

Selective degradation of reverse gyrase and DNA fragmentation induced by alkylating agent in the archaeon *Sulfolobus solfataricus*

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ABSTRACT

Reverse gyrase is a peculiar DNA topoisomerase, specific of hyperthermophilic *Archaea* and *Bacteria*, which has the unique ability of introducing positive supercoiling into DNA molecules. Although the function of the enzyme has not been established directly, it has been suggested to be involved in DNA protection and repair. We show here that the enzyme is degraded after treatment of *Sulfolobus solfataricus* cells with the alkylating agent MMS. MMS-induced reverse gyrase degradation is highly specific, since (i) neither hydroxyurea (HU) nor puromycin have a similar effect, and (ii) topoisomerase VI and two chromatin components are not degraded. Reverse gyrase degradation does not depend on protein synthesis. Experiments *in vitro* show that direct exposure of cell extracts to MMS does not induce reverse gyrase degradation; instead, extracts from MMS-treated cells contain some factor(s) able to degrade the enzyme in extracts from control cells. *In vitro*, degradation is blocked by incubation with divalent metal chelators, suggesting that reverse gyrase is selectively degraded by a metal-dependent protease in MMS-treated cells. In addition, we find a striking concurrence of extensive genomic DNA degradation and reverse gyrase loss in MMS-treated cells. These results support the hypothesis that reverse gyrase plays an essential role in DNA thermoprotection and repair in hyperthermophilic organisms.

INTRODUCTION

Alkylating agents induce extensive damage of DNA, RNA, lipids and proteins, thus affecting virtually every cell process. Therefore it is expected that organisms cope with alkylating damage activating a highly coordinated response to carry out repair, removal and replacement of damaged molecules (1,2). Alkylating damage in DNA can be repaired following specific mechanisms, such as excision by the base excision repair (BER) pathway and direct demethylation by DNA methyltransferases, as well as by other repair pathways, such as nucleotide excision repair (NER) and homologous recombination (HR) (3,4). In addition, genetic and expression analysis showed that the cell response to alkylation damage involves a high fraction of genes involved in all cell processes (5–8).

Methylating agents are widely occurring in nature in all environments, including thermophilic niches, as by-products of biomass decaying and burning (3,9). *Archaea*, including those adapted to thermophilic environments, encode for homologs of eukaryal enzymes involved in NER, BER and HR (10); several of them have been characterized biochemically and structurally [reviewed in (11,12)]. However, the effects of methylating agents have been studied in none of the *Archaea* or thermophilic organisms.

Reverse gyrase is a peculiar enzyme found only in hyperthermophilic microorganisms, which induces positive supercoiling in an ATP-dependent reaction. The enzyme has a modular organization, with a N-terminal domain showing the signature of Superfamily II helicases, and a C-terminal topoisomerase domain. This latter belongs to the Topo IA family of DNA topoisomerases, ubiquitous in *Bacteria* and *Eucarya*. Whereas the C-terminal domain is able to perform an ATP-independent DNA relaxation reaction, neither the whole protein nor the isolated N-terminal domain shows helicase

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activity (13–16). In the hyperthermophilic archaeon *Sulfolobus solfataricus*, reverse gyrase activity is negatively regulated by one of the main chromatin components of this strain, Sul7d (17), whereas it is stimulated by the single strand binding protein SSB (18).

Because reverse gyrase is the only hyperthermophile-specific protein identified so far (19), and positive supercoiling is known to stabilize DNA, the enzyme has been associated to adaptation to high temperature. Recently, reverse gyrase was reported to have ‘DNA chaperone’ activity *in vitro*, preventing heat-induced breakage and aggregation of the double strand (20). Genetic analysis in *Thermococcus kodakaraensis* showed that a strain disrupted for reverse gyrase gene was viable, but its growth was significantly retarded specifically at high temperature, and suggested that the enzyme plays a role in maintenance of genome stability (21).

We have previously shown that reverse gyrase is recruited to DNA in *S.solfataricus* after ultraviolet (UV) irradiation (22), suggesting that the enzyme is involved, directly or indirectly, in the cell response to DNA damage.

We therefore sought to investigate the effect of other damaging agents on reverse gyrase. We show here that the enzyme is specifically depleted from cells treated with the alkylating agent MMS. This phenomenon is highly selective, since none of the other proteins tested (Topoisomerase VI, the two non-specific DNA binding proteins Sul7d and Smj12, and one subunit of the chaperone prefoldin) showed reduction of their amount. Likewise, the replication inhibitor hydroxyurea (HU) did not induce reverse gyrase loss. Depletion of reverse gyrase from MMS-treated extracts is not due to transcriptional down-regulation, as shown by quantitative RT-PCR, and occurs in the absence of protein synthesis. Experiments *in vitro* shows that reverse gyrase depletion is not induced by direct exposure of cell extracts to MMS; however, extracts from MMS-treated cells contain some factor(s) able to degrade the enzyme in extracts from control cells. This degradation is blocked by the presence of divalent metal chelators [EDTA or 1,10 phenantroline (PHE)], strongly suggesting that reverse gyrase is selectively degraded by a metal-dependent protease activated in MMS-treated cells. In addition, we show that genomic DNA is extensively degraded after MMS treatment, and we found striking coincidence between timing and dose-dependence of DNA and reverse gyrase degradation. Our results are discussed in light of the potential protective role of reverse gyrase against damage to DNA.

MATERIALS AND METHODS

Cultures growth, treatment and extract preparation

S.solfataricus P2 cultures were grown at 80°C as described previously (23) until exponential phase (about 0.4 OD₆₀₀); compounds were added to an aliquot of the culture, and the rest was used as a control. Incubation continued for different duration at 80°C. Aliquots of treated and control cultures were withdrawn and used for preparation of total and fractionated protein extracts as described (22).

Western blot

Total and fractionated extracts were analysed using the Amersham ECLPlus kit and a ChemiDoc apparatus (BioRad).

Polyclonal antibodies against *Sulfolobus acidocaldarius* reverse gyrase (24), *S.solfataricus* Sul7d (25), topoisomerase VI B subunit (P. Forterre and M. Gabelle, Orsay, unpublished data) and the β -subunit of prefoldin (A. D’Amaro and M. Ciaramella, unpublished data) were raised in rabbit, and against *S.solfataricus* Smj12 (25) was raised in goat.

Reverse gyrase assays

Reverse gyrase activity was assayed in total protein extracts and quantified as reported (18,22). Plasmid pGEM7 was from Promega. For quantifications, the fluorescence of each band in ethidium bromide-stained gels was measured under UV light with a Chemidoc apparatus and the QuantityOne software (BioRad). The specific linking difference (σ) was determined using the equation $\sigma = \Delta Lk/Lk_0$. σ values of the topoisomers whose intensity was >30% of the intensity of the most intense topoisomer were considered for the calculation of the mean σ value.

Real-time RT-PCR

Total RNA was prepared as previously described (23), extensively digested with DNase (Qiagen) and the absence of DNA was assessed by the lack of PCR amplification with each sets of primers described below. Total cDNA was obtained using M-MLV Reverse Transcriptase and random hexamers (Ambion). cDNA was then amplified in a BioRad LightCycler using the DyNAmo HS Syber Green qPCR Kit (Finnzymes). Oligonucleotides used for amplification were the following: GACCCTTGCCACCATACACT; TCCCCTTGTTCTATG-GATGC for TopR1; CTGCCATGTGAGGCTTGTTTAC; CTCTGATTACTCGCAAGCCTTC for TopR2; GAATG-GGGGTGATACTGTCG; TTACAGCCGGGACTACAGG for the 16S rRNA gene. Optimal melting temperatures for each primer pair were determined by performing real-time analysis with a temperature gradient ranging over 10°C; negative controls with no template cDNA were always included. PCR conditions were: 15 min at 95°C for initial denaturation, followed by 40 cycles of 10 s at 95°C, 25 s at 56°C and 35 s at 72°C, and a final step of 10 min at 72°C. Product purity was controlled by melting point analysis of setpoints with 0.5°C temperature increase from 72° to 95°C. PCR products were analysed on 2% agarose gels and visualized by ethidium bromide staining.

