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Genome stability: recent insights in the topoisomerase reverse gyrase and thermophilic DNA alkyltransferase

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Abstract Repair and defence of genome integrity from endogenous and environmental hazard is a primary need for all organisms. Natural selection has driven the evolution of multiple cell pathways to deal with different DNA damaging agents. Failure of such processes can hamper cell functions and induce inheritable mutations, which in humans may cause cancerogenicity or certain genetic syndromes, and ultimately cell death. A special case is that of hyperthermophilic bacteria and archaea, flourishing at temperatures higher than 80 °C, conditions that favor genome instability and thus call for specific, highly efficient or peculiar mechanisms to keep their genome intact and functional. Over the last few years, numerous studies have been performed on the activity, function, regulation, physical and functional interaction of enzymes and proteins from hyperthermophilic microorganisms that are able to bind, repair, bypass damaged DNA, or modify its structure or conformation. The present review is focused on two enzymes that act on DNA catalyzing unique reactions: reverse gyrase and DNA alkyltransferase. Although both enzymes belong to evolutionary highly conserved protein families present in organisms of the three domains (*Eucarya*, *Bacteria* and *Archaea*), recently characterized members from hyperthermophilic archaea show both common and peculiar features.

Keywords DNA repair · Genome stability · Archaea · Hyperthermophiles · DNA topoisomerase · DNA alkyltransferase

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Reverse gyrase, a DNA topoisomerase with multiple activities

DNA topoisomerases are ubiquitous enzymes essential to regulate the topologic state of the DNA double helix and solve entanglement problems occurring during all DNA activities (replication, transcription, recombination and repair). They are classified in a few families according to their structure, specific activity and cellular function (Champoux 2001; Wang 2002). Type IA DNA topoisomerase family enzymes are present in all organisms with a few exceptions (Brochier-Armanet et al. 2008), and catalyze the relaxation of negatively supercoiled substrates, a reaction not energy consuming, and thus not depending on nucleotide hydrolysis; they play many roles in the cells, including regulation of the supercoiling level, transcription, recombination and repair. Among Type IA topoisomerases, reverse gyrase shows peculiar structure and function. Structurally, it is a chimeric protein, where the topoisomerase module is fused to a N-terminal domain, which shows evolutionary relatedness with the SF2 helicase family. This is a large protein family, whose members are widespread in all organisms and fulfill a variety of functions related to genome activities (Byrd and Raney 2012). Their hallmark is the ability to unwind nucleic acids and/or translocate along them in an ATP hydrolysis-dependent reaction. The association of two domains with distinct

activities confers on reverse gyrase a novel ability, performed by neither protein families, i.e., ATP-dependent positive supercoiling of topologically closed DNA molecules (reviewed in D'Amaro et al. 2007; Nadal 2007; Perugino et al. 2009).

The massive genomic sequence data currently available confirmed, as anticipated by Forterre more than a decade ago (Forterre 2002), that reverse gyrase is associated to life at high temperature. Indeed, its gene is invariably present in all bacteria and archaea living above 80 °C (hyperthermophiles), in some thermophiles (whose optimal growth temperature is between 65 and 80 °C), such as the bacteria *Thermus commune*, *T. thermophilus* HB8 and *Caldicellulosiruptor saccharolyticus* and in the moderate thermophile *Nautilia profundicola* (optimal growth temperature 65 °C; Campbell et al. 2009); in contrast, the reverse gyrase gene has not been found in any mesophilic organism (Brochier-Armanet and Forterre 2007; Heine and Chandra 2009). Thus, reverse gyrase is a distinctive marker of hyperthermophiles; however, whether it is essential to allow life at high temperature is still matter of debate. Positive DNA supercoiling increases the amount of links between the two DNA strands, a conformation which grants increased resistance of the duplex to heat-induced denaturation and might, thus, be suitable to life in hyperthermophilic environments (Valenti et al. 2011). This prediction has been confirmed by the observation that plasmids isolated from hyperthermophiles show, on average, higher linking number as compared with plasmids isolated from mesophilic bacteria and archaea (Forterre et al. 1996; Lopez-Garcia 1999). Moreover, in vitro experiments suggest that reverse gyrase might protect DNA against heat-induced depurination and degradation, independently of its topoisomerase activity (Kampmann and Stock 2004). On the other hand, other observations raise doubt about this hypothesis: the hyperthermophilic bacterium *Thermotoga maritima* was found to harbor both gyrase and reverse gyrase, yet its plasmids are negatively supercoiled (Guipaud et al. 1997); in addition, negatively supercoiled DNA is stable in vitro up to 107 °C as long as it is covalently closed (Marguet and Forterre 1994).

