis by the dimeric MSH protein complexes is a critical aspect of MMR and is believed to modulate the interactions of MSH2–MSH6 and MSH2–MSH3 with the mismatched DNA and other downstream factors (5–7). Several models have been proposed regarding the role of ATP in the recognition of mismatches by the MSH protein complexes, most of which agree on the basic principle of an ATP-dependent movement of the MSH heterodimers along the mismatched DNA following mismatch recognition. Thus, the presence of ATP reduces the steady-state affinity of MutSα for mismatched DNA (8). Opinions have differed regarding the fate of the ATP molecule. Some authors have

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suggested that hydrolysis of the ATP molecule and the energy generated thereby is necessary for the translocation of MSH proteins (6,9), while others have suggested that ATP binding alone can do the same (10,11). Formation of higher-order structures of the MSH2–MSH6 dimer with proteins, such as MLH1–PMS1 and PCNA, in the presence of ATP and mismatched DNA has also been reported previously (11–13).

Because of the essential role of ATP in MMR, it is likely that defects in ATP binding and hydrolysis severely affect the pathway. Mutations in the ATP-binding site of MSH2–MSH6 result in dominant negative alleles that exhibit a strong mutator phenotype (14,15). Cadmium (Cd^{2+}) was shown recently to impair this essential DNA repair pathway in yeast, as well as in human cells *in vivo* (16).

Cd^{2+} is a ubiquitous metal with no known biological function, to which humans are exposed mainly through occupation, environmental contamination and from cigarette smoke (17). The deleterious effects of Cd^{2+} reported to date include generation of reactive oxygen species, inhibition of DNA repair, depletion of glutathione, alteration of apoptosis and enhanced peripheral arterial disease (18,19). Cd^{2+} has also been reported to have a high affinity for protein sulfhydryl groups, can compete with and replace Zn^{2+} in proteins, and can bind to DNA at random, causing single-strand DNA breaks (20,21).

In light of the reported inhibitory effect of Cd^{2+} on the DNA MMR machinery, we took a biochemical approach to further define the role of Cd^{2+} on MMR inhibition. Our results described here demonstrate the direct effects of Cd^{2+} on the MSH2–MSH6 dimer. We observed inhibition of ATP binding, concomitant with inhibition of the ATPase activity, as well as inhibition of the mispaired DNA-binding activity of MSH2–MSH6 in the presence of Cd^{2+}, with the inhibition of the ATPase activity being significantly more pronounced. This inhibitory effect was also observed to be dose and exposure time dependent. In addition, a comparison of the inhibitory effect of Cd^{2+} on the ATPase activity of MSH2–MSH6 with *N*-ethyl maleimide (NEM), a sulfhydryl group inactivating compound, indicated that their methods of inhibition are different. However, Cd^{2+} was seen to bind to cysteine, a sulfur containing amino acid. Zn^{2+}, a member of the same group in the periodic table as Cd^{2+}, and a known antagonist of the mutagenic effects of Cd^{2+} (18,22), enhanced the inhibitory effect of Cd^{2+} *in vitro*. However, Zn^{2+} reduced the appearance of an increased mutator phenotype in yeast cells exposed to Cd^{2+}. We propose a mechanism of inhibition whereby Cd^{2+} allows MSH2–MSH6 to bind to a mispair but prevents ATP hydrolysis, effectively abrogating the pathway at this stage.

MATERIALS AND METHODS

Purification of MSH2–MSH6

Saccharomyces cerevisiae MSH2–MSH6 was purified by chromatography on PBE94, single-stranded DNA (ssDNA) cellulose and Q Sepharose as described previously (23). Purity was estimated to be at least 90% by Coomassie-stained gels.

ATPase assay

The measurement of hydrolysis of [γ-^{32}P]ATP into ADP and Pi by the MSH2–MSH6 was carried out as described previously (24). Briefly, the reaction was carried out in Buffer A containing 20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 5% glycerol, 5.7 μg ml^{-1} activated calf thymus DNA and 2 mM [γ-^{32}P]ATP. MSH2–MSH6 was pre-incubated with Cd^{2+} at 4°C for 10 min, mixed with the reaction buffer and incubated at 37°C for 30 min. Aliquots (1 μl) of each reaction were spotted onto a polyethyleneamide-TLC plate (Sigma). ATP and Pi were separated by chromatography in 1 M formic acid and 0.5 M LiCl. Products were analyzed in a PhosphorImager and quantitated using the ImageQuant software. One unit of ATPase activity was defined as the amount of protein that hydrolyzed 1 pmol of ATP to ADP and Pi under the above mentioned conditions.

To assay for the inhibition of MSH2–MSH6 ATPase activity by Cd^{2+} in the presence of Zn^{2+}, MSH2–MSH6 (5 μg) was pre-incubated with 50 μM Cd^{2+}, 50 μM Zn^{2+} and a combination of both in Buffer B (50 mM Tris–HCl, pH 7.5, 100 mM NaCl and 0.5 mM EDTA) in a total volume of 100 μl for 10 min. To remove excess metal ions, the protein–metal mixtures were dialyzed against 500 ml Buffer B for 3 h with one change of buffer. The protein contents of the dialyzed mixtures were recovered and quantified by Bradford (Bio-Rad). MSH2–MSH6–Cd^{2+} complex (40 nM) was pre-incubated with 50 μM Zn^{2+}. In addition, undialyzed MSH2–MSH6 protein (40 nM) was pre-incubated with 50 μM Zn^{2+}, 50 μM Cd^{2+} and a combination of both. The dialyzed and undialyzed metal-treated samples were then incubated with Buffer A at 37°C for 30 min and spotted on a TLC plate. Chromatography was carried out as described above.

To test the effect of Cd^{2+} on sulfhydryl groups, the inhibition of MSH2–MSH6 ATPase activity was compared with that caused by NEM, a sulfhydryl alkylating agent. MSH2–MSH6 (5 μg) was pre-incubated with Cd^{2+} (0.5 mM) in Buffer B at 4°C for 10 min as described above. To remove excess Cd^{2+}, the protein was then dialyzed against 500 ml Buffer B for 3 h with one change of buffer. The protein contents of the dialyzed mixtures were measured. The dialyzed Cd^{2+}-treated MSH2–MSH6 protein (40 nM) was then pre-incubated with 5 mM NEM. In addition, 40 nM undialyzed MSH2–MSH6 protein was pre-incubated with 0.5 mM Cd^{2+}, 5 mM NEM and a combination of both at 4°C for 10 min. The reaction mixtures were then incubated at 37°C for 30 min with the Buffer A, spotted on a TLC plate and analyzed as described above.

To determine whether reducing agents containing sulfhydryl groups could reverse the inhibitory effect of Cd^{2+} on the ATPase activity, MSH2–MSH6 protein (160 nM) was pre-incubated with excess of either Cd^{2+} (0.5 mM) or NEM (5 mM) at 4°C for 30 min, after which 2 mM DTT was added and the mixture was further incubated for 30 min. In addition, to test the capacity of DTT to prevent sulfhydryl group inhibition, MSH2–MSH6 was pre-incubated first with 2 mM DTT for 30 min, after which either Cd^{2+} (0.5 mM) or NEM (5 mM) was added and the mixture was incubated for an additional 30 min. As a control, MSH2–MSH6 was pre-incubated with either Cd^{2+} (0.5 mM) or NEM (5 mM) for 30 min. The reaction mixtures were then incubated with Buffer A at 37°C for 30 min and spotted on a TLC plate and assayed as described above.