The expression values of reverse gyrase genes were normalized to the values determined for the 16S rRNA gene. Absolute expression levels were calculated as Target/16S ratio in controls and MMS-treated cells, respectively. Relative mRNA expression levels (controls/MMS-treated cells ratio) were calculated as (Target/16S ratio in MMS-treated cells)/(Target/16S ratio in controls). Data reported are from two independent RNA preparations; each cDNA was then used for two independent amplifications and in each amplification samples were in triplicate.

Preparation and analysis of genomic DNA

A protocol was set up for the extraction of total DNA without bias for high-molecular weight molecules, in order to extract with similar efficiency long as well as degraded DNA fragments. Cultures grown as described above (80 ml) were centrifuged and the cell pellet was resuspended in 0.3 ml of

lysis buffer [20 mM Tris-HCl (pH 8), 5 mM EDTA, 1% SDS). The lysate was extracted five times with a phenol-chlorophorm (1:1) suspension equilibrated in 10 mM Tris-HCl (pH 8), 1 mM EDTA. Nucleic acids were then ethanol precipitated and RNA was digested by RNase A (1 mg/ml). DNA was analysed by electrophoresis in 1% agarose gel in Tris-Acetate buffer containing 1 µg/ml ethidium bromide. Gels were photographed under UV light using the Chemidoc system (BioRad).

RESULTS

S.solfataricus is highly sensitive to MMS

S.solfataricus P2 cultures were grown at 80°C in rich medium with shaking; under these conditions, the replication cycle of this strain is about 7 h and it takes about 24 h from dilution to reach the exponential phase. Different concentrations of MMS were added and culture density was followed spectrophotometrically (Figure 1A). Whereas the drug at 0.17 mM only had a minor effect, all higher MMS concentrations induced slight reduction of absorbance. After 24 h, absorbance resumed exponential increase if 0.35 mM was used; in contrast, at higher MMS concentrations culture density remained essentially constant over the next 48 h. Absorbance was also measured 2 and 6 h after MMS addition, showing values similar to controls at 0.17 mM MMS; instead, when 0.35, 0.7 and 1.4 mM MMS were used, the cell density remained essentially similar to that measured at the time of MMS addition (data not shown).

The same results were obtained if after 30 min of incubation with MMS at different concentrations, the cultures were centrifuged and fresh medium was added (data not shown). These data demonstrate that *S.solfataricus* is remarkably more sensitive to MMS than mesophilic model organisms: for instance, wild-type *Escherichia coli* strains are fully resistant to 1.5 mM and *Saccharomyces cerevisiae* to 5 mM MMS (26,27).

Phase-contrast microscopy showed that, consistent with OD reduction, the total cell number was highly reduced 24 h after treatment with 0.35–1.4 mM MMS. In addition, MMS-treated cells show heterogeneous aspect, with most cells of reduced size with respect to controls and several larger cells with disordered interior that might be indicative of problems in DNA replication and cell division (data not shown).

Total protein content from controls and MMS-treated cultures was analysed by SDS gel electrophoresis. No dramatic change in the protein pattern was apparent under the conditions used (Figure 1B).

MMS treatment induces reverse gyrase loss

We have previously shown that reverse gyrase is translocated from the cytoplasm to DNA in *S.solfataricus* after UV irradiation (22). We therefore wondered whether MMS also affected reverse gyrase level or distribution. Cultures grown at 80°C were treated with different amounts of MMS, cells were lysed after 2 h and lysates were fractionated, obtaining a soluble supernatant and a pellet containing genomic DNA with tightly associated chromosomal proteins (22).

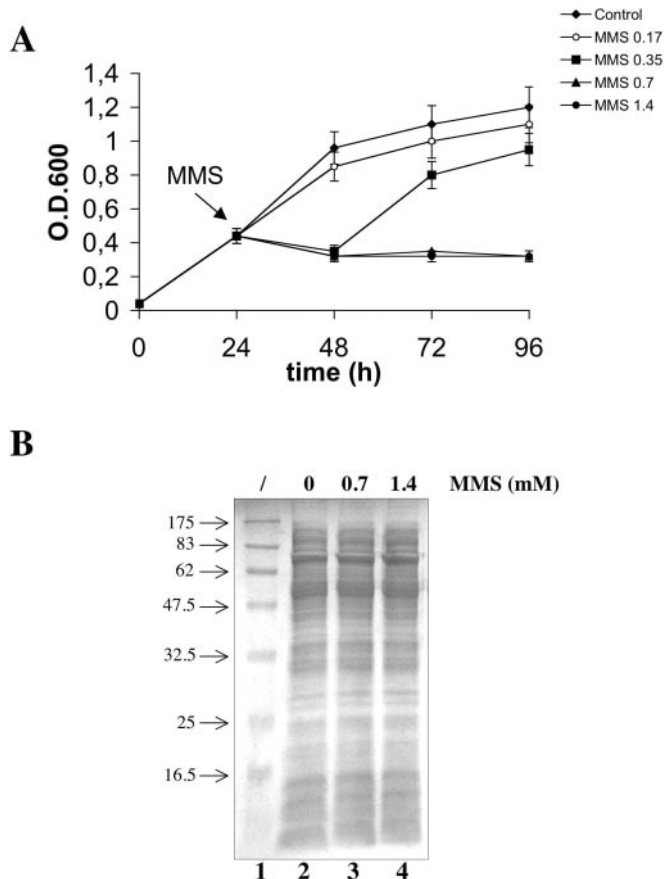


Figure 1. Effects of MMS treatment on *S.solfataricus*. (A) *S.solfataricus* P2 cultures were grown at 80°C until the exponential phase; at the time indicated by the arrow, cultures were split and MMS was added to each aliquot at the indicated concentrations; one aliquot was mock-treated. Absorbance at 600 nm was measured at indicated time points. Values from five independent experiments are reported. (B) Coomassie-stained SDS-PAGE of total proteins (60 µg/lane) extracted 2 h after treatment with indicated MMS concentrations. Lane 1, molecular weight marker (kDa).

We analysed the amount and distribution of reverse gyrase in cell extracts by western blotting using a polyclonal antibody directed against the *S.acidocaldarius* reverse gyrase which recognizes the *S.solfataricus* protein (Figure 2A).

As shown previously (22), under the experimental conditions used to prepare these extracts, in control cells about 90% of reverse gyrase was found in the soluble fraction, and only about 10% in the pellet. A similar distribution was observed at 0.35 mM (Figure 2A) or lower MMS concentrations (data not shown). In contrast, 2 h after MMS treatment the protein was dramatically reduced in supernatants at 0.7 mM MMS and disappeared at 1.4 mM MMS; only a slight reduction was observed in the pellets. The same filter was probed with an antibody directed against the B subunit of topoisomerase VI, an archaeal-specific type II topoisomerase (28): topoisomerase VI was mainly associated to the insoluble fraction, and no variations in its level or distribution were observed after MMS treatment. As a control of loading we also used an antibody directed against the β -subunit of the chaperone prefoldin, which showed no significant variations (Figure 2A).