The role of reverse gyrase in hyperthermophiles' vitality was directly tested by knockout experiments of the reverse gyrase gene in two species, giving however contradictory results. Deletion of the single reverse gyrase gene in the euryarchaeon *Thermococcus kodakarensis* was not lethal (Atomi et al. 2004); however, whereas the wild type continued vigorous growth up to 100 °C, the mutated strain showed slow growth already at 90 °C, thus suggesting that reverse gyrase might contribute to the ability of *T. kodakarensis* to thrive at very high temperature. Different results were obtained for the crenarchaeon *Sulfolobus islandicus,* which, like other crenarchaea, encodes two reverse gyrases; attempts to obtain stable knockout mutants of either gene were unsuccessful, strongly suggesting that both genes are essential (Zhang et al. 2013). The reasons for these differences are currently not clear; whereas it seems unlikely that reverse gyrase plays completely different roles in different hyperthermophiles, it is possible that the extent of its contribution to adaptation to high temperature might vary among different species; further experiments are required to demonstrate unambiguously whether and how reverse gyrase contributes to organisms' adaptation to hyperthermophilic environmental conditions.

Phylogenetic analysis showed that reverse gyrase gene was probably present in the last common ancestor of archaea, thus it could have originated in a very ancient archaeon or a precursor of the archaeal domain, where ancestor topoisomerase and helicase coding modules were fused; this analysis also suggested that reverse gyrase emergence coincided with the origin of the first hyperthermophilic archaeon; later on, the reverse gyrase gene was transferred to bacteria through lateral gene transfer, allowing the appearance of hyperthermophilic bacteria (Brochier-Armanet and Forterre 2007).

Structural and functional analyses have been performed on several bacterial and archaeal reverse gyrases, revealing common themes, subtle differences among different enzymes, but also leaving many unanswered questions. The C-terminal domain catalyzes the true topoisomerase reaction, i.e., hydrolysis of a phosphodiester bond on one DNA strand, formation of a transient covalent intermediate between a conserved tyrosine in the enzyme active site and a DNA 5′ end, strand passage, religation, and release of the product with a changed linking number (ΔLk). Typical type IA topoisomerases induce reduction of ΔLk (relaxation of negative torsional stress), a reaction not energy consuming, and thus not depending on ATP. In contrast, the reverse gyrase reaction proceeds beyond: instead of releasing the relaxed product, the enzyme continues its activity using the ATP hydrolysis energy to induce DNA overlinking (positive supercoiling; Fig. 1a). This is a complex and unique reaction, which occurs only at high temperature (>60 °C) and requires the coordination of the two domains, but the details of the mechanisms are not completely understood (Déclais et al. 2000; Valenti et al. 2008; D'Amaro et al. 2007; Nadal 2007; Perugino et al. 2009; Heine and Chandra 2009).

The crystallographic structure of two reverse gyrases, from the archaeon *Archaeoglobus fulgidus* and the bacterium *T. maritima*, respectively, has been resolved (Rodríguez and Stock 2002; Rudolph et al. 2013), revealing a typical, well-conserved type IA topoisomerase fold for the C-terminal domain, comprising four subdomains set in a toroidal shape around a central cavity for DNA passage. The structure of the N-terminal domain of the two reverse