The effect of different amino acids on Cd^{2+}-induced inhibition of MSH2–MSH6 was performed as follows. Stock solution of amino acids (0.1 M) was made in water, and the pH was the adjusted to 7.0. Cysteine is oxidized to cystine at

neutral pH and the latter has a very low solubility in water. Therefore, the pH of the cysteine solution was kept at pH 6.0. Tryptophan was dissolved in dimethyl sulfoxide (DMSO) as its solubility in water was very low. DMSO is inhibitory toward the ATPase activity of MSH2–MSH6, so a control reaction in the absence of Cd^{2+} was carried out. Tyrosine could not be dissolved in water, DMSO or ethanol and was not tested. Cd^{2+} (0.5 mM) was pre-incubated with 10 mM amino acid for 30 min at 4°C and then with MSH2–MSH6 (160 nM) at 4°C for 10 min. This mixture was then incubated with Buffer A at 37°C for 30 min and spotted on TLC plates as described above.

ATP binding to MSH2–MSH6

The ATP-binding assay was carried out with a Hoefer 25 mm filtration apparatus, pre-cooled to 4°C (25). Nitrocellulose membranes (25 mm) (Whatman) were briefly (30 s) soaked in 0.4 M KOH, extensively rinsed with distilled water and then equilibrated with Buffer C (25 mM Tris–HCl, pH 7.6, 100 mM NaCl and 10% v/v glycerol) at 4°C. $MgCl_2$ was left out of the buffer mixture to ensure ATP binding and not hydrolysis. MSH2–MSH6 (100 nM) was pre-incubated with 0–0.5 mM Cd^{2+} at 4°C for 10 min in a total volume of 250 µl in Buffer C. An aliquot (10 µl) of the mixture was taken out and incubated with 2 mM [γ^{32}P]ATP in Buffer A at 37°C for 30 min to assay for ATP hydrolysis activity as described above. The remaining mixture was further incubated with 1 µM [α^{32}P]ATP for 10 min. Control reactions in the absence of Cd^{2+} and MSH2–MSH6 were also carried out. The binding mixture was then applied to the equilibrated membranes, filtered under vacuum and washed extensively (20 ml) with Buffer C. The filter membranes were dried at room temperature and radioactivity was quantitated in a liquid scintillation counter.

Yeast mutator assays

The *lys2-10A* reversion assay was used to assay mutator phenotype in yeast. Individual colonies of the strain RDKY3590 (a gift from Dr R. Kolodner; Genotype: *a*, *ura3-52*, *leu2Δ1*, *trp1Δ63*, *hom3-10* and *lys210A*) was grown in synthetic complete medium (3 ml) supplemented with 2% glucose in presence of Cd^{2+} (1 µM) or Zn^{2+} (0–1 mM) or a combination of both. The cells were grown to saturation overnight with shaking at 30°C, washed twice with sterile water and resuspended in 1 ml sterile water. Dilutions were plated on YPD to quantify survival and on medium lacking lysine, to determine mutation frequency. The appearance of revertant colonies was an indication of a mutator phenotype. Mutation rates were determined by fluctuation analysis using at least five independent colonies per experiment (26). Each fluctuation test was repeated at least three times.

Duplex DNA substrates

Substrates for MMR assays were prepared by annealing 200 pmol each of oligonucleotides (Invitrogen) 5′-ATTTCCTTCAGCAGATAGGAACCATACTGATTCACAT-3′ (HFRO 1107), 5′-ATGTGAATCAGTATGGTTTCTATCTGCTGAAGGAAAT-3′ (HFRO 1108) and 5′-ATGTGAATCAGTGTTCCTATCTGCTGAAGGAAAT-3′ (HFRO 1109). HFRO 1108 and HFRO 1109 were annealed to HFRO 1107, yielding a G:T heteroduplex and a G:C homoduplex, respectively, by heating at 95°C for 5 min in 100 µl annealing buffer (0.5 M NaCl, 10 mM Tris–HCl, pH 7.5, 1 mM EDTA) and slow cooling to 25°C over 3 h. To remove ssDNA, electrophoresis of the DNA duplex was carried out in a 12% polyacrylamide gel in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0) at 150 V for 1 h under non-denaturing conditions. The band containing the double-stranded DNA was excised from the gel and DNA was extracted by phenol–chloroform extraction and ethanol precipitation. The purified oligonucleotide (20 pmol) was 5′ end labeled with [γ-^{32}P]ATP (Amersham) by incubating with T4 polynucleotide kinase (NEB) at 37°C for 30 min in a reaction volume of 50 µl. Unincorporated [γ-^{32}P]ATP was removed by purification in a G-25-Sephadex column (Roche). To test for the removal of ssDNA, aliquots of the duplex DNA were resolved in a 4.5% polyacrylamide gel under non-denaturing conditions.

Gel mobility shift assay of DNA binding

The purified [γ-^{32}P]ATP labeled duplex DNA (G:T and G:C) was used as substrates for this assay. MSH2–MSH6 (80 nM) was pre-incubated with 0–0.5 mM Cd^{2+} at 4°C for 10 min and then incubated with 100 fmol G:T or G:C substrate in a Buffer D (20 mM HEPES-KOH, 5 mM $MgCl_2$, 50 µg ml^{-1} BSA and 50 mM NaCl) in a total volume of 20 µl at 4°C for 15 min. The reaction was stopped with 4 µl of stop mixture (20% Ficoll and 0.75% bromophenol blue). Gel electrophoresis of 12 µl of the mixture was carried out under non-denaturing conditions in a 4.5% polyacrylamide gel (60:1 bisacrylamide) containing 5% glycerol in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0) at 10 V/cm at 4°C. Gels were dried and exposed on Kodak BioMax film. Analysis using a PhosphorImager and the ImageQuant software was carried out.

Statistical analysis

Data analysis and graphing was performed using the GraphPad Prism 4 software package. Specific analysis for each experiment is indicated in each Figure legend. In most cases, the mean of at least three experiments is plotted together with the standard deviation. Linear regression was used for best curve fitting when necessary and is indicated.