Reverse gyrase depletion was both time- and MMS concentration-dependent: indeed, after 30 min of treatment,

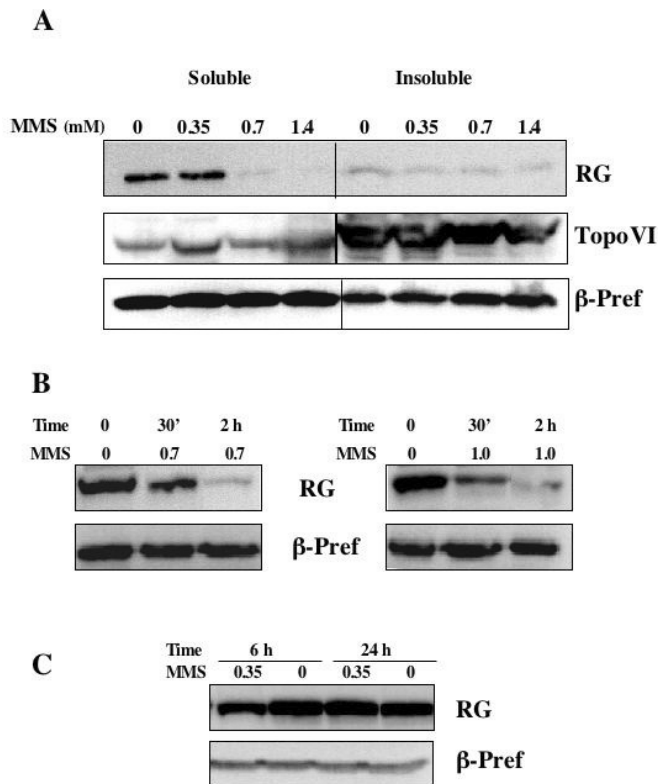


Figure 2. MMS treatment induces reverse gyrase loss. (A) Western blot of soluble and insoluble protein extracts (200 μ g/lane) prepared from cultures treated with MMS at the indicated concentrations. Extracts were prepared 2 h after MMS or mock treatment. The same filter was stripped and probed with antibodies against the indicated proteins. (B and C) Reverse gyrase level in soluble extracts (200 μ g/lane) prepared from controls and cultures treated with the indicated MMS concentrations (mM) at the indicated time points. The same filters were stripped and probed with the anti β -prefoldin antibody.

the protein was reduced to about 50 or 20% of the control, respectively, if 0.7 or 1 mM MMS was used (Figure 2B).

Although no reduction of reverse gyrase content was observed after 2 h of treatment with 0.35 mM MMS, the level of the enzyme was transiently reduced after 6 h and returned to normal levels 24 h after MMS addition (Figure 2C).

Reverse gyrase activity in cell extracts is reduced upon MMS treatment

We next sought to determine whether the residual reverse gyrase persisting at lower MMS concentrations was active. The positive supercoiling activity of reverse gyrase can be assayed by 2D gel electrophoresis. Incubation of *S. solfataricus* cell extracts with a negatively supercoiled plasmid in the presence of ATP results in production of topoisomers with increasing linking number. We measured reverse gyrase activity in extracts from cultures treated with different MMS concentrations (Figure 3). The activity was similar to that found in the control at 0.35 mM MMS and it was greatly reduced at 0.7 mM. At 1 mM MMS a weak relaxing activity was present in cell extracts, but no positive topoisomers were produced. Thus the enzyme activity correlates well with its amount, providing no evidence of enzyme inactivation.

Effect of MMS on other proteins affecting DNA structure

To obtain a more general picture of changes in chromatin composition associated with alkylating DNA damage, we analysed the effect of MMS on two DNA binding proteins involved in the control of DNA structure in *S. solfataricus*, namely Sul7d and Smj12. Sul7d (formerly called Sso7d) is a 7 kDa architectural protein, specific of the *Sulfolobus* genus and is one of the main chromatin components in these strains. This protein induces DNA bending, compaction and negative supercoiling, (17) and it negatively regulates reverse gyrase activity (17,18). Smj12 is a member of a large family of helix–turn–helix DNA binding proteins found in *Archaea* and *Bacteria* that binds DNA non-specifically and induces positive supercoiling in cooperation with topoisomerases (25). It has no effect on reverse gyrase activity (18).

The total amount of these proteins did not change after exposure to MMS (Figure 4A). However, interestingly, both Sul7d and Smj12 showed significant changes in their distribution after MMS treatment. In control cells, both proteins were mainly associated to the chromatin fraction; however, at both MMS concentration tested, a significant, MMS concentration-dependent amount of both proteins was translocated to the soluble fraction, showing a parallel reduction in the chromatin fraction.

The mechanism and biological meaning of the translocation of two binding proteins affecting DNA conformation from DNA to the cytosol fraction are currently under study. For the purpose of this work, the results reported in this paragraph confirm that reverse gyrase loss is a specific event and does not concern DNA-interacting proteins in general.

HU does not induce reverse gyrase loss

The effect of MMS on reverse gyrase is very different from that seen with UV radiation that induces translocation of the enzyme to DNA but not changes in the overall protein level. In addition, we have previously shown that actinomycin D, a transcription inhibitor which blocks DNA topoisomerases and elicits a transcriptional response similar to that induced by UV (23), does not induce any change in reverse gyrase level or distribution (22).

We therefore checked the effect of other agents blocking cell growth with different mechanisms, namely HU and puromycin (see below). HU is a ribonucleotide reductase inhibitor, known to induce a replication block in both bacteria and eukaryotes by reducing the deoxynucleotide pool. Its effect in *Archaea* has not been reported so far. Treatment of *S. solfataricus* cultures with 10 mM HU induced a block of absorbance increase (Figure 4B). Phase-contrast microscopy of HU-treated cultures showed reduction of cell number and heterogeneous phenotypes similar to that observed with high MMS concentrations (data not shown).

However, no variation of reverse gyrase level or distribution was observed after 2 h of treatment with the same HU concentration (Figure 4C). In addition, HU did not affect significantly the level and distribution of Sul7d, Smj12 or TopoVI. Taken together with previous results and those obtained with puromycin reported in the next paragraph, this finding shows that reverse gyrase loss is highly specific