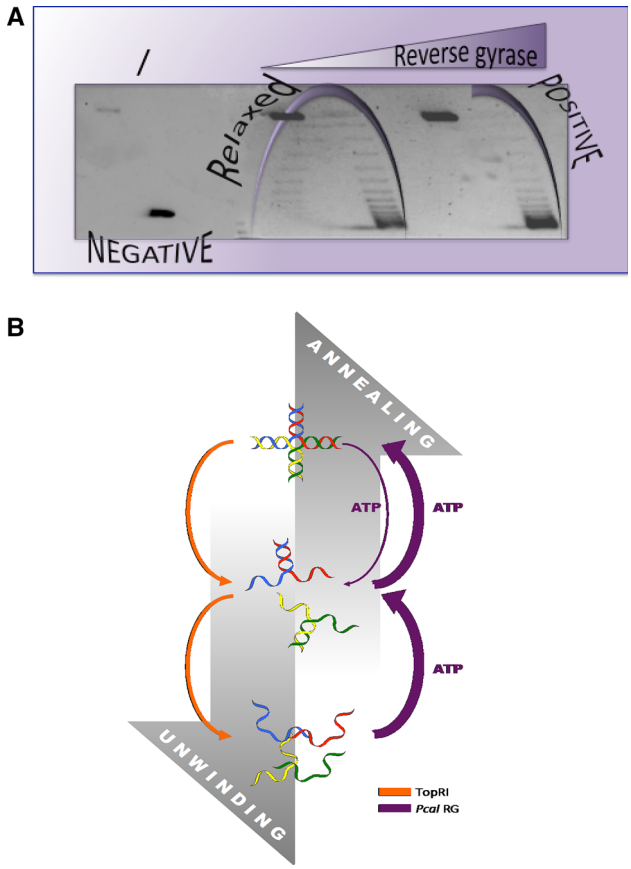


Fig. 1 **a** Positive supercoiling activity of reverse gyrase analyzed by two-dimensional gel electrophoresis. Typical 2D gel showing the migration of a negatively supercoiled plasmid either not incubated (*left panel*) or incubated with increasing reverse gyrase concentrations in the presence of 1 mM ATP. Each band represents a topoisomer with different linking number. Relaxed topoisomers migrate along the left branch of the arc and positive ones along the right branch (Valenti et al. 2008; Jamroze et al. 2014). **b** The unwinding and annealing activities of reverse gyrases on synthetic HJ. The cartoon highlights the differences between the *S. solfataricus* TopR1 and the *P. calidifontis* PcalRG enzymes; whereas TopR1 induces ATP-independent unfolding of HJ up to ss oligonucleotides, PcaRG induces ATP-dependent unwinding of the HJ to forks if used at low protein/DNA ratio, while increased enzyme concentrations induce ATP-stimulated annealing (Valenti et al. 2010; Jamroze et al. 2014). The *arrows* of different colors indicate the activities of the two enzymes; for PcalRG, thin and wide *arrows* indicate lower and higher enzyme concentrations, respectively

gyrases is less conserved; whereas both show the overall folding of ATP-binding domain of the SF2 family, very different structures are seen for a small domain called latch, which links the helicase and the topoisomerase domains and is somehow involved in their cooperation. Primary sequence of this region is poorly conserved among all reverse gyrases and shows variable length and sequence. These differences may reflect the different functions that the latch seems to have in different reverse gyrases: for example, it is essential for positive supercoiling in the *Thermoanaerobacter tengcongensis* and *T. maritima* reverse gyrases (Li et al. 2011; Ganguly et al. 2011), but not in the *A. fulgidus* enzyme (Rodriguez 2002, 2003); it participates in DNA binding in the *T. maritima* (Ganguly et al. 2011), but not in the *T. tengcongensis* reverse gyrase (Li et al. 2011). These differences are at moment difficult to rationalize. Mutational analysis of the N-terminal domain has identified motives essential for the ATPase- and DNA-binding activity (Bouthier de la Tour et al. 2008; Valenti et al. 2008; Li et al. 2011). In-depth studies showed that, upon nucleotide binding and hydrolysis, the N-terminal domain of the *T. maritima* reverse gyrase undergoes conformational modifications, which generates a signal somehow transferred to the C-terminal domain, coupling ATP hydrolysis with topoisomerase activity to allow positive supercoiling (Del Toro et al. 2008; 2011a, b).

Considerable variability in the details and requirements of the reaction is seen among different reverse gyrases, for instance, as regards optimal temperature, processivity, ionic strength, nucleotide preference, and so on. Indeed, of the two reverse gyrases of *S. solfataricus,* TopR2 is less thermoactive (its maximal reaction temperature is 75 °C) and very processive, i.e., it is able to perform multiple supercoiling cycles before disengaging from DNA and attacking a new substrate molecule, thus producing highly positively supercoiled products (Bizard et al. 2011). In contrast, TopR1 is active at higher temperatures (up to 90 °C) and is typically distributive, i.e., shows Gaussian distribution of the linking numbers of its products, whose average supercoiling degree is dependent on DNA/protein ratio (Valenti et al. 2008; Bizard et al. 2011). Very recently, a novel reverse gyrase from the crenarchaeon *Pyrobaculum calidifontis* (PcalRG) was characterized, which shows even more peculiar features (Jamroze et al. 2014). PcalRG is the most thermostable reverse gyrase found thus far, with significant activity even at 100 °C, is very active, processive and robust. Interestingly, it is also quite insensitive to ionic strength, as it is active from 0 to 1.2 M NaCl, an odd result considering that DNA–protein and, in particular, DNA–topoisomerases interactions are usually very sensitive to ionic strength.