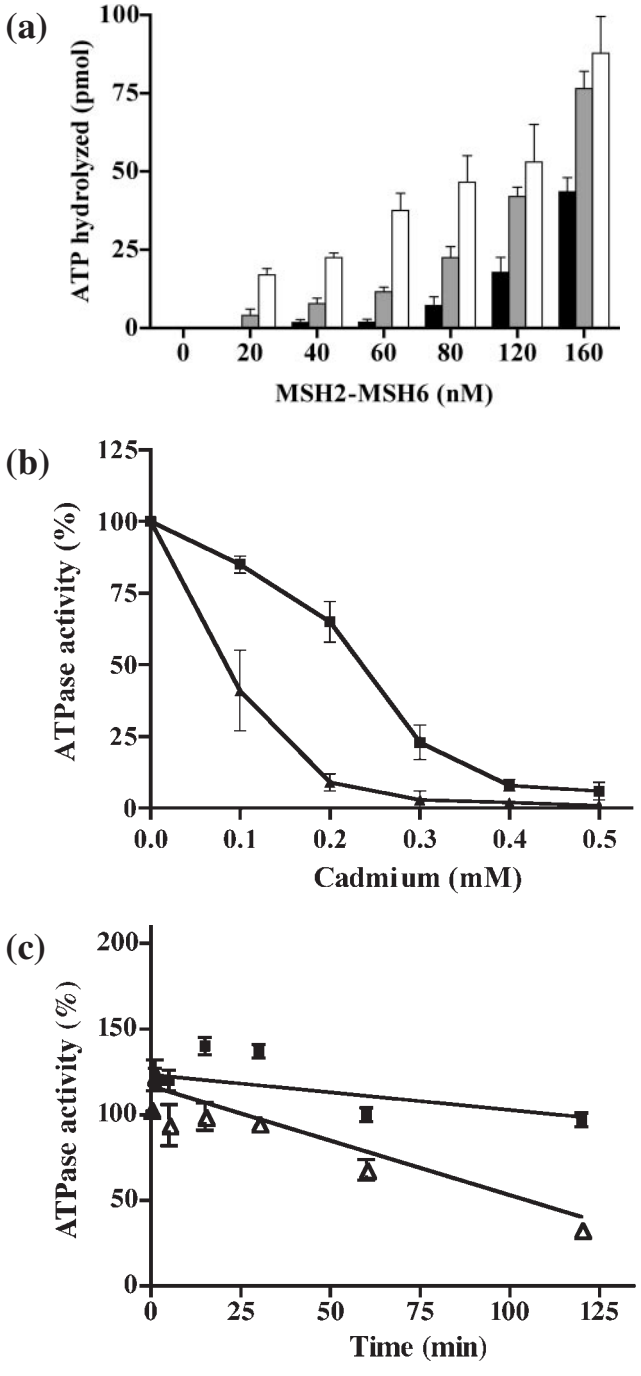
RESULTS

Effect of Cd^{2+} on the ATPase and DNA-binding activities of MSH2–MSH6

Cd^{2+} exposure resulted in a strong mutator phenotype in yeast cells, as determined with frameshift mutation reporters (homonucleotide runs in the *LYS2* gene that revert by −1 frameshifts), indicating that it reduces the MMR capacity (16). The initial mismatched DNA recognition and binding by the MSH proteins are crucial steps of the MMR pathway. In addition, ATP binding to the MSH proteins and hydrolysis are key control points (27). Therefore, we hypothesized that at a biochemical level, Cd^{2+} may have an effect on these two very important initial steps of the pathway.

The ATP hydrolytic activity of increasing amounts of purified MSH2–MSH6 protein (0–160 nM) was tested in the presence of 100 µM Cd^{2+} (Figure 1a, gray bars) as described in

Materials and Methods. A parallel reaction was carried out where MSH2–MSH6 (0–160 nM) was pre-incubated with Cd^{2+} for 10 min, before assaying for ATPase activity (Figure 1a, black bars). A control reaction of the ATP hydrolysis by MSH2–MSH6 (0–160 nM) in the absence of Cd^{2+} was also carried out (Figure 1a, white bars). The results indicate that Cd^{2+} severely inhibited the ATPase activity of MSH2–MSH6 at different levels of the protein (Figure 1a). In addition, pre-incubation with Cd^{2+} greatly enhanced the inhibitory effect, e.g. at 60 nM of MSH2–MSH6, Cd^{2+} pre-incubation resulted in only 6% (2.3 pmol ATP hydrolyzed) of the activity remaining, as opposed to 34% (13 pmol) of activity remaining when no pre-incubation was performed.

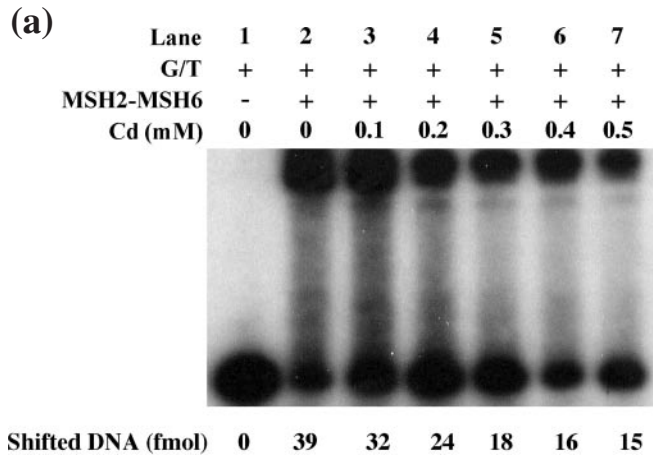


To assay for the effect of increasing Cd^{2+} concentration on a fixed amount of MSH2–MSH6, concentrations of 0–0.5 mM Cd^{2+} were pre-incubated with 80 and 160 nM MSH2–MSH6 and assayed for ATPase activity as described above. The results (Figure 1b) indicated that the Cd^{2+}-mediated inhibition of MSH2–MSH6 is dose dependent. At lower concentrations (Figure 1b, closed triangles), MSH2–MSH6 is significantly more sensitive to Cd^{2+} inhibition with 50% inhibition obtained with a Cd^{2+} concentration of 87 μM versus 240 μM for the higher concentration (Figure 1b, closed squares) of MSH2–MSH6 tested. At higher concentrations of Cd^{2+}, the ATPase activity was almost completely abolished. At the lower concentration of MSH2–MSH6 used (Figure 1b, closed triangles), only 3% of the activity remained at 200 μM of Cd^{2+}, while 8% activity remained at 500 μM for higher concentrations of MSH2–MSH6 (Figure 1b, closed squares).

A time-course study of the inhibition of Cd^{2+} (100 μM) with MSH2–MSH6 (160 nM) conducted at various time points over 120 min pre-incubation indicated that the inhibition is time dependent (Figure 1c). After 120 min pre-incubation with Cd^{2+} (Figure 1c, open triangles), MSH2–MSH6 displayed only 30% of the activity observed with 1 min pre-incubation.

MSH2–MSH6 binds to DNA containing mismatches (23). To determine the effect of Cd^{2+} on the mismatched DNA-binding activity, MSH2–MSH6 was pre-incubated with 0.1–0.5 mM Cd^{2+} and aliquots were taken and assayed for DNA binding by gel mobility shift assay. DNA with mispairs (G:T) or fully paired (G:C) were used as substrates. Cd^{2+} inhibited the mismatched DNA-binding activity of MSH2–MSH6 (Figure 2a) in a dose-dependent manner. At the conditions tested, the binding of MSH2–MSH6 to the G:C substrate was negligible and was not significantly affected by Cd^{2+} treatment (data not shown). A comparison of the effect of 0–0.5 mM Cd^{2+} on the ATPase and DNA-binding activity of MSH2–MSH6 was carried out (Figure 2b). The data indicate that while Cd^{2+} causes a reduction in the mismatched DNA-binding capacity of MSH2–MSH6 (Figure 2b, black bars), it has a significantly more pronounced inhibitory effect on the ATPase activity of MSH2–MSH6 (Figure 2b, white bars). At concentrations of Cd^{2+} of 200 μM, 70% of the DNA-binding activity of MSH2–MSH6 remained, compared with only 8% of the ATPase activity, a 9-fold difference. Similar effect was observed when higher concentrations of Cd^{2+} were used (Figure 2b). As a control, the DNA-binding