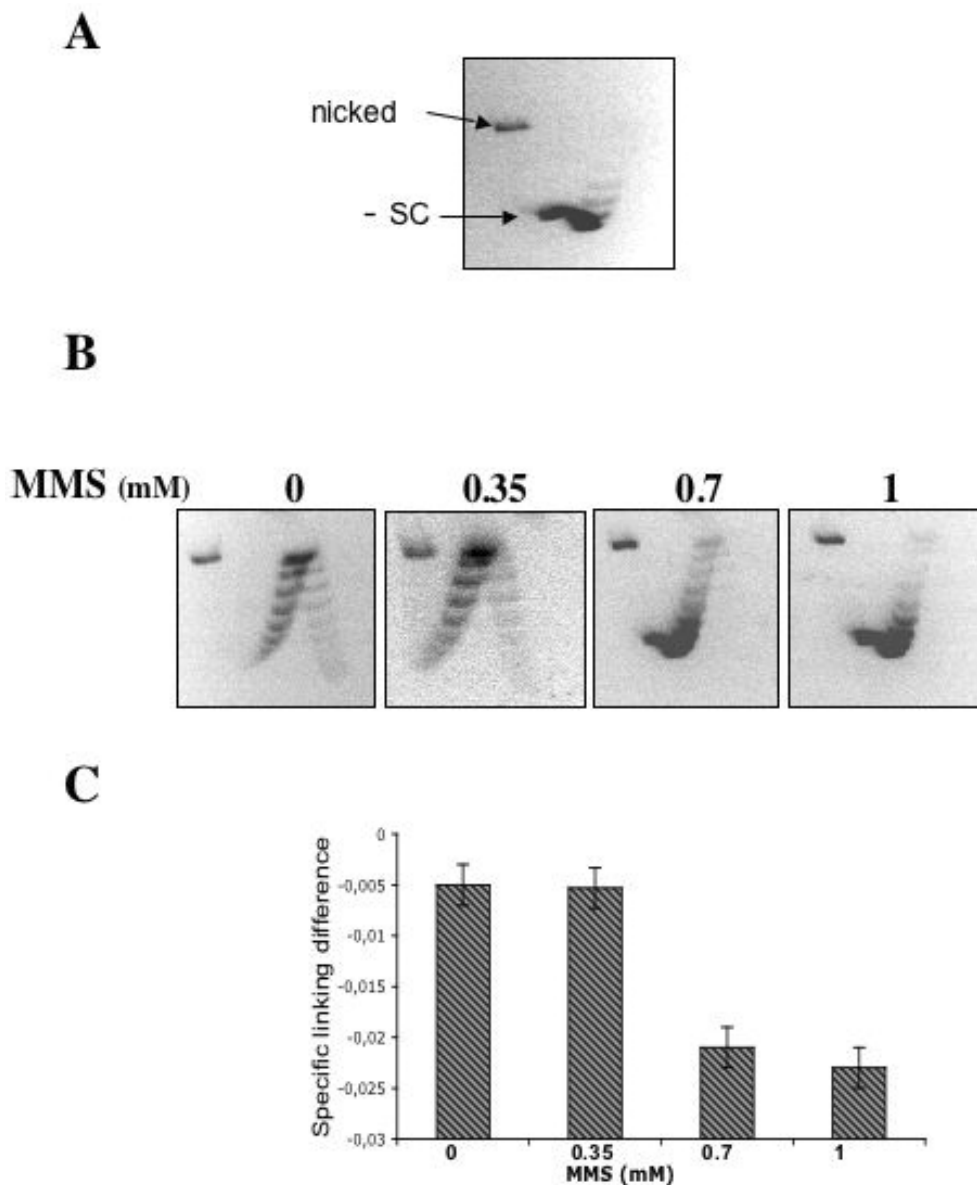


Figure 3. Reverse gyrase activity in cell extracts after MMS treatment. (A) 2D gel showing the migration of the substrate, pGEM7 plasmid DNA; arrows indicate the position of the nicked and negatively supercoiled (–SC) plasmid forms. (B) Positive supercoiling activity in soluble cell extracts prepared from cultures treated with MMS at the indicated concentrations. Extracts were prepared 2 h after MMS or mock treatment. Each sample contained 5 μ g of protein (final concentration 0.25 mg/ml). Negative and positive topoisomers migrate at the left and right part of the arc, respectively. Representative gel out of three independent assays is shown. (C) Quantification of reverse gyrase activity; the mean specific linking difference (mean σ) was calculated as described in Materials and Methods. Values are the mean of three independent experiments.

for MMS and is not a consequence of replication block and/or cell death in general.

Reverse gyrase loss is a post-translational event

To check whether reverse gyrase disappearance in MMS-treated cells depends on transcriptional down-regulation, we analysed the level of the reverse gyrase mRNA by quantitative RT–PCR. Reverse gyrase is encoded by two genes in *S.solfataricus* P2 (29). We therefore designed a set of specific primers for each gene and performed quantitative real-time RT–PCR on cDNA prepared from RNA extracted after 2 h of treatment with different MMS

concentrations. The mRNA for both TopR1 and TopR2 was present and even slightly increased in MMS-treated with respect to control cells (Figure 5A), thus showing that reverse gyrase disappearance is not due to transcriptional silencing.

We then checked whether reverse gyrase disappearance after MMS treatment requires active protein synthesis. To this aim we used puromycin, an antibiotic that interferes with protein synthesis and blocks DNA replication in the hyperthermophilic archaeon *Pyrococcus abyssi* (30). Treatment with 50 μ g/ml of puromycin induced a block of culture density increase (Figure 5B). Cell cultures were pre-treated with puromycin for 30', then MMS at 1 mM was added and proteins were extracted after 2 h of incubation at 80°C (Figure 5C).

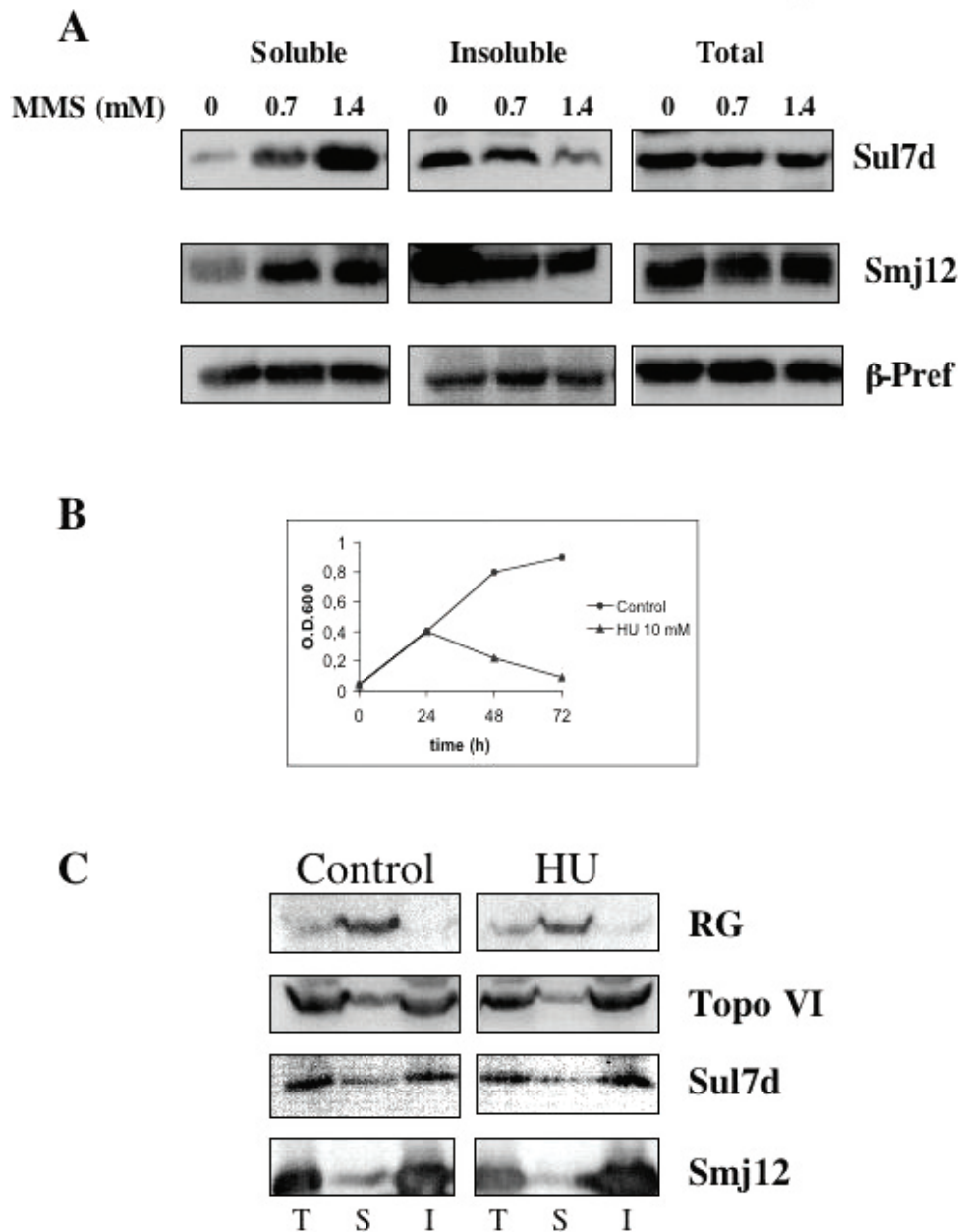


Figure 4. (A) Chromatin proteins are not depleted after MMS treatment. Western blot of total, soluble and insoluble protein extracts (200 μ g/lane) prepared from cultures treated with MMS at the indicated concentrations. Extracts were prepared 2 h after MMS or mock treatment. The same filter was stripped and probed with antibodies against the indicated proteins. (B) *S.solfataricus* P2 cultures were grown at 80°C until the exponential phase; at the time indicated by the arrow, cultures were split and 10 mM HU was added to one aliquot; absorbance at 600 nm was measured at indicated time points. (C) Western blot of total (T), soluble (S) and insoluble (I) protein extracts (200 μ g/lane) prepared from cultures treated with 10 mM HU. Extracts were prepared 2 h after HU or mock treatment. The same filter was stripped and probed with antibodies against the indicated proteins.

In cultures treated with puromycin alone, reverse gyrase amount was reduced to about 60% of the control. From this result we could draw two conclusions: (i) puromycin did not induce complete reverse gyrase loss, emphasizing again the specificity of MMS action; (ii) the reverse gyrase half life is about 2 h, thus the enzyme disappearance in MMS-treated cells is not simply a consequence of the physiological protein turnover in the absence of protein synthesis, but rather is the result of an active degradation.

When MMS was added to cells pre-treated with puromycin, reverse gyrase was almost completely absent after 2 h of

incubation, as in cells treated with MMS without puromycin (Figure 5C). This result shows that degradation of reverse gyrase in MMS-treated cells does not depend on MMS-induced synthesis of a protein factor.