As explained above, DNA cleavage is a step of the catalytic mechanism of reverse gyrase (and topoisomerases in general), and normally is followed by religation of the ends after DNA passage. However, under particular conditions, the normal cycle is interrupted and reverse gyrase can either be frozen in a covalent complex with DNA or release cleaved products (Jaxel et al. 1999). Cleavage affects one strand, shows some sequence preference and occurs in the absence of nucleotides, while is inhibited by ATP, thus suggesting that the nucleotide stimulates DNA religation or improves the coordination between cleavage and religation. This activity has been shown for several

reverse gyrases, but is prominent in PcalRG with both covalently closed and linear substrates and depends on the catalytic tyrosine of the topoisomerase domain (Jamroze et al. 2014).

Consistent with their distinct evolutionary origin and function, the isolated C- and N-terminal domains of reverse gyrase are stable and functional; when incubated together they interact physically and reconstitute the positive supercoiling activity. This was demonstrated for the first time in Duguet laboratory for the *Sulfolobus acidocaldarius* enzyme (Déclais et al. 2000), and similar results were obtained for the *S. solfataricus* reverse gyrase (Valenti et al. 2008). Interestingly, in the parasite *Nanoarchaeum equitans*, the reverse gyrase gene is naturally split in two open reading frames, corresponding to the two domains (Waters et al. 2003), which interact forming a functional heterodimeric enzyme (Capp et al. 2010).

Given its chimeric structure, a long-lasting question is whether reverse gyrase might function analogously to helicase–topoisomerase complexes. Indeed, helicases of the RecQ family and topoisomerase 3 enzymes interact in many organisms and play important functions in DNA replication, recombination, and repair (reviewed in Larsen and Hickson 2013). By using their coordinate DNA unwinding, annealing and topoisomerase activities, these complexes are able to catalyze complex reactions such as reversal of replication fork, branch migration and resolution of model recombination intermediates (Bussen et al. 2007; Plank et al. 2006). However, until recently, the analogy between reverse gyrase and RecQ–Topoisomerase 3 complexes has remained hypothetical, since only mesophilic RecQ–Topo3 complexes were available, which obviously were unable to catalyze positive supercoiling, a reaction strictly requiring high temperature; conversely, no helicase activity could be demonstrated for any reverse gyrase (see below). Results obtained in the last few years allowed to clarify similarities and differences between reverse gyrase and RecQ–Topoisomerase 3 complexes. First, studies on a hyperthermophilic RecQ-like helicase, Hel112, and Topoisomerase 3, SsTop3, from *S. solfataricus*, allowed to directly test whether these complexes might catalyze the positive supercoiling reaction. Hel112 shares both DNA helicase and annealing activity with eukaryotic RecQ homologs (De Felice et al. 2007; Valenti et al. 2012), whereas SsTop3 catalyzes the typical Topoisomerase 3 DNA relaxation reaction and displays strong annealing activity (Chen and Huang 2006). The two proteins were shown to interact directly; however, despite its structural similarity with reverse gyrase, the SsTop3–Hel112 complex was unable to induce positive supercoiling even when incubated at temperature and conditions that would allow this reaction by reverse gyrase (Valenti et al. 2012). This result suggests that the positive supercoiling activity is a peculiar property of reverse gyrase and cannot be reconstituted by the association of canonical helicases and topoisomerases, leaving the question of what makes reverse gyrase such a peculiar enzyme still open.