Figure 1. Effect of cadmium on the ATPase activity of MSH2–MSH6. (**a**) Effect of 100 μM Cd^{2+} on increasing concentrations (0–160 nM) of MSH2–MSH6. Pre-incubation of Cd^{2+} with MSH2–MSH6 (black bars) at 4°C for 10 min followed by incubation with the reaction mixture containing ATP at 37°C for 30 min. Direct incubation of MSH2–MSH6 (gray bars) with Cd^{2+} and ATP in reaction mixture at 37°C for 30 min without any pre-incubation. ATPase activity of MSH2–MSH6 in the absence of Cd^{2+} (white bars). Each bar corresponds to the average of three experiments. Standard deviation is indicated at the top of each bar. (**b**) Effect of increasing concentrations of Cd^{2+} (0–0.5 mM) on 160 nM (closed squares) and 80 nM (closed triangles) of MSH2–MSH6. Pre-incubation of MSH2–MSH6 with Cd^{2+} at 4°C for 10 min was carried out. The average of three experiments for each concentration of Cd^{2+} is presented for MSH2–MSH6 at 160 nM and the average of four experiments for MSH2–MSH6 at 80 nM. Standard deviation is included. The curves were not fitted. (**c**) Time-course study over 120 min pre-incubation of the ATPase activity of MSH2–MSH6 (160 nM) in the presence (closed squares) and the absence of Cd^{2+} (open triangles). The average of three experiments for each time point is presented. Standard deviation is included. Curves were fitted by linear regression analysis.



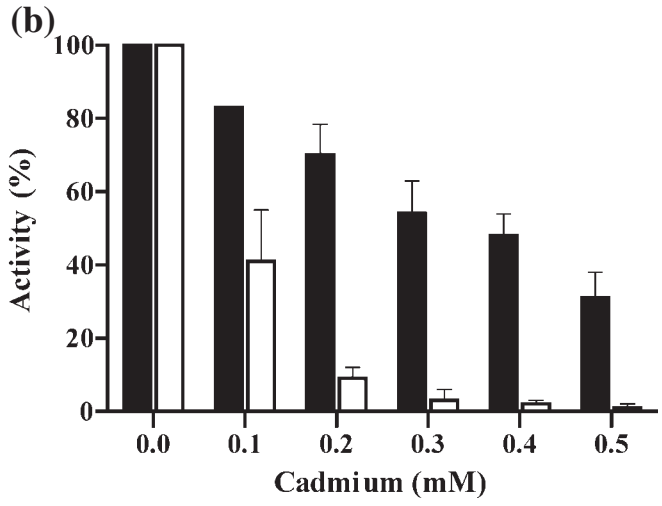


Figure 2. Effect of cadmium on the DNA-binding activity of MSH2–MSH6. (**a**) MSH2–MSH6 was pre-incubated with Cd^{2+} at 4°C for 10 min, mixed with reaction mixture containing labeled G/T mispaired DNA and further incubated at 4°C for 15 min in 20 µl final volume. Gel was run with 10 µl of the mixture, and autoradiographed. Quantitation was carried out with a phosphorImager. (**b**) Comparison of the ATPase activity (white bars) and DNA mobility shift activity (black bars) of MSH2–MSH6 (80 nM) in the presence of 0–0.5 mM Cd^{2+}. Activity is presented as a percentage of the activity of MSH2–MSH6 in the absence of Cd^{2+} taken as 100%. The average of three experiments for each concentration of Cd^{2+} is presented. Bars also include standard deviation.

capacity of the ssDNA-binding protein, replication protein A, was tested and found to not be inhibited by Cd^{2+} (data not shown).

Effect of Cd^{2+} on ATP-binding activity of MSH2–MSH6

To assay the effect of Cd^{2+} on the ATP-binding activity of MSH2–MSH6, a nucleotide filter binding assay was carried out. MSH2–MSH6 (100 nM) was incubated with 1 µM [α^{32}P]ATP in the presence or absence of Cd^{2+} and filtered through nitrocellulose membrane as described in Materials and Methods. ATP binding to MSH2–MSH6 resulted in retention of radioactive signal on the membrane, which was measured in a scintillation counter. To compare the ATP-binding activity (Figure 3, white bars) of MSH2–MSH6 with its ATP

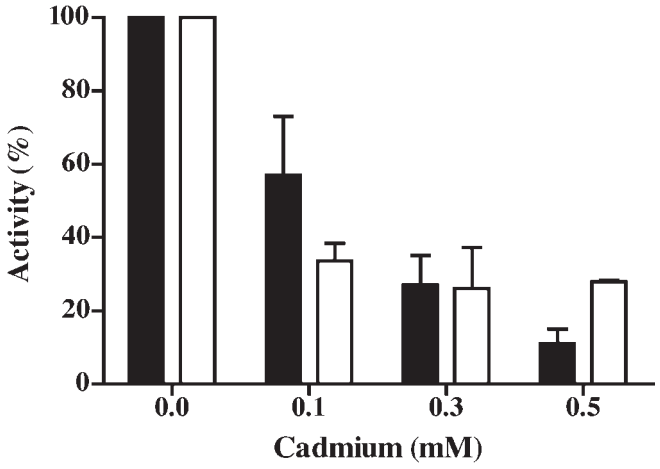


Figure 3. Effect of cadmium on the ATP hydrolysis and ATP-binding activities of MSH2–MSH6. MSH2–MSH6 was pre-incubated with Cd^{2+} (0–0.5 mM) at 4°C for 10 min. Aliquots were withdrawn and assayed for ATP hydrolysis (black bars) and ATP binding (white bars) as described in Materials and Methods. Results are expressed as a percentage of the activity of MSH2–MSH6 in the absence of Cd^{2+}. For ATP hydrolysis 100% corresponds to 200 pmol ATP hydrolyzed, while for ATP binding, 100% of the activity corresponds to 2 pmol ATP bound. The average of three experiments for each concentration of Cd^{2+} is presented. Standard deviation is included.

hydrolysis activity (Figure 3, black bars) in the presence of Cd^{2+}, an aliquot of the protein was simultaneously assayed for hydrolysis activity. The data indicate that the MSH2–MSH6 ATP-binding activity was inhibited in the presence of Cd^{2+} (Figure 3). The ATP-binding activity of MSH2–MSH6 was as sensitive as the hydrolysis activity to Cd^{2+} at every concentration. These results are consistent with a mechanism of inhibition of the MSH2–MSH6 ATPase activity as a consequence of reduced ATP binding.

Effect of Zn^{2+} on the Cd^{2+}-induced mutator phenotype of yeast

Yeast cells show a strong mutator phenotype after exposure to Cd^{2+} (16). To test the effect of Zn^{2+} on the Cd^{2+}-induced mutator phenotype, the mutation rate in the presence of Cd^{2+} (1 µM) and Zn^{2+} (0–1000 µM) was determined using a yeast strain carrying the *lys2-10A* allele as a reporter for mutation accumulation. The results (Figure 4a and b) indicate that 1 µM Cd^{2+} is highly mutagenic to yeast, increasing the mutation rate to 4.2×10^{-6}, a 221-fold effect compared with non-treated cells (background rate is 1.9×10^{-8}). This inhibition is comparable with that observed in *msh2* (9×10^{-6}) and *mlh1* (1×10^{-5}) strains (data not shown). The presence of Zn^{2+} causes a dose-dependent decrease in the mutator phenotype of the *lys2-10A* cells. At a concentration of Zn^{2+} of 1 µM, equal to that of Cd^{2+}, the mutator phenotype was reduced to 43% (rate of 1.8×10^{-6}) to that observed with Cd^{2+} alone. At 1000-fold higher Zn^{2+} concentration than Cd^{2+}, the appearance of a mutator phenotype was completely prevented yielding a mutation rate of 3.9×10^{-8}, only 2-fold higher than untreated cells (Figure 4b). Cell viability did not considerably change at the concentration of Cd^{2+} used. A determination of the toxic concentrations of Cd^{2+} resulted in survival of 100% at 0 µM, 113% at 1 µM, 82% at 10 µM and 18% at 100 µM.

