The archaeal topoisomerase reverse gyrase is a helix-destabilizing protein that unwinds four-way DNA junctions

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SUMMARY

Four-way junctions are non-B DNA structures that originate as intermediates of recombination and repair (Holliday junctions) or from the intra-strand annealing of palindromic sequences (cruciforms). These structures have important functional role, but may also severely interfere with DNA replication and other genetic processes; therefore, they are targeted by regulatory and architectural proteins and dedicated pathways exist for their removal. Whereas it is well known that resolution of Holliday junctions occurs either by recombinases or specialized helicases, less is known on the mechanisms dealing with secondary structures in nucleic acids. Reverse gyrase is a DNA topoisomerase, specific of microorganisms living at high temperature, which comprises a type IA topoisomerase fused to a SF2 helicase-like module and catalyzes ATP-hydrolysis dependent DNA positive supercoiling. Reverse gyrase is likely involved in regulation of DNA structure and stability, and might also participate in the cell response to DNA damage. By applying FRET technology to multiplex fluorophore gel imaging, we show here that reverse gyrase induces unwinding of synthetic four-way junctions as well as forked DNA substrates, following a mechanism independent of both the ATPase and the strand-cutting activity of the enzyme. The reaction requires high temperature and saturating protein concentrations. Our results suggest that reverse gyrase works like an ATP-independent helix-destabilizing protein specific for branched DNA structures. The results are discussed in light of reverse gyrase function and their general relevance for protein-mediated unwinding of complex DNA structures.

INTRODUCTION

Four-way junctions are complex DNA structures that play major biological role as intermediates in DNA rearrangements of various kinds. In particular, the Holliday junction (HJ) is the central intermediate in DNA recombination and repair of collapsed replication forks, whereas cruciforms or stem-loop structures are four-way junctions due to intra-strand base pairing of inverted repeats in DNA, RNA or DNA-RNA hybrids. These structures have regulatory role but may also pose a threat to genome stability and cause replication, transcription and translation stall, calling for specific mechanisms for their removal. Two classes of well-characterized enzymes are able to resolve HJ: resolvases, which are responsible for its cleavage and formation of crossover products (1, 2); and specialized helicases, which promote ATP hydrolysis-dependent branch migration of the junction (3-5). In archaea, representatives of the two classes of enzymes are the resolvase Hjc (6-8) and the helicase Hjm (9), respectively. Diverse non-enzymatic architectural proteins whose action induces structural modification of DNA, and in particular DNA supercoiling, share the ability to bind four-way structures (10). For instance, the SMC subunits of the cohesin and condensin complexes and the human DEK protein (which is involved in acute myeloid leukemias and in several autoimmune diseases), both inducing positive supercoiling of DNA upon binding, show high affinity for cruciform DNA (11, 12). In addition, at least some DNA topoisomerases were shown to interact with their substrates preferentially at level of DNA crossovers and to cleave four-way junctions in vitro (13,14).

Reverse gyrase is a unique DNA topoisomerase, specific of microorganisms living above 80 °C. In contrast to all other topoisomerases, this enzyme introduces positive supercoils into DNA molecules, an activity possibly involved in genome stabilization and protection against thermal denaturation (for reviews, see (15, 16). Reverse gyrase comprises two evolutionarily highly conserved protein modules: a C-terminal type IA topoisomerase domain and an N-terminal domain similar to SF2 helicases, including an ATP-binding domain. ATP hydrolysis is essential for the positive supercoiling reaction, although true helicase activity has never been demonstrated. Several results suggest that reverse gyrase might participate in the cell response to DNA damage. In the crenarchaeon Sulfolobus solfataricus, reverse gyrase is recruited to DNA after UV irradiation (17), interacts with the single strand binding protein, SSB and the translesion DNA polymerase, PolY (18, 19) and is degraded after treatment with MMS (20). Reverse gyrase structure and its involvement in genome stability are reminiscent of the evolutionarily conserved complexes comprising Topoisomerase III and members of the RecQ helicase family, which have essential functions in recombination and repair (for a recent review, see ref 5).

We show here that reverse gyrase is able to bind and unwind branched DNA structures, such as four-way junctions and forks. Unwinding is independent on the ATPase and cleavage activity of the enzyme, thus suggesting the existence of a previously undescribed, non-enzymatic mechanism to process such important structures.
Experimental Procedures

Proteins. All chromatographic separations were performed on AKTA FPLC systems (GE Healthcare Buckinghamshire, UK); protein concentration was determined with a Bio-Rad Protein Assay Kit (Bio-Rad Pacific, USA), and purity was assessed by SDS-PAGE. All proteins were diluted in RG buffer (20 mM phosphate buffer pH 6.5, 150 mM NaCl, 20% glycerol).