A second set of recent results gave further insight in the analogy between reverse gyrase and Topo3–RecQ complexes: previously, several reverse gyrases tested for processive helicase activity failed to show such ability (Déclais et al. 2000; Valenti et al. 2008; Capp et al. 2010). Interestingly, the *S. solfataricus* TopR1 was shown to destabilize in vitro short synthetic substrates mimicking the recombination intermediate Holliday junction (HJ) structure (Fig. 1b; Valenti et al. 2010). Mutational analysis showed that the enzyme does not behave like an helicase, as HJ unfolding does not depend on ATP binding or hydrolysis, rather the binding of TopR1 to the central core of the HJ induces a structural distortion that facilitates the junction unfolding at high temperature (Valenti et al. 2010). More recently, the full-length *T. maritima* reverse gyrase and its isolated N-terminal domain were shown to induce transient unwinding of short double-stranded oligonucleotides; however, unwinding could be observed only in particular conditions, namely, under single-turnover conditions and in the presence of the non-hydrolyzable ATP analog ADP·BeFx, but not ATP (Ganguly et al. 2013). This observation could be explained assuming that the *T. maritima* reverse gyrase does unwind DNA duplex, but the reaction is rapidly reversed in the presence of ATP, whereas the non-hydrolyzable analog allows unwound products accumulation (see also below).

A significant advance in this story was the discovery that the *P. calidifontis* PcalRG, as the *S. solfatarics* TopR1, is able to process synthetic HJ; however, PcalRG shows a real helicase activity, namely, ATP hydrolysis-dependent unwinding (Jamroze et al. 2014; Fig. 1b). Interestingly, whereas lower concentrations support ATP-dependent unwinding, at higher enzyme/DNA ratios the reaction is reversed toward annealing of single-strand DNA, which is also stimulated by ATP (Jamroze et al. 2014; Fig. 1b). As already mentioned, combined unwinding and annealing activities are shared by eukaryotic and archaeal helicases, which use these activities in various reactions, including branch migration of HJ structures (Larsen and Hickson, 2013). At moment, it is not clear if the unwinding/annealing activity of PcalRG is a peculiarity of this specific enzyme or is a general feature of reverse gyrases. Two observations suggest that annealing is part of the normal enzyme cycle: Hsieh and Plank demonstrated that the *A. fulgidus* reverse gyrase induces ATP-stimulated annealing of complementary single-stranded DNA circles as well as positively supercoiled DNA containing a single-stranded bubble (Hsieh and Plank, 2006); moreover, the fact that transient unwinding by the *T. maritima* reverse gyrase

cannot be detected in the presence of ATP suggests that the nucleotide stimulates rapid annealing of the unwound intermediate (Ganguly et al. 2013). If confirmed for other reverse gyrases, the double unwinding–annealing activity could help clarify the mechanism of the positive supercoiling reaction. Indeed, an enzyme catalyzing unwinding of a topologically closed DNA circle would create two domains in the molecule, one positive in front of the enzyme and one negative behind; these latter can be relaxed by the topoisomerase activity, resulting in positive supercoiling of the final product (Jaxel et al. 1996; Plank and Hsieh 2009).

Several studies showed that reverse gyrase interacts with proteins involved in DNA damage repair, tolerance, or prevention, implying reverse gyrase in the cell response to DNA damage (Napoli et al. 2004; Valenti et al. 2006, 2009). In *S. solfataricus*, reverse gyrase activity is stimulated by the single-strand binding protein, SSB, through physical interaction (Napoli et al. 2005). Experiments in vivo in this archaeon showed that reverse gyrase is recruited to chromatin after UV irradiation (Napoli et al. 2004). SSB stimulates reverse gyrase binding to UV-irradiated DNA (Napoli et al. 2005), thus it is possible that reverse gyrase is directed to damaged DNA through its interaction with SSB, which has high affinity for DNA lesions (Cubeddu and White 2005). A third partner of the complex was identified as the translesion DNA polymerase, PolY (Valenti et al. 2009). In functional assays, reverse gyrase inhibits PolY, and this inhibition depends on both ATPase and topoisomerase activities of reverse gyrase, suggesting that the positive supercoiling activity is required for PolY inhibition. *In vivo*, both reverse gyrase and PolY were shown to be degraded after the treatment of *S. solfataricus* cells with the alkylating agent methyl methanesulfonate (MMS), which also induced degradation of genomic DNA (Valenti et al. 2006, 2009). Lethal doses of MMS induced irreversible degradation of reverse gyrase, PolY and DNA, a phenomenon strikingly reminiscent of DNA damage-induced apoptosis in higher eukaryotes, leading to the attractive hypothesis that analogous mechanism controlling cell death in the presence of irreparable DNA damage may exist in archaea. In this context, reverse gyrase may play an important role in the control of PolY activity, which is a potentially mutagenic enzyme. Another interactor of reverse gyrase is the *S. solfataricus* Sul7d, one of the main chromatin components in this species (Guagliardi et al. 1997). Sul7d stabilizes DNA against denaturation and induces DNA-bending and negative supercoiling, antagonizing reverse gyrase positive supercoiling activity (Napoli et al. 2002).