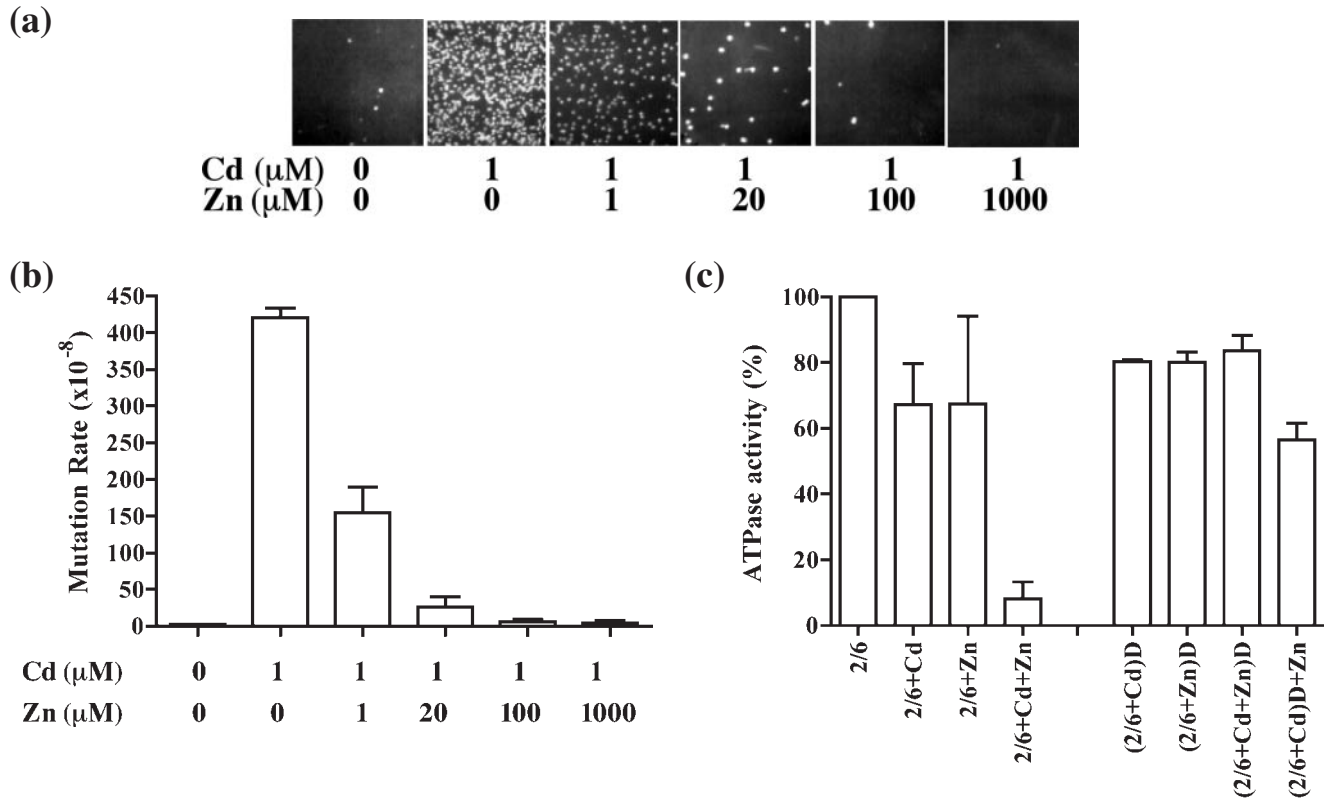


Figure 4. Effect of zinc on the cadmium-induced inhibition of MMR. (**a**) Yeast mutator assay using a strain carrying the *lys2-10A* allele was preformed in the presence of 1 μM Cd^{2+} and 0–1000 μM Zn^{2+}. The appearance of *Lys*$^+$ revertant colonies indicates a mutator phenotype. (**b**) Effect of Zn^{2+} on the mutator rate of the *lys2-10A* strain in the presence of Cd^{2+}. Each bar corresponds to the average of three sets of experiments using five independent colonies per set. Rates are calculated as described in Materials and Methods and standard deviation is included at the top of each bar. (**c**) MSH2–MSH6 (40 nM) was pre-incubated with 50 μM Cd^{2+} (2/6+Cd), 50 μM Zn^{2+} (2/6+Zn) or a combination of both (2/6+Cd+Zn) at 4°C for 10 min and assayed for ATPase activity as described in Materials and Methods. To remove excess metal ions, the mixture was dialyzed (denoted by a D in the Figure) extensively and assayed for ATPase activity. (2/6+Cd)D indicates that MSH2–MSH6 was treated with Cd^{2+} followed by dialysis; (2/6+Zn)D indicates that MSH2–MSH6 was treated with Zn^{2+} followed by dialysis; (2/6+Cd+Zn)D indicates that MSH2–MSH6 was treated with Cd^{2+} and Zn^{2+} followed by dialysis; and (2/6+Cd)D+Zn indicates that MSH2–MSH6 was treated with Cd^{2+} followed by dialysis, and then treated with Zn^{2+}. A comparison of the ATPase activity of the dialyzed and undialyzed MSH2–MSH6 is shown. The activity of untreated, undialyzed MSH2–MSH6 was used as 100% and corresponds to 26 pmol ATP hydrolyzed. Each set of experiments was repeated three times and the average is presented together with the standard deviation.

These data are the average of three experiments, and the standard deviation range was no more than 20% at each concentration. This result reinforces the fact that at the concentration of Cd^{2+} used (1 μM), we are scoring for cadmium-induced mutagenesis and not toxicity. Similarly, the concentrations of Zn^{2+} utilized in our experiments did not greatly reduce the viability of the cells. Survival results of three independent experiments were 100% at 0 mM, 97% at 0.1 mM, 78% at 1 mM and 6.4% at 10 mM Zn^{2+}. Because mutation rates were determined by fluctuation analysis using the method of Lea and Coulson (26), the small reduction in viability observed with Zn^{2+} is intrinsic in the calculation. Combination of Zn^{2+} and Cd^{2+} resulted in survival similar to those obtained by Zn^{2+} alone (data not shown). Thus, we conclude that Zn^{2+} can competitively reduce the Cd^{2+}-induced mutagenesis *in vivo*.