Reverse gyrase is degraded by a metal-dependent protease

Two possibilities can be suggested to explain the results shown so far: either exposure to MMS induces some modification in reverse gyrase that renders the enzyme prone to degradation,

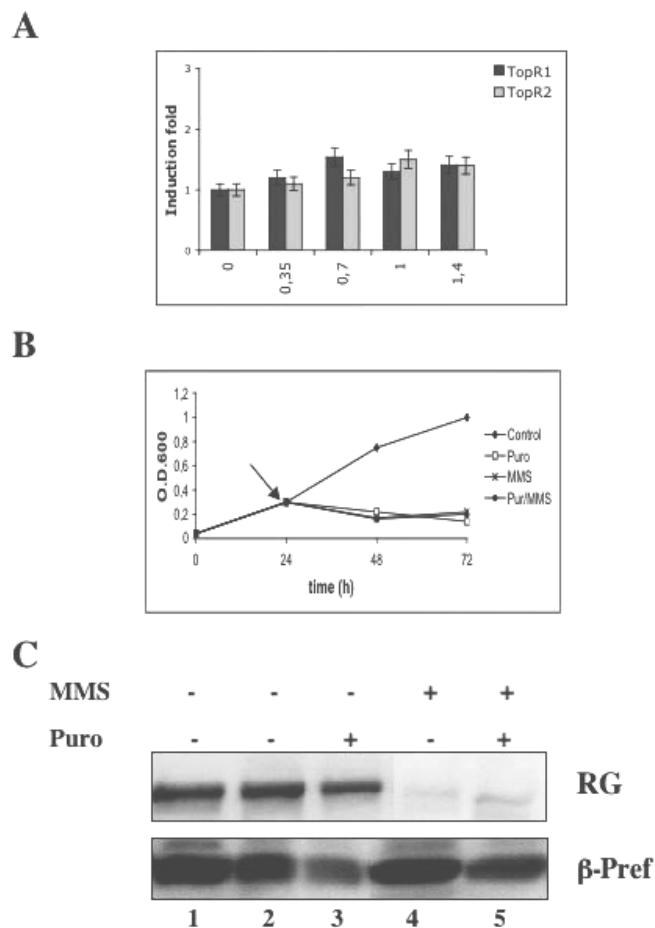


Figure 5. (A) Reverse gyrase loss is a post-translational event. Relative mRNA levels for TopR1 and TopR2 in cells treated with the indicated MMS concentrations (mM) were determined using real-time RT-PCR. Total RNA was extracted after 2 h of treatment. Data were normalized to the expression level of 16S rRNA and expressed as the relative mRNA level compared with the average expression level in control cells. (B) *S. solfataricus* P2 cultures were grown at 80°C until the exponential phase; at the time indicated by the arrow, cultures were split and 50 µg/ml of puromycin was added to one aliquot at the time point indicated by the arrow. After 30 min, control and puromycin-treated aliquots were split again and 1 mM MMS was added to one half of each aliquot. Absorbance at 600 nm was measured at the indicated time points. (C) Western blot of soluble protein extracts (200 µg/lane) prepared from cultures treated with indicated drugs. Lane 1, extracts from controls before puromycin addition; lane 2, extracts from controls after 2.5 h of mock treatment; lane 3, extracts from cultures treated with puromycin for 2.5 h; lane 4, extracts from cultures treated with 1 mM MMS for 2 h; lane 5, extracts from cultures treated with puromycin for 30 min followed by 1 mM MMS for 2 h. The same filter was probed with antibodies against the indicated proteins.

or the drug activates a pre-existing factor that induces degradation of the enzyme. To distinguish between these possibilities, we attempted to mimic reverse gyrase degradation *in vitro*, by adding 1 mM MMS to cell extracts prepared from control cells and analysing the residual reverse gyrase amount after incubation at 80°C (Figure 6A). No variation of reverse gyrase level was observed, suggesting that its degradation is not a direct effect of some MMS-induced chemical modification (e.g. methylation).

We then mixed extracts from control cells, containing reverse gyrase, with equal amounts of extracts from cells treated with 1 mM MMS for 1 h, which contain very low

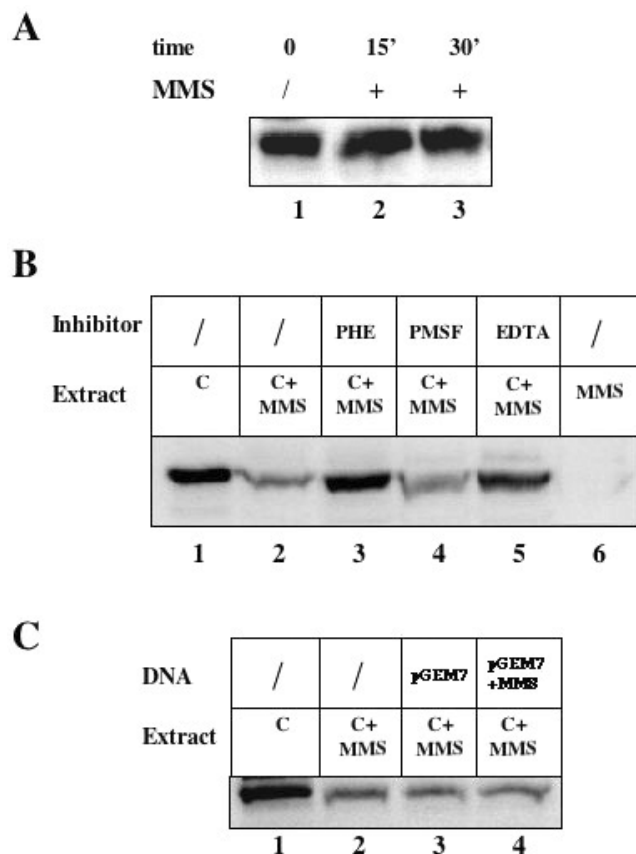


Figure 6. Reverse gyrase is degraded by a metal-dependent protease. (A) Soluble protein extracts (200 µg/lane) prepared from control cells were incubated at 80°C with 0 (lane 1) or 1 mM MMS for 15 min (lane 2) or 30 min (lane 3) and reverse gyrase amount was analysed by western blotting. (B) Extracts from control cells (200 µg/lane) and extracts from cultures treated with 1 mM MMS for 2 h (200 µg/lane), alone or mixed, were incubated at 70°C for 30 min. Lane 1, extract from control cells; lane 2, extract from control cells + extract from MMS-treated cells; lane 3, as lane 2 with 10 mM 1,10 PHE; lane 4, as lane 2 with 4 mM PMSF; lane 5, as lane 2 but with 5 mM EDTA; lane 6, extracts from MMS-treated cells. The residual reverse gyrase content was analysed by western blotting. (C) Extracts from control cells (200 µg/lane) and extracts from cultures treated with 1 mM MMS for 2 h (200 µg/lane) were mixed and incubated at 70°C for 30 min with or without pGEM7 plasmid DNA (Promega). Lane 1, extract from control cells; lane 2, extract from control cells + extract from MMS-treated cells; lane 3, as lane 2 with 500 ng of untreated pGEM7 plasmid DNA; lane 4, as lane 2 with 500 ng of plasmid DNA pre-treated for 15 min with 1 mM MMS.

levels of the enzyme. The mix was incubated for 30 min at 70°C and analysed by western blotting (Figure 6B). Reverse gyrase content in the extract mix was significantly reduced, thus showing that extracts from MMS-treated cells contain some factor(s) able to induce partial degradation of the enzyme in extracts from control cells. Degradation was also observed if extracts prepared after 30 min or 2 h from MMS addition, and drug concentrations between 0.7 and 1.4 mM were used (data not shown). Degradation was prevented by incubating the extract mix with EDTA or 1,10 PHE, but not phenylmethylsulfonyl fluoride (PMSF). In addition, degradation was not blocked if we added to the mix excess of purified plasmid DNA, either untreated or pre-treated with MMS (Figure 6C). Together, these results show that MMS-induced selective reverse gyrase degradation

requires some factor present in MMS-treated cells, that a metal-dependent protease is involved in this degradation and DNA does not protect the enzyme. MMS might induce post-translational activation of an endogenous protease that selectively cleaves reverse gyrase. Alternatively, MMS might activate pre-existing factor(s) that trigger(s) some reverse gyrase modification turning the enzyme into a protease-sensitive form.