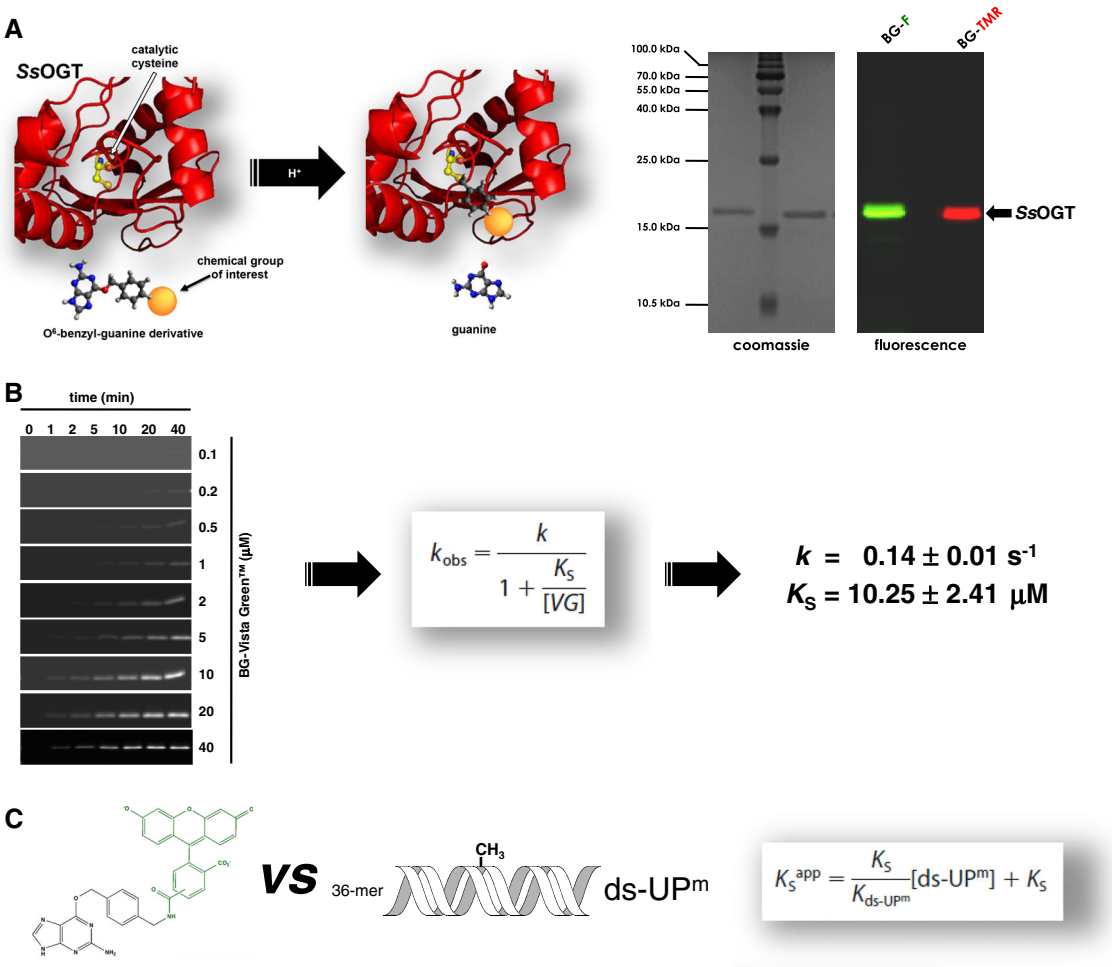
Although three decades have passed since the discovery of reverse gyrase (Kikuchi and Asai 1984a), many unanswered questions remain on the function and mechanism of this enzyme, and studies on newly characterized members are adding on the complexity of the picture. Hopefully, more structural and biochemical studies will expand our knowledge of reverse gyrase in the near future.

DNA alkyltransferase: a DNA repair protein with peculiar mechanism

Alkylating agents are among the most common mutagens and are particularly harmful for the genome of hyperthermophilic organisms, because high temperatures accelerate the conversion of alkylated bases into abasic sites, which can cause rupture of the phosphodiester bond and ultimately DNA fragmentation. Indeed, it was previously reported that *S. solfataricus* shows sensitivity to UV radiation doses comparable to that of *Escherichia coli* (Salerno et al. 2003; Romano et al. 2007), whereas it was very sensitive to lower doses of the alkylating agent MMS (Valenti et al. 2006, 2009).