Role of Zn^{2+} in Cd^{2+} inhibition of MSH2–MSH6 activities

Cd^{2+} and Zn^{2+} belong to the same group on the periodic table. Zn^{2+} is an essential trace element that is required for several cellular functions and has been shown to antagonize the mutagenic effects of Cd^{2+} (18). In the present study, we determined the effect of Zn^{2+} on the inhibition of the ATPase activity of MSH2–MSH6 by Cd^{2+}. MSH2–MSH6 protein was pre-incubated with either 50 μM Cd^{2+} or 50 μM Zn^{2+} or a combination of both as described in Materials and Methods. The mixture was then dialyzed (denoted by D in Figure 4c) to remove metal ions and tested for ATPase activity. The results (Figure 4c) indicate that at the concentrations used, Zn^{2+} and Cd^{2+} resulted in the inhibition of the ATPase activity of MSH2–MSH6 by ∼35% (Figure 4c, 2/6+Cd and 2/6+Zn). However, when the metal ions were used in a combination, the inhibitory effect was greatly enhanced resulting in ∼90% inhibition (Figure 4c, 2/6+Cd+Zn). Dialysis resulted in recovery of the activity to 80–90% of original activity for the single metals [Figure 4c, (2/6+Cd)D and (2/6+Zn)D] and metal combination [Figure 4c, (2/6+Cd+Zn)D], indicating that at the concentrations of metal ions used, the inhibition was reversible. In fact, the addition of Zn^{2+} to dialyzed Cd^{2+}-treated MSH2–MSH6 yielded an inhibition similar to that obtained by Zn^{2+} alone [Figure 4c, (2/6+Cd)D+Zn]. When Cd^{2+} was tested at a higher concentration (0.5 mM), a stronger inhibitory effect was observed, which could only be partially

recovered by dialysis (data not shown), suggesting that higher concentration of Cd^{2+} results in a more severe and irreversible inactivation of MSH2–MSH6.

Effect of sulfhydryl groups on Cd^{2+}-induced inhibition of MSH2–MSH6

Cd^{2+} has been reported to have a high affinity for sulfhydryl groups (28) and has been proposed to specifically bind free sulfhydryl groups in MMR proteins resulting in inhibition of enzyme activities (20).

To determine whether Cd^{2+} inhibits the ATPase activity of MSH2–MSH6 by inactivating sulfhydryl groups, we compared the inhibitory effects of Cd^{2+} with that exerted by NEM. As described in Materials and Methods, MSH2–MSH6 was pre-incubated with 0.5 mM Cd^{2+}, 0.5 mM NEM or both, and the ATPase activity was determined. As expected, both agents (Figure 5a, 2/6+Cd and 2/6+NEM) significantly inhibited the ATPase activity of MSH2–MSH6 at the concentrations tested (Cd to 5% and NEM to 17% of original activity). When in combination (Figure 5a, 2/6+Cd+NEM), both inhibitors resulted in an increased inactivation of the ATPase activity (2% activity remaining), suggesting that Cd^{2+} and NEM most likely act at different sites of MSH2–MSH6. When MSH2–MSH6 was pre-incubated with Cd^{2+} or NEM, followed by dialysis (denoted by D in Figure 5a) recovery of the activity (from 5 to 14% of remaining activity) was observed only for the Cd^{2+}-treated sample [Figure 5a, (2/6+Cd)D], while the NEM treated sample [Figure 5a, (2/6+NEM)D] did not significantly change (from 17 to 19% of activity), suggesting that inhibition by alkylation of sulfhydryl groups by NEM is stable. The dialyzed Cd^{2+}-treated MSH2–MSH6 sample was still inhibited by NEM [Figure 5a, (2/6+Cd)D+NEM], indicating that the target sites for NEM-mediated inhibition are still available, and suggesting that both Cd^{2+} and NEM interact with different sites in MSH2–MSH6.

To further investigate whether Cd^{2+} inhibition was in part due to binding to, or oxidation of free sulfhydryl groups, we tested the effect of DTT, a sulfhydryl group-containing disulfide bond reducing agent, on the inhibition of the ATPase activity of MSH2–MSH6. For this purpose, the protein was either pre-incubated with DTT and then treated with either Cd^{2+} or NEM, or first treated with Cd^{2+} or NEM followed by the addition of DTT. The ATPase activity was scored and compared to that obtained in reactions where MSH2–MSH6 was not incubated with DTT. When DTT was added after treatment of MSH2–MSH6 with Cd^{2+}, only a slight recovery of the activity (from 4 to 12% of the activity remaining) was observed (Figure 5b, 2/6+Cd and 2/6+Cd+DTT). Similarly, when DTT was added to NEM treated MSH2–MSH6 (Figure 5b, 2/6+NEM and 2/6+NEM+DTT), only a slight recovery of the activity was obtained (from 7 to 16% of the activity remaining). However, DTT was very efficient at preventing the