MMS induces extensive DNA degradation

MMS generates primarily methylation of guanine in position N7 and, at lesser extent, O-6 methylguanine and N-3 methyladenine adducts; alkylated bases are heat labile and may give rise to strand breaks if incubated above 50°C (31).

To monitor the effect of MMS treatment on genomic DNA, *S.solfataricus* cultures were treated with different MMS concentrations, aliquots were harvested after various times, total DNA was purified and analysed by agarose gel electrophoresis. No genomic DNA degradation was observed in controls or in cells treated for 2 h with 0.35 mM MMS. In contrast, in cells treated with higher MMS concentrations for 2 h, DNA was extensively degraded in a concentration-dependent manner (Figure 7A and B). When 1.4 mM MMS was used, DNA degradation was evident after 30 min and almost complete after 2 h of MMS treatment (Figure 7B). After 24 h from treatment DNA was completely degraded (data not shown). Degradation was also detectable, at a lesser extent, 30 min after addition of 0.7 or 1.0 mM MMS (data not shown). DNA extracted from the soluble and insoluble fractions of controls and MMS-treated cells showed a pattern similar to that observed for total DNA, even if its amount was unequally distributed, with more than 99% in the pellets and only minor contamination in the supernatants (data not shown).

Although DNA was intact after 2 h of treatment with 0.35 mM MMS, it was slightly degraded after 6 h (Figure 7C); however, degradation was greatly reduced 24 h after MMS addition.

Genomic DNA degradation observed in *S.solfataricus* might be a consequence of methylation of DNA bases during growth at 80°C. Indeed incubation with 1 mM MMS for 30 min at 80°C of DNA purified from untreated cells resulted in extensive degradation (data not shown). Interestingly, there is striking coincidence in both timing and extent of DNA and reverse gyrase degradation after MMS treatment (see Discussion).

DISCUSSION

The major conclusion of this work is that MMS induces two dramatic events in *S.solfataricus*: (i) specific degradation of reverse gyrase and (ii) concomitant genomic DNA degradation. Although at present we cannot establish a causal relationship between these two events, it is tempting to speculate that they are in some way connected. DNA and reverse gyrase are degraded at the same MMS concentrations and with overlapping timing; both reverse gyrase and DNA are extensively degraded whenever growth is irreversibly arrested (MMS concentrations above 0.35 mM). At 0.35 mM MMS degradation of both DNA and reverse gyrase is partial and transient, and

occurs in parallel with the transient growth arrest; when normal growth resumes reverse gyrase content returns to normal levels and DNA is not fragmented.

Kampmann and Stock (20) reported that reverse gyrase of *Archaeoglobus fulgidus* protects DNA from thermodegradation *in vitro*. They suggested a model in which reverse gyrase might protect DNA by stabilizing apurinic sites, and/or protecting DNA ends from thermodegradation. Our results *in vivo*, although do not directly prove, support and extend this model: indeed at high temperature alkylated bases give rise with high frequency to abasic sites, that are easily converted to single strand or double strand breaks and might trigger DNA denaturation. Reverse gyrase might counteract degradation at low MMS concentrations; however, at higher MMS concentrations the enzyme is degraded and DNA is no longer protected from the combined effects of MMS and heat. This model predicts that the enzyme should be recruited to DNA at low MMS concentrations, as seen with UV irradiation (22); however, this is not the case (Figure 2A). One explanation of this result is that, under the experimental conditions used, the enzyme can be found associated with DNA only if it is trapped in covalent intermediates or otherwise very stable complexes. For some reason these complexes might be formed with substrates containing large helix-distorting UV-induced lesions, but not abasic sites.

In an alternative model, reverse gyrase might be degraded to allow DNA relaxation and exposure of single strand regions required for access of repair/replication complexes. However, two observations might argue against this model: (i) reverse gyrase is not degraded in the case of UV damage (22); (ii) reverse gyrase is degraded only at higher MMS concentrations, when cells are committed to die, and not at lower MMS concentrations, when they resume from the damage. We thus favour a model in which reverse gyrase has a positive, rather than negative, role in the cell response to MMS-induced (and possibly other types, of) DNA damage.

In any case, heat-induced conversion of unrepaired alkylation lesions into DNA breaks may comprise one of the reasons for the high MMS-mediated cytotoxicity in *S.solfataricus*.

Our observations raise a number of questions that remain to be addressed. First of all, which is the signal for reverse gyrase degradation in MMS-treated cells? We have provided evidence that degradation depends on a metal-dependent protease yet to be identified. Since proteins are substrates for MMS alkylation, reverse gyrase might be alkylated by MMS and then targeted for degradation. The removal of chemically damaged proteins seems logical to promote recovery from protein damage. However, we have shown that reverse gyrase is not degraded if extracts are directly exposed to MMS and, conversely, extracts from MMS-treated cells induce degradation of the enzyme in control extracts in the absence of MMS. We conclude that (i) alkylation is not the reason for reverse gyrase degradation, and (ii) degradation requires the activation of some pathway *in vivo*. Additionally, we reasoned that if reverse gyrase is alkylated, it is likely that such modification would alter the enzyme activity besides its stability. However, we could not detect significant change in enzyme specific activity in MMS-treated cells compared to controls, thus providing no evidence of (partial) enzyme inactivation. If the enzyme is a target for MMS-induced alkylation remains to be established.