DNA alkyltransferases (called AGT, MGMT or OGT) are small (17–20 kDa) proteins conserved in most (although not all) eukaryotes, bacteria and archaea, that catalyze repair of DNA alkylation damage (mainly at the O6 position of guanine) by a one-step mechanism involving the transfer of the alkylic group from DNA to a cysteine residue in the protein active site (reviewed in Mishina et al. 2006; Tubbs et al. 2007; Pegg 2011). This reaction results in direct repair of DNA, but also irreversible alkylation and inactivation of the protein. For this reason, AGTs are called "kamikaze" (suicide) proteins. In humans and yeast, once alkylated, AGTs are rapidly degraded by the ubiquitin–proteasome pathway (Xu-Welliver and Pegg 2002; Hwang et al. 2009).

The 3D structure of a number of AGTs has been resolved, including those from humans (Daniels et al. 2000, 2004; Duguid et al. 2005) and the hyperthermophilic archaea *Thermococcus kodakarensis* (formerly named *Pyrococcus kodakaraensis KOD1*; Hashimoto et al. 1999) *Methanococcus jannaschii* (Roberts et al. 2006) and *Sulfolobus tokodaii* (PDB entry 1WRJ). Although the primary sequence conservation among AGTs from evolutionary distant organisms is limited, their 3D structures are overall similar. They consist of a C-terminal domain containing the active site pocket and the DNA-binding region, and an N-terminal domain, which plays structural roles (Qingming et al. 2005). The DNA-binding region shows a classical helix-turn-helix (HTH) fold, common to many DNA-binding proteins. However, whereas usually the HTH motif contacts the DNA major groove, in AGT this region forms specific interactions with the DNA minor groove. The AGT



ds-UPm	k	K_S	$K_{ds\text{-}UP^m}$
µM	s^{-1}	µM	µM
0	0.14 ± 0.01	10.25 ± 2.41	0.77 ± 0.01
0.63	0.17 ± 0.01	21.14 ± 1.73	
1.00	0.15 ± 0.02	25.68 ± 2.14	
1.25	0.13 ± 0.01	27.73 ± 3.81	

Fig. 2 The *S. solfataricus* DNA alkyltransferase, SsOGT. **a** (*Left*) Model of the active site pocket showing the catalytic cysteine and the covalent linkage with the fluorescent O6-benzyl-guanine (O6-BG) derivative leading to protein labeling; (*right*) example of a SDS gel showing purified SsOGT labeled with either fluorescein (*green*) or TAMRA (*red*) derivatives of O6-BG. **b** Application of the fluorescent assay method to calculate kinetic constants. (*Left*) Example of a time course experiment using different concentrations of the fluorescent substrate/inhibitor and fixed SsOGT amounts; (*middle*) The equation used to calculate the kinetic parameters (*Right*). **c** Application of the assay to calculate SsOGT affinity for DNA by competition with the fluorescent substrate/inhibitor. (*Up-left*) The fluorescent substrate/inhibitor (BG-Vista Green™); (*Up-middle*) the DNA substrate, a 36-mer oligonucleotide containing a single-methylated guanine; (*Up-left*) the equation used to calculate the affinity for DNA (*Bottom*). Data are from Perugino et al. (2012)

structure revealed a unique mechanism for DNA repair: the protein actively flips out the alkylated base, while a conserved arginine residue forms a "finger", which is pulled into the DNA helix and interacts with the unpaired base, stabilizing the protein–DNA complex and allowing the transfer of the alkylic adduct to the catalytic cysteine (Daniels et al. 2004; Yang et al. 2009). The exact mechanism of how the protein

searches for the lesion is less clear. Upon binding, the protein induces DNA bending, which widens the minor groove and displaces the methylated base, thus it is likely that AGT recognizes such structural distortion; it remains to be established whether, to find the lesion, the protein detects the destabilized base pair or it passively captures the extruded base while scanning the double helix. Since AGT does not require nucleotide hydrolysis or other energy source, another interesting question is how DNA scanning is supported; human AGT binds to DNA cooperatively and slides along DNA in 5′ to 3′ direction; such cooperative binding could facilitate rapid directional scanning (Tessmer et al. 2012).