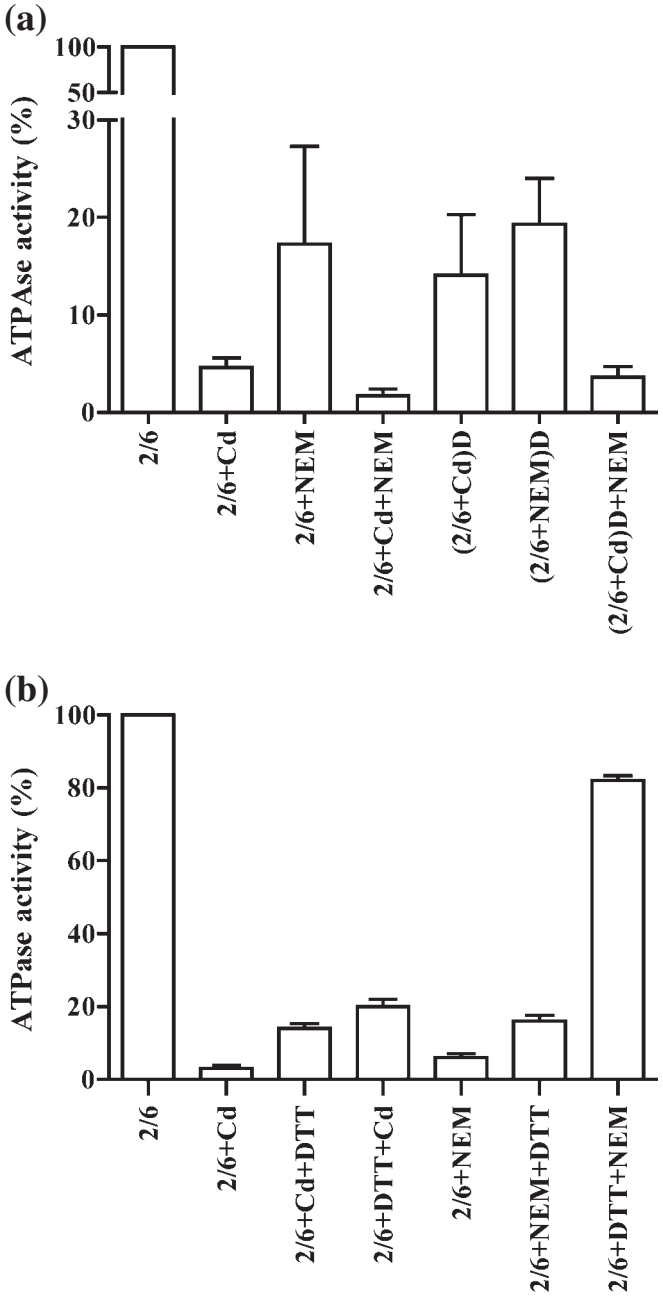


Figure 5. Role of sulfhydryl groups as targets in the inhibition of the ATPase activity of MSH2–MSH6 by cadmium. (**a**) Comparison of the inhibition of ATPase activity by NEM and Cd^{2+}. MSH2–MSH6 (40 nM) was pre-incubated with 0.5 mM Cd^{2+} (2/6+Cd), 0.5 mM NEM (2/6+NEM) or a combination of both (2/6+Cd+NEM) at 4°C for 10 min and assayed for ATPase activity. To remove excess NEM and Cd^{2+}, the pre-incubation mixture was extensively dialyzed (denoted as D in the Figure) and assayed for ATPase activity. (2/6+Cd)D indicates that MSH2–MSH6 was treated with Cd^{2+} followed by dialysis; (2/6+NEM)D indicates that MSH2–MSH6 was treated with NEM followed by dialysis; and (2/6+Cd)D+NEM indicates that MSH2–MSH6 was treated with Cd^{2+} followed by dialysis, and the treated with NEM. The comparison of the ATPase activities is shown here. The average of three experiments is presented, bars include the standard deviation. (**b**) Effect of DTT (2 mM) on the inhibition of the MSH2–MSH6 ATPase activity by Cd^{2+} (0.5 mM) and NEM (5 mM). MSH2–MSH6 (160 nM) was pre-incubated with Cd^{2+} for 15 min (2/6+Cd), Cd^{2+} for 15 min followed by DTT for 15 min (2/6+Cd+DTT), DTT for 15 min followed by Cd^{2+} for 15 min (2/6+DTT+Cd), DTT+Cd), NEM for 15 min (2/6+NEM), NEM for 15 min followed by DTT for 15 min (2/6+NEM+DTT), and DTT for 15 min followed by NEM for 15 min (2/6+DTT+NEM). The mixtures were then assayed for ATPase activity as described in Materials and Methods. The average of three experiments is presented, including the standard deviation.

inactivation of MSH2–MSH6 by NEM when present in the reaction at the time of addition of the alkylating agent, resulting in a recovery of 81% of the original ATPase activity (Figure 5b, 2/6+DTT+NEM). Conversely, only a slight recovery of activity (from 4 to 20%) was observed when the protein was pre-incubated with DTT and then treated with Cd^{2+} (Figure 5b, 2/6+DTT+Cd), suggesting that DTT does not effectively bind to Cd^{2+} as it does to NEM. These results suggest that Cd^{2+} inhibits the ATPase activity of MSH2–MSH6 via a mechanism distinct from binding to or oxidation of sulfhydryl groups.

Effect of amino acids on Cd^{2+} inhibition

To determine whether a specific amino acid residue is a target of Cd^{2+}, we tested if amino acids could prevent the inhibition the MSH2–MSH6 ATPase activity by Cd^{2+}. For this purpose, Cd^{2+} (0.5 mM) was pre-incubated with 10 mM of each of the essential amino acids (except Tyr) for 30 min at 4°C before the addition of MSH2–MSH6 (160 nM) at 4°C for 10 min. The mixture was then assayed for ATPase activity as described above. Of all the amino acids assayed, cysteine was the most effective at preventing Cd^{2+} inhibition of the ATPase, resulting in 88% of the activity remaining, versus 7% of remaining activity when no amino acid was added (Table 1). Interestingly, histidine was also effective in binding to Cd^{2+} and preventing the complete inactivation (33% activity remaining) of the MSH2–MSH6 ATPase activity (Table 1). Other amino acids had no or a marginal effect on preventing the inhibition by Cd^{2+}.

Table 1. Effect of the essential amino acids on the inhibition of MSH2–MSH6 by cadmium

Amino acid added[a]	% ATPase activity (±SD) $-Cd^{2+}$[b]	$+Cd^{2+}$
None	100	7 (2.3)
Alanine	138 (8.3)[c]	9 (1.6)
Arginine	94 (2.0)	5 (1.0)
Asparagine	120 (11.5)	4 (1.2)
Aspartic acid	121 (3.8)	10 (1.0)
Cysteine	122 (6.0)	88 (2.7)
Glutamine	125 (3.4)	6 (0.9)
Glutamic acid	133 (2.3)	14 (5.0)
Glycine	102 (5.0)	8 (1.0)
Histidine	113 (4.0)	33 (2.0)
Isoleucine	100 (1.0)	6 (0.7)
Leucine	102 (9.8)	8 (1.1)
Lysine	99 (7.0)	7 (0.5)
Methionine	110 (16.2)	9 (1.0)
Proline	105 (7.3)	7 (1.8)
Phenylalanine	104 (0.6)	8 (0.9)
Serine	107 (4.0)	8 (0.1)
Threonine	121 (0.4)	10 (0.5)
Tyrosine	ND	ND
Tryptophan	107 (10.7)	12 (2.5)
Valine	97 (1.6)	8 (0.5)

[a]Amino acids (10 mM) were pre-incubated with Cd^{2+} (0.5 mM) for 30 min at 4°C and then with 160 nM MSH2–MSH6 at 4°C for 10 min. The mixture was then incubated with 2 mM [γ^{32}-P]ATP at 37°C for 30 min and spotted on a TLC plate as described in Materials and Methods.
[b]Activity expressed as percentage of the ATPase activity of MSH2–MSH6 in the absence of Cd^{2+} and amino acids (100% activity corresponds to 200 pmol ATP hydrolyzed).
[c]Numbers in parenthesis represent standard deviation.

DISCUSSION

Cd^{2+} is a naturally occurring type I human carcinogen that is used widely in the industry. Since Cd^{2+} does not appear to cause a direct mutagenic effect on DNA, it is likely that the carcinogenicity of Cd^{2+} is via aberrant gene activation, suppressed apoptosis or altered DNA repair (18). Cd^{2+} was shown to inhibit the eukaryotic MMR pathway (16,22). It was also shown to inhibit a number of other proteins involved in DNA repair, such as apurinic/apyrimidinic (AP) endonuclease 1 (Ape1), bacterial formamidopyrimidine–DNA glycosylase (Fpg protein), mammalian XPA protein, as well as the H_2O_2 induced activation of poly(ADP–ribose)polymerase (29–31). It has also been shown to deregulate the expression of genes involved in controlling cell growth and division (32). Metal ions, such as nickel, selenium and arsenic, have also been proposed as DNA repair inhibitors (33–35). In the current study, we have elucidated the biochemical nature of Cd^{2+}-induced inhibition of MMR.

The primary findings of this study are (i) Cd^{2+} inhibits the ATP binding and hydrolysis activities of the MMR protein MSH2–MSH6 and is less inhibitory toward the DNA-binding activity of the protein. (ii) The inhibition is concentration and exposure time dependent. (iii) Zn^{2+} does not protect from the inhibitory effect of Cd^{2+} *in vitro*, rather, it exacerbates the inhibition. However, *in vivo*, the presence of Zn^{2+} reduces the appearance of mutator phenotype induced by Cd^{2+} in yeast. (iv) The mechanism of inhibition is distinct from that of NEM, a sulfhydryl group modifier. (v) Cysteine, a sulfur containing amino acid, and histidine are capable of binding to Cd^{2+} and preventing its inhibitory effect on the ATPase activity of MSH2–MSH6.