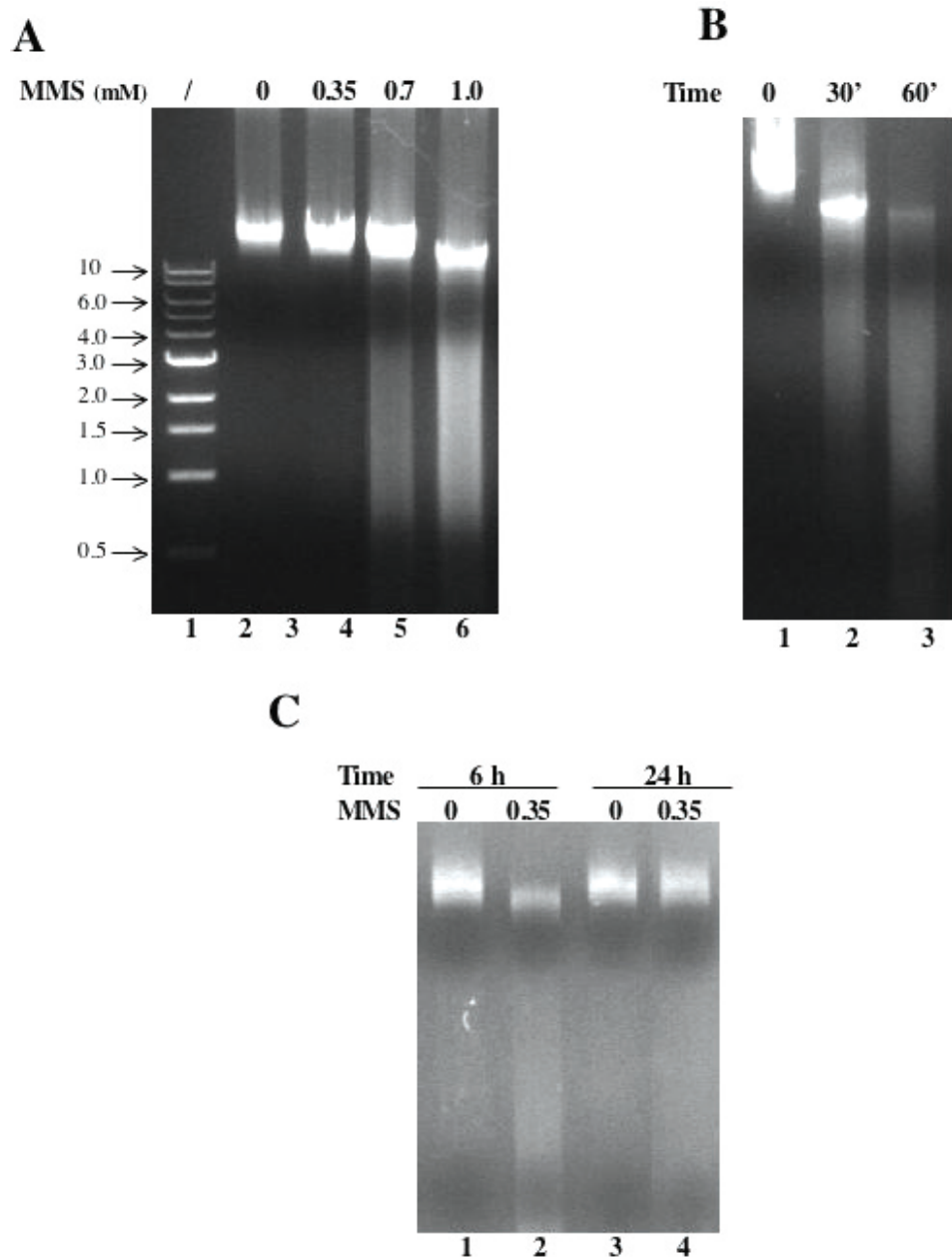


Figure 7. MMS induces degradation of genomic DNA. (A) Cultures were grown as in Figure 1, MMS was added at the indicated concentrations (mM) and genomic DNA was extracted after 2 h of incubation at 80°C. Samples were run on 1% agarose gels in Tris–Acetate buffer. Lane 1, molecular weight marker (kb). (B) Cultures were treated with 1.4 mM MMS and DNA was extracted after indicated time spans of incubation at 80°C. (C) Cultures were treated with 0.35 mM MMS and DNA was extracted after 6 or 24 h of incubation at 80°C.

The signal for reverse gyrase degradation is not the cell cycle or replication arrest *per se*, since HU, puromycin (this work), UV and actinomycin D (22) all fail to induce reverse gyrase degradation. Furthermore, this phenomenon is highly specific as it does not concern any of the other proteins tested, including Topo VI and several DNA binding proteins (this work and data not shown). However, we cannot rule out the possibility that other proteins may be specifically affected by MMS treatment.

No intermediate degradation products of reverse gyrase were observed even at early stage after MMS addition.

Different hypothesis might explain this result: intermediates might be very unstable and do not accumulate; proteolysis might give rise to products that are no longer recognized by the antibody or of very low molecular weight (<6 kDa). At moment we cannot distinguish among these possibilities; the isolation of the protease involved in this process will shed light on the mechanism of reverse gyrase degradation.

Although the majority of reverse gyrase is found in the soluble fraction of cell extracts, whereas only a small amount is associated with the insoluble fraction, the first one was

degraded with higher efficiency (Figure 2). At least two hypotheses could explain this observation: DNA-bound reverse gyrase might be inherently resistant to degradation, or the MMS-triggered factor responsible for reverse gyrase degradation could not access the enzyme in the 'nucleoidal' compartment. The addition of purified DNA failed to protect reverse gyrase from MMS-induced degradation *in vitro* (Figure 6C), thus arguing against a protective effect of DNA on the enzyme. On the other hand, it is not clear which is the difference between the soluble and the insoluble reverse gyrase fractions. For instance, the latter could be trapped in a covalent intermediate with DNA and this complex might be resistant to degradation.

The selective degradation of proteins involved in DNA repair after induction of DNA damage and/or cell cycle arrest has been reported in other cases, although not in *Archaea*. In human cells MMS activates a cellular protease to degrade the DNA methyltransferase DNMT1 (32), and EXO1b, a human mismatch repair-associated exonuclease, is significantly reduced upon treatment of cells with agents that cause replication fork arrest (33). In addition, Rad51 recombinase, the mismatch repair protein MLH1 and the Bloom syndrome helicase (BLM) are all cleaved by caspases following different apoptotic stimuli (34–37). The biological meaning of these events is not understood; it has been suggested that some protein complexes that can repair DNA need to be dismantled for apoptosis to proceed (34).

BLM is a RecQ family helicase that interacts with Topoisomerase III; such interaction, which is conserved from bacteria to humans, is essential for cellular processes, such as processing of DNA after exposure of cells to DNA damaging agents (38,39). Interestingly, proteolytic cleavage of BLM during apoptosis inhibits its interaction with Topoisomerase III, but the resulting protein is still active as helicase (37). This finding suggests that the BLM–TopoIII complex, rather than the BLM protein itself, is a relevant player in the apoptotic process.

The analogies between reverse gyrase and RecQ–TopoIII complexes are manifold. Reverse gyrase comprises a helicase-like and a topoisomerase IA module; it performs an SSB-stimulated positive supercoiling reaction (18); it is recruited to DNA after UV irradiation (22) and is degraded after induction of cell death by MMS (this work). The RecQ–TopoIII complex of *E.coli* is able to perform strand passage inducing negative supercoiling (40,41); this reaction is stimulated by single strand binding proteins. Members of the RecQ family localize to specific nuclear foci after induction of DNA damage in yeast and mammals (42,43); BLM is cleaved during apoptosis (36,37). It is tempting to speculate that reverse gyrase degradation has some role analogous to BLM proteolysis.

It is well known that induction of apoptosis in higher eukaryotes is characterized by two landmarks: DNA fragmentation and activation of proteases. The activation of specific proteases induced by DNA damage, cell cycle arrest or death signals has never been reported in *Archaea*, nor are homologs of eukaryotic caspases present in their genomes. It would be of outstanding interest to identify the protease responsible for reverse gyrase degradation and its function in the biology of *S.solfataricus*.

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REFERENCES

- Begley,T.J. and Samson,L.D. (2004) Network responses to DNA damaging agents. *DNA Repair*, **3**, 1123–1132.
- Sedgwick,B. (2004) Repairing DNA-methylation damage. *Nature Rev. Mol. Cell Biol.*, **5**, 148–157.
- Drablos,F., Feyzi,E., Aas,P.A., Vaagbo,C.B., Kavli,B., Bratlie,M.S., Pena-Diaz,J., Otterlei,M., Slupphaug,G. and Krokan,H.E. (2004) Alkylation damage in DNA and RNA-repair mechanisms and medical significance. *DNA Repair*, **3**, 1389–1407.
- Kaina,B., Ochsm,K., Grosch,S., Fritz,G., Lips,J., Tomicic,M., Dunkern,T. and Christmann,M. (2001) BER, MGMT, and MMR in defense against alkylation-induced genotoxicity and apoptosis. *Prog. Nucleic Acid Res. Mol. Biol.*, **68**, 41–54.
- Volkert,M.R., Gately,F.H. and Hajec,L.I. (1989) Expression of DNA damage-inducible genes of *Escherichia coli* upon treatment with methylating, ethylating and propylating agents. *Mutat. Res.*, **217**, 109–115.
- Said,M.R., Begley,T.J., Oppenheim,A.V., Lauffenburger,D.A. and Samson,L.D. (2004) Global network analysis of phenotypic effects: protein networks and toxicity modulation in *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA*, **101**, 18006–18011.
- Lee,W., St Onge,R.P., Proctor,M., Flaherty,P., Jordan,M., I., Arkin,A.P., Davis,R.W., Nislow,C. and Giaever,G. (2005) Genome-wide requirements for resistance to functionally distinct DNA-damaging agents. *PLoS Genet.*, **1**, e24.
- Islaih,M., Halstead,B.W., Kadura,I.A., Li,B., Reid-Hubbard,J.L., Flick,L., Julie,L., Altizer,J.L., Deahl,J.T., Monteith,D.K. *et al.* (2005) Relationships between genomic, cell cycle, and mutagenic responses of TK6 cells exposed to DNA damaging chemicals. *Mutat. Res.*, **578**, 100–116.
- Hamilton,J.T., McRoberts,W.C., Keppler,F., Kalin,R.M. and Harper,D.B. (2003) Chloride methylation by plant pectin: an efficient environmentally significant process. *Science*, **301**, 206–209.
- Aravind,L., Walker,D.R. and Koonin,E.V. (1999) Conserved domains in DNA repair proteins and evolution of repair systems. *Nucleic Acids Res.*, **27**, 1223–1242.
- White,M.F. (2003) Archaeal DNA repair: paradigms and puzzles. *Biochem. Soc. Trans.*, **31**, 690–693.
- Kelman,Z. and White,M.F. (2005) Archaeal DNA replication and repair. *Curr. Opin. Microbiol.*, **8**, 669–676.
- Forterre,P., Bergerat,A. and Lopez-Garcia,P. (1996) The unique DNA topology and DNA topoisomerases of hyperthermophilic archaea. *FEMS Microbiol. Rev.*, **18**, 237–248.
- Duguet,M. (1997) When helicase and topoisomerase meet! *J. Cell. Sci.*, **110**, 1345–1350.
- Declais,A.C., Marsault,J., Confalonieri,F., de La Tour,C.B. and Duguet,M. (2000) Reverse gyrase, the two domains intimately cooperate to promote positive supercoiling. *J. Biol. Chem.*, **275**, 19498–19504.
- Rodriguez,A.C. and Stock,D. (2002) Crystal structure of reverse gyrase: insights into the positive supercoiling of DNA. *EMBO J.*, **21**, 418–426.
- Napoli,A., Zivanovic,Y., Bocs,C., Buhler,C., Rossi,M., Forterre,P. and Ciaramella,M. (2002) DNA bending, compaction and negative supercoiling by the architectural protein Sso7d of *Sulfolobus solfataricus*. *Nucleic Acids Res.*, **30**, 2656–2662.
- Napoli,A., Valenti,A., Salerno,V., Nadal,M., Garnier,F., Rossi,M. and Ciaramella,M. (2005) Functional interaction of reverse gyrase with single-strand binding protein of the archaeon *Sulfolobus*. *Nucleic Acids Res.*, **33**, 564–576.