Recombinant His-tagged *S. solfataricus* TopR1 reverse gyrase (hereafter called RG) as well as deletion and site-specific mutants were purified as described previously (21).

Native reverse gyrase (nRG) was purified from *S. solfataricus* P2 cells. Eight L of cell culture were grown at 80°C as described previously (22) until exponential phase (0.4-0.6 OD₆₀₀). Cells were harvested by centrifugation and soluble protein extract was prepared as described (17). Soluble extract was incubated for 30’ at 37°C with 20 U/ml of Benzonase (Novagen) dialyzed against Heparin buffer (20 mM Tris-HCl pH 7.0) and loaded onto heparin column (HiPrep 16/10 Heparin FF, GE Healthcare). Proteins were eluted with a linear gradient of NaCl (0 to 1 M NaCl). Throughout purification, fractions were analysed by SDS-PAGE and western blotting using the anti-His antibody (GE Healthcare); positive fractions were pooled, concentrated with Amicon Ultra system (Millipore) and stored at 20°C with the addition of 40% glycerol. Activity was checked by DNA relaxation assay.

DNA topoisomerase assays. Positive supercoiling assays were performed at 70 °C as reported (23) using either RG or nRG and pQE31 plasmid (Qiagen) as substrate. Samples were analysed by 2D agarose gel electrophoresis, analysed and quantified as reported (23). Relaxation assays were performed at 55 °C as reported (24) using recombinant purified *E. coli* Topo3 and pQE31 (Qiagen). Samples were analysed by mono-dimensional 1.2 % agarose gels in TBE buffer 1X, stained and analysed as described before (23).

Western blots were performed as previously described (19) using the anti RG polyclonal antibody which recognises both recombinant TopR1 and TopR2 (A. Valenti, data not shown) as well as reverse gyrase in *S. solfataricus* cell extracts (19).

DNA substrates. Oligonucleotides used to prepare DNA substrates, either unmodified or with Cy3, Cy5, TAMRA or BHQ2 modification (listed in Table 1) were purchased from PRIMM (Italy). DNA substrates used for gel-FRET assay were prepared by annealing oligonucleotides in the appropriate combinations: HJ, A1-A2-A3-A4; IM-HJ, A1-A2-A5-A6; Q-HJ, A3-A4-A8-A9; Fork, A1-A2; 40 bp-double strand (ds), A2-A7. For each substrate, stoichiometric amounts of purified oligonucleotides were annealed in TE buffer, heated for 5 min at 95°C followed by slow cooling to RT. The annealed substrates were loaded on an 8% (w/v) polyacrylamide gel in 0.5X TBE buffer (pH 8.3). After gel migration the bands were excised from the gel and incubated o/n at 37°C with shaking in soak solution (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% SDS). Eluted DNA substrates were ethanol purified and quantified using a Nanodrop™ 2000 (Thermo-Scientific) instrument. Substrates used...
for EMSA and for KMnO₄ probing were prepared as described above using the same oligonucleotides but without fluorophores. Prior to annealing, the A2 oligonucleotide was radiolabelled at the 5'-end using [32P]-ATP and T4 polynucleotide kinase.

**Gel-FRET assay.** DNA substrates (20 nM) were incubated in HJ-buffer (2.5 mM Tris-HCl pH8.0, 0.25 mM β-mercaptoethanol, 5 mM NaAc, 0.5 mM MgCl₂) at indicated temperatures for 30 min without or with different amounts of the indicated proteins. To minimize variations among samples within each experiment, a single mix with all common components was set up; in both negative controls and samples, the total buffer concentration was kept constant by correcting with the appropriate amount of RG buffer. Reactions were terminated by adding 5X STOP solution (0.5% SDS, 40 mM EDTA, 0.5 mg/ml Proteinase K, 20% glycerol), immediately loaded on 8% polyacrylamide gel containing 0.1% SDS and run in TBE 0.5X Buffer at 150V/cm. Reaction substrates and products were visualised by gel imaging on Versadoc 4000™ (BioRad) using the preset laser excitation and emission setting for Cy5 and Cy3 fluorophores: red led and filter at 695 nm for Cy5, green led and filter at 605 nm for Cy3, green led and filter at 695 nm for FRET. Each assay was performed at least three times.