The direct inhibition of eukaryotic MMR proteins by metal ions has not been reported widely. We have shown in this study that Cd^{2+} strongly inhibits the ATPase activity of the MSH2–MSH6 heterodimer. This could be a possible mechanism of inhibition of several of the transition metal ions that have been designated as carcinogens. In addition, we have shown that Cd^{2+} is less inhibitory toward the DNA-binding activity of MSH2–MSH6, suggesting that ATP hydrolysis defective protein may still recognize and bind to mispaired DNA. A similar effect was seen with vanadate, an inhibitor of bacterial MutS, which inhibited the DNA-binding activity by ~60%, while the ATPase activity was almost completely abolished (36).

The antagonistic effect of Zn^{2+} on Cd^{2+} has been well documented, but has mostly focused on Cd^{2+} toxicity (18). However, cells exposed to low Cd^{2+} concentrations (1 μM) do not present a detectable reduction in viability but display a significant increase in the mutator phenotype, indicating that at these low levels, Cd^{2+} is mutagenic. We demonstrate that the mutagenic effect of Cd^{2+} *in vivo* can be efficiently prevented by Zn^{2+}. This is a very important observation, since carcinogenesis by Cd^{2+} is believed to occur through chronic exposure to low levels of the metal. A recent report presents similar results for human cells (22). A potential mechanism of protection may involve a direct effect of Zn^{2+} on MSH2–MSH6 protein, since it has been suggested that Cd^{2+} may exert its carcinogenicity by replacing Zn^{2+} from zinc finger proteins as well as other binding domains that may be essential for DNA repair (37). However, Zn^{2+} failed to prevent the

inhibitory effect of Cd^{2+} on the MSH2–MSH6 ATPase activity *in vitro*. Furthermore, Zn^{2+} enhanced the inhibitory effect of Cd^{2+} and was inhibitory on its own. No Zn^{2+} finger has been described in MSH2–MSH6 and this is consistent with the lack of protection effect by Zn^{2+} at the protein level. The mechanism by which Zn^{2+} inhibits MSH2–MSH6 is not known at this time. Other reports of the inhibitory effects of Zn^{2+} have been published. For example, the Mg^{2+} dependent ($Na^{+}K^{+}$) ATPase activity, which is essential for cell excitability, was shown to be competitively inhibited by Zn^{2+} in electrocytes from *Electrophorus electricus* (38), and the (Ca^{2+}) ATPase activity of human erythrocyte plasma membrane was also shown to be inhibited by Zn^{2+} ions (39).

A potential mechanism that explains the reduction of the Cd^{2+}-induced mutagenesis by Zn^{2+} may involve common transporters, whereby the presence of Zn^{2+} ions prevents the Cd^{2+} ions from being transported into the cells. Zn^{2+} homeostasis is carefully controlled by several mechanisms (40). Zn^{2+} uptake into the cell is carried out by two membrane transporters, Zrt1 and Zrt2 (41), which have high affinity (K_m = 10 nM) and low affinity (K_m = 100 nM) for Zn^{2+}, respectively. The expression of these transporters is activated by transcription factor Zap1 when Zn^{2+} levels are low (40). Conversely, exposing the cell to high levels of Zn^{2+} (zinc shock) results in a rapid loss of Zrt1 uptake activity and protein (40). This inactivation occurs through zinc-induced endocytosis of the protein and subsequent degradation in the vacuole (40), allowing the cell to tolerate exposure to high levels of Zn^{2+}. In yeast, Cd^{2+} uptake has been shown to be mediated by the zinc transporter Zrt1 (42,43). This observation is consistent with our findings. We believe that when cells are exposed to high levels of Zn^{2+} (zinc shock), the Zrt1 transporter is internalized preventing, or significantly reducing the entry of Cd^{2+}. Under these conditions, the levels of intracellular Cd^{2+} would be too low to inactivate MMR and cause a mutator phenotype. This is consistent with our finding that increasing concentrations of Zn^{2+} reduce the mutator phenotype. This mechanism also explains why exposure of cells to excess Zn^{2+} did not result in a mutator phenotype *in vivo*, although Zn^{2+} can inactivate the MSH2–MSH6 ATPase activity *in vitro*. Intracellular Zn^{2+} is compartmentalized and its level is tightly controlled (40) in such a way that it is unlikely that normally Zn^{2+} reaches concentrations that interfere with MMR. High levels of Zn^{2+} are not normally toxic to the cell. However, at very high levels Zn^{2+} is toxic and results in reduced viability, indicating the inactivation of other essential pathways.

Cd^{2+} has been reported to react with thiol groups, particularly glutathione (44). In our studies, we have shown that Cd^{2+} and NEM (a sulfhydryl group alkylating agent) have distinct mechanisms of inhibition of the ATPase activity of MSH2–MSH6 and that the inhibitory effect of NEM but not Cd^{2+} can be reduced by DTT. Cd^{2+} seems to be a more effective inhibitor of the ATPase activity of MSH2–MSH6, since higher concentrations of NEM were necessary to exert the same effect. However, when individual amino acids were assayed for their ability to bind Cd^{2+}, cysteine was seen to bind very strongly. These data suggest that Cd^{2+} may be recognizing additional features in the amino acid residue cysteine, besides its free sulfhydryl group.

Interestingly, histidine was also found to bind to Cd^{2+}. The role of histidine residues in ATP hydrolysis catalysis has been documented. In fact, a mutation of the His728 residue in *Taq* MutS was shown to almost completely inhibit its ATPase activity (45). Mutation of the equivalent conserved His in hMSH6 (H1248D) was found in an HNPCC patient, while the same in yMSH6 (H1096A) was found to increase the mutation rates (46,47). The binding of Cd^{2+} to an essential His residue at the ATP binding/hydrolysis site of yeast MSH2–MSH6 could be a possible mechanism of inhibition of this protein and, consequently, of the MMR pathway.

Cys and His are components of zinc finger proteins, and binding of Cd^{2+} to such proteins has been proposed as a mechanism of inhibition for DNA repair proteins XPA and Fpg (30). Although Zn^{2+} finger proteins are known to bind to DNA, our studies indicate that Cd^{2+} does not inhibit the mismatched DNA binding of MSH2–MSH6 as strongly as its ATPase activity. A conserved Phe residue in *E.coli* MutS, human MSH6 and yeast MSH6 has been shown to be essential for DNA binding (15,48,49). Phe, however, did not bind to Cd^{2+} in our studies. Thus, it is possible that a zinc finger motif is not involved in DNA binding in the MSH proteins. In bacterial MutS, the ATP-binding domains of both MutS subunits interact with each other and with the DNA-binding regions of both proteins (50,51). This leads to a close connection between ATP/ADP binding and DNA binding that is transferred throughout the protein and affects either activities. Based on this fact, it is possible that Cd^{2+} binding to Cys and His residues of potential zinc finger domains in MSH2–MSH6 causes a mild defect in DNA binding that is transmitted to the ATP-binding sites of the proteins effectively blocking its ATP binding and hydrolysis activities.

In conclusion, we propose that in the presence of Cd^{2+}, the MSH2–MSH6 protein binds to mispaired DNA. However, as the ATP binding and hydrolysis activities of the protein are abolished, the pathway stalls at this stage resulting in a dominant negative effect and a mutator phenotype. It remains to be determined whether other components of the MMR pathway (e.g. MSH2–MSH3, MLH1–PMS1, EXO1, etc.) are similarly inactivated by Cd^{2+}.

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