19. Forterre, P. (2002) A hot story from comparative genomics: reverse gyrase is the only hyperthermophile-specific protein. *Trends Genet.*, **18**, 236–237.
20. Kampmann, M. and Stock, D. (2004) Reverse gyrase has heat-protective DNA chaperone activity independent of supercoiling. *Nucleic Acids Res.*, **32**, 3537–3545.
21. Atomi, H., Matsumi, R. and Imanaka, T. (2004) Reverse gyrase is not a prerequisite for hyperthermophilic life. *J. Bacteriol.*, **186**, 4829–4833.
22. Napoli, A., Valenti, A., Salerno, V., Nadal, M., Garnier, F., Rossi, M. and Ciaramella, M. (2004) Reverse gyrase recruitment to DNA after UV irradiation in *Sulfolobus solfataricus*. *J. Biol. Chem.*, **279**, 33192–33198.
23. Salerno, V., Napoli, A., White, M.F., Rossi, M. and Ciaramella, M. (2003) Transcriptional response to DNA damage in the archaeon *Sulfolobus solfataricus*. *Nucleic Acids Res.*, **31**, 6127–6138.
24. Nadal, M., Couderc, E., Dugué, M. and Jaxel, C. (1994) Purification and characterization of reverse gyrase from *Sulfolobus shibatae*. Its proteolytic product appears as an ATP-independent topoisomerase. *J. Biol. Chem.*, **269**, 5255–5263.
25. Napoli, A., Kvaratskeli, M., White, M.F., Rossi, M. and Ciaramella, M. (2001) A novel member of the bacterial-archaeal regulator family is a non-specific DNA-binding protein and induces positive supercoiling. *J. Biol. Chem.*, **276**, 10745–10752.
26. Dinglay, S., Trewick, S.C., Lindahl, T. and Sedgwick, B. (2000) Defective processing of methylated single-stranded DNA by *E. coli* AlkB mutants. *Genes Dev.*, **14**, 2097–2105.
27. Johnson, R.E., Torres-Ramos, C.A., Izumi, T., Mitra, S., Prakash, S. and Prakash, L. (1998) Identification of APN2, the *Saccharomyces cerevisiae* homolog of the major human AP endonuclease HAP1, and its role in the repair of abasic sites. *Genes Dev.*, **12**, 3137–3143.
28. Bergerat, A., de Massy, B., Gabelle, D., Varoutas, P.C., Nicolas, A. and Forterre, P. (1997) An atypical topoisomerase II from Archaea with implications for meiotic recombination. *Nature*, **386**, 414–417.
29. She, Q., Singh, R.K., Confalonieri, F., Zivanovic, Y., Allard, G., Awayez, M.J., Chan-Weiher, C.C., Clausen, I.G., Curtis, B.A., De Moors, A. *et al.* (2001) The complete genome of the crenarchaeon *Sulfolobus solfataricus* P2. *Proc. Natl Acad. Sci. USA*, **98**, 7835–7840.
30. Myllykallio, H., Lopez, P., Lopez-Garcia, P., Heilig, R., Saurin, W., Zivanovic, Y., Philippe, H. and Forterre, P. (2001) Bacterial mode of replication with eukaryotic-like machinery in a hyperthermophilic archaeon. *Science*, **292**, 803.
31. Lundin, C., North, M., Erixon, K., Walters, K., Janssen, D., Goldman, A.S. and Helleday, T. (2005) Methyl methanesulfonate (MMS) produces heat-labile DNA damage but no detectable *in vivo* DNA double-strand breaks. *Nucleic Acids Res.*, **33**, 3799–3781.
32. Chuang, L.S., Tan, E.H., Oh, H.K. and Li, B.F. (2002) Selective depletion of human DNA-methyltransferase DNMT1 proteins by sulfonate-derived methylating agents. *Cancer Res.*, **62**, 1592–1597.
33. El-Shemerly, M., Janscak, P., Hess, D., Jiricny, J. and Ferrari, S. (2005) Degradation of human exonuclease 1b upon DNA synthesis inhibition. *Cancer Res.*, **1**, 3604–3609.
34. Huang, Y., Nakada, S., Ishiko, T., Utsugisawa, T., Datta, R., Kharbada, S., Yoshida, K., Talanian, R.V., Weichselbaum, R., Kufe, D. *et al.* (1999) Role for caspase-mediated cleavage of Rad51 in induction of apoptosis by DNA damage. *Mol. Cell Biol.*, **19**, 2986–2997.
35. Chen, F., Arseven, O.K. and Cryns, V.L. (2004) Proteolysis of the mismatch repair protein MLH1 by caspase-3 promotes DNA damage-induced apoptosis. *J. Biol. Chem.*, **279**, 27542–27548.
36. Bischof, O., Galand, S., Farzaneh, F., Kohwi-Shigematsu, T. and Campisi, J. (2001) Selective cleavage of BLM, the bloom syndrome protein, during apoptotic cell death. *J. Biol. Chem.*, **276**, 12068–12075.
37. Freire, R., d'Adda Di Fagagna, F., Wu, L., Pedrazzi, G., Stagljar, I., Hickson, I.D. and Jackson, S.P. (2001) Cleavage of the bloom's syndrome gene product during apoptosis by caspase-3 results in an impaired interaction with topoisomerase III α . *Nucleic Acids Res.*, **29**, 3172–3180.
38. Khakhar, R.R., Cobb, J.A., Bjergbaek, L., Hickson, I.D. and Gasser, S.M. (2003) RecQ helicases: multiple roles in genome maintenance. *Trends Cell Biol.*, **13**, 493–450.
39. Hickson, I.D. (2003) RecQ helicases: caretakers of the genome. *Nature Rev. Cancer*, **3**, 169–178.
40. Harmon, F.G., DiGate, R.J. and Kowalczykowski, S.C. (1999) RecQ helicase and topoisomerase III comprise a novel DNA strand passage function: a conserved mechanism for control of DNA recombination. *Mol. Cell*, **3**, 611–620.
41. Harmon, F.G., Brockman, J.P. and Kowalczykowski, S.C. (2003) RecQ helicase stimulates both DNA catenation and changes in DNA topology by topoisomerase III. *J. Biol. Chem.*, **278**, 42668–42678.
42. Bischof, O., Kim, S.H., Irving, J., Beresten, S., Ellis, N.A. and Campisi, J. (2001) Regulation and localization of the Bloom syndrome protein in response to DNA damage. *J. Cell Biol.*, **153**, 367–380.
43. Laursen, L.V., Ampatzidou, E., Andersen, A.H. and Murray, J.M. (2003) Role for the fission yeast RecQ helicase in DNA repair in G2. *Mol. Cell Biol.*, **23**, 3692–3705.