**EMSA.** RG binding assays were performed as previously described (21) using either HJ [32P]-labelled at the 5’end of the A2 oligonucleotide, or [32P]-labelled A2 oligonucleotide double stranded oligonucleotides (21 and Tab 1). Incubation was for 10 min at 37 °C. Each assay was performed at least three times. Radioactivity was determined by autoradiography with a Storm PhosphoImager and quantified with the IQ-Mac software (Molecular Dynamics, GE Healthcare, Buckinghamshire, UK).

**Modification of DNA by Potassium Permanganate.** DNA probing by KMnO₄ was performed following the published procedure (6) using HJ [32P]-labelled at the 5’end of the A2 oligonucleotide and 0.6 μM RG. After electrophoresis, radioactivity was determined by autoradiography with a Storm PhosphoImager (GE Healthcare).

**Fluorescence measurements.** Steady state fluorescence measurements were performed on a K2 fluorometer (ISS, Champaign, IL, USA) equipped with a 2-cell temperature controlled sample holder. Fluorescence emission spectra between 560 and 650 nm were recorded at fixed excitation wavelength of 550 nm, with slit width of 1.0 nm. Each emission curve obtained with different RG concentrations was normalized to the value obtained for the free junction in the presence of corresponding volumes of RG buffer and was corrected for lamp fluctuations and instrumental variations. All measurements were performed at 55°C. The effect of temperature on the fluorescence emission of TAMRA-A8, BHQ2-A9 and Q-HJ was almost negligible.

**RESULTS**

**Reverse gyrase unwinds four-way structures**

We developed a Förster Resonance Energy Transfer (FRET)-based electrophoretic technique (gel-FRET) to enable monitoring of assembly and disassembly of a four-way junction. We designed a synthetic junction (HJ) containing a central 4-nucleotide core homology and four arms of 20 bp each, in which the 3’ and 5’ ends of one arm were labelled with the Cy3 and Cy5 fluorophores, respectively (Supplementary Fig. S1A and Tab. 1). With respect to conventional radioactive methods, this technique offers the possibility to discriminate among different products, follow the fate of different strands and determine the pathway of HJ processing in a single experiment (Fig 1A). Because reverse gyrase is a highly thermophile enzyme, whose activity is virtually undetectable below 50 °C (21), we determined the optimal incubation conditions for the HJ (Supplementary Fig. S1B); we chose incubation for 30 min at 55 °C that secured stability of the HJ and prevented spontaneous annealing of the denatured junction.

The archaeon *S. solfataricus* encodes two isoforms of reverse gyrase, called TopR1 and TopR2, which are indistinguishable in size, activity and immunoreactivity (A. Valenti, unpublished results). We purified both the recombinant his-tagged TopR1 reverse gyrase over-expressed in *E. coli* (hereafter called RG; 21; see also Supplementary Fig. 4S), as well as the native protein directly from *S. solfataricus* (nRG; Supplementary Fig. 4S).

Addition of RG to HJ, and incubation at 55 °C, followed by gel electrophoresis and multiplex fluorophore imaging showed RG concentration-dependent reduction of the HJ and appearance of faster products that, based on their migration and fluorophore labelling, were identified as the three forks, two full-length ss and two ss cleavage products (Fig 1B and Supplementary Fig 2S; see also below). Intermediate oligonucleotide combinations were also present. Supersaturating concentrations of protein (P/DNA ratio >10) were required for efficient processing of HJ; whereas at lower RG concentration the forks were the main products, ss bands became gradually more evident with increasing RG concentration, suggesting that the reaction proceeds through formation of relatively stable fork intermediates, which are then processed to ss products.
When the nRG purified from *S. solfataricus* was used, identical results were obtained (Fig. 2A), thus ruling out any suspicion that the HJ unwinding activity might be due to a contaminant, or some artifact of our his-tagged protein preparation. The high amounts of both RG and nRG needed for efficient HJ processing might raise doubt on the physiological significance of this activity; we thus determined the intracellular amount of reverse gyrase by quantitative western blot (Fig. 2B). Reverse gyrase turned out to be a rather abundant protein, accounting for about 0.37% of the total soluble *S. solfataricus* protein. Since exact data on the absolute intracellular protein content of this archaeon are not available in the literature, we could assume that, consistently with the relative size of the two genomes, *S. solfataricus* proteome is about half that of *E. coli*, that is 10^6 - 10^7 protein molecules/cell (25). Thus, a conservative estimation would predict that reverse gyrase ranges between 2 \times 10^5 (about 4% of 5 \times 10^5) and 2 \times 10^6 (4% of 5 \times 10^5) molecules/cell. This means that there is enough reverse gyrase in the cell to have 20-200 protein molecules binding simultaneously to 100 four-way junctions, which is likely far above the number of these structures in a cell.

### Fluorescence measurements

The gel-FRET experiments allow endpoint analysis and require extensive purification steps after the reaction to remove the protein, which might in principle affect the results. In order to follow the reaction in real time, avoid artefacts due to protein removal, and quantify RG activity using an independent technique, we carried out fluorescence measurements. To overcome possible fluorophore quenching by the protein, leading to fluorescence reduction, we decided to synthesize a molecular beacon, an identical HJ (Q-HJ) in which two strands were labelled with the TAMRA fluorophore and its quencher BHQ2, respectively (Fig 3A and Tab S1). In this case, separation of the labelled strands is monitored by fluorescence increase. Fig 3B shows that the fluorescence emission spectrum of the Q-HJ was almost completely quenched due to the fact that BHQ2 and TAMRA are located within Forster distance. Addition of RG resulted in a marked increase of the fluorescence emission spectrum of the Q-HJ, which showed major sites located 3-8 bases from the cleavage sites by RG on the A1 and A2 strands of HJ, which showed major sites located 3-8 bases from the strand crossing; importantly, exactly the same cleavage sites were also found on ss DNA (data not shown). Consistent results were obtained using a 32P-labelled HJ (Tab 1), indicating that the fluorophores do not affect the normal behaviour of RG and HJ in this reaction (data not shown).

Reversing the reaction (data not shown). When the nRG purified from *S. solfataricus* was used, identical results were obtained (Fig. 2A), thus ruling out any suspicion that the HJ unwinding activity might be due to a contaminant, or some artifact of our his-tagged protein preparation. The high amounts of both RG and nRG needed for efficient HJ processing might raise doubt on the physiological significance of this activity; we thus determined the intracellular amount of reverse gyrase by quantitative western blot (Fig. 2B). Reverse gyrase turned out to be a rather abundant protein, accounting for about 0.37% of the total soluble *S. solfataricus* protein. Since exact data on the absolute intracellular protein content of this archaeon are not available in the literature, we could assume that, consistently with the relative size of the two genomes, *S. solfataricus* proteome is about half that of *E. coli*, that is 10^6 - 10^7 protein molecules/cell (25). Thus, a conservative estimation would predict that reverse gyrase ranges between 2 \times 10^5 (about 4% of 5 \times 10^5) and 2 \times 10^6 (4% of 5 \times 10^5) molecules/cell. This means that there is enough reverse gyrase in the cell to have 20-200 protein molecules binding simultaneously to 100 four-way junctions, which is likely far above the number of these structures in a cell.

### Reverse gyrase does not work like helicases or resolvases in HJ processing

Based on its known biochemical activities, one could anticipate that, by virtue of its helicase-like domain, reverse gyrase might promote branch migration of the HJ, a typical ATP-dependent reaction catalysed by several helicases. In addition, exploiting the cleavage-religation activity of its topoisomerase domain (21), RG might also act as recombinases, namely by cleavage, exchange and religation of two DNA strands. We thus sought to elucidate the HJ processing reaction mechanism.

Both RG and its isolated N-terminal domain show DNA-dependent ATPase activity, which is essential for the positive supercoiling reaction (19, 21); however, HJ processing by RG was unaffected by addition of 5 mM ATP (Fig 4A), 0.5 mM MgCl_2, which is required for ATPase activity, or 1 mM EDTA, which inhibits ATPase activity (data not shown). At 37 °C, RG was unable to process HJ, and ATP addition was ineffective in stimulating the reaction (Fig 4A), suggesting that the energy derived from nucleotide hydrolysis cannot compensate for the temperature. In addition, the ATPase deficient K116A mutant of RG (19) efficiently processed HJ (Fig 4C). Because it is possible that under our conditions we are looking at single turn-over reactions, we wondered whether ATP might affect product release and enzyme recycling under different circumstances. However, ATP failed to stimulate HJ processing in reactions containing lower enzyme concentrations (Supplementary Fig. 4SA); in addition, the kinetics of the reaction was very rapid and was not affected by ATP (Supplementary Fig. 4SB). Thus, the nucleotide does not appear to stimulate enzyme recycling.

Taken together, these results suggest that RG processes HJ by an ATP-independent mechanism, which is thus distinct from that used by helicases.

In order to test whether RG might work like HJ resolvases, we used the Y965F mutant, carrying a substitution of the catalytic tyrosine (Fig 4B), which impairs both DNA relaxation and positive supercoiling (19). Surprisingly, the Y965F protein was able to process HJ efficiently, although, as expected, no cleavage products were obtained (Fig 4D), suggesting that HJ resolution by RG does not require DNA cleavage