DNA alkyltransferase activity has been found in cell extracts of several hyperthermophilic archaea (Skorvaga et al. 1998), but only a few AGTs from extremely thermophilic organisms, including the bacterium *Aquifex aeolicus* and the archaea *A. fulgidus, T. kodakaraensis* and, more recently, *S. solfataricus* have been characterized in some details. They were all shown to be highly thermostable and thermoactive proteins catalyzing repair O6-methylguanine adducts in vitro (Shiraki et al. 2001; Kanagula and Pegg Kanugula and Pegg 2003; Nishikori et al. 2004a, b, 2005; Perugino et al. 2012). In particular, the AGT from *S. solfataricus,* called SsOGT, was identified and deeply characterized thanks to the development of a novel functional assay. The assay is based on the use of fluorescent derivatives of the AGT inhibitor O6-benzylguanine (O6-BG). Upon reaction, the benzylic group of this molecule with its fluorescent conjugate becomes covalently bound to the protein through the active site cysteine, with a 1:1 stoichiometry, allowing the measure protein activity directly from its fluorescence intensity analyzed by SDS–PAGE (Perugino et al. 2012; Fig. 2a). The assay allows the determination of kinetics parameters for the AGT reaction as well as of the protein affinity for different substrates and proved to be efficient also with a mesophilic AGT (Miggiano et al. 2013).

As a typical thermophilic protein, SsOGT showed temperature optima at 70 °C, but also considerable activity at temperature as low as 25 °C. Interestingly, the AGT from *T. kodakarensis* (formerly named *Pyrococcus* sp KOD1), when expressed in an *E. coli* strain deficient for alkyl transferase activity, was able to complement the alkylating agents hypersensitive phenotype (Leclere et al. 1998). These results suggest that thermophilic AGTs, in contrast to most thermophilic proteins, are correctly folded and active at mesophilic temperatures, well below the normal growth temperature of their natural sources. SsOGT was also quite tolerant to different reaction conditions, as it was active in broad pH and ionic strength intervals and in the presence of low concentrations of detergents (Perugino et al. 2012). The reasons for this loose stringency in the requirement of the reaction conditions are currently unclear. Moreover, SsOGT was insensitive to high concentrations of EDTA, suggesting that, unlike the human AGT, it does not contain any structural Zn^{2+} ion (Perugino et al. 2012).

Primary sequence comparison of SsOGT with other AGTs and 3D structure model showed overall similar fold and highly conserved aminoacid residues in the active site pocket, as well as in the HTH region. Multiple mutations in residues predicted to contact DNA completely abolished DNA binding, whereas mutation of the arginine "finger" R102 residue reduced DNA-binding affinity, thus confirming the involvement of these residues in DNA binding. Both mutants were able to catalyze dealkylation of O6-BG, but not of methylated DNA, showing that dealkylation and DNA binding are two distinct activities of SsOGT (Perugino et al. 2012). Like the human AGT, SsOGT binds dsDNA in a cooperative manner (Perugino et al. 2012), thus suggesting that it might use the same mechanism to scan DNA searching for the lesion, although direct evidence is lacking.

Data on the function of AGT in hyperthermophiles are scarce. *S. solfataricus* cultures treated with non-lethal doses of MMS are able to recover growth after a transient arrest, thus suggesting that mechanisms to signal and repair alkylation damage are effective in this organism (Valenti et al. 2006). Transcription of the SsOGT gene was induced by treatment of the cultures with alkylating agents, which is consistent with involvement of the protein in repair of this type of damage. Interestingly, increase of SsOGT RNA level did not correspond to increase in intracellular protein level: instead, SsOGT was degraded in response to alkylation damage. Experiments combining the native and recombinant protein showed that SsOGT degradation follows its alkylation (Perugino et al. 2012). This phenomenon is reminiscent of the mechanism of the eukaryotic AGT turnover, which is subject to conformational changes induced by alkylation that render the protein prone to ubiquitination and degradation by the proteasome (Xu-Welliver and Pegg 2002; Hwang et al. 2009). Although the players and mechanism of AGT degradation in archaea have not yet been clarified, this result thus adds to the body of evidence suggesting striking evolutionary conservation of basic pathways for maintenance of the genetic information from archaea to eucaryotes. Hopefully, more studies on AGT from hyperthermophilic archaea will help clarify the extent of this analogy and elucidate the mechanism of AGT degradation in archaea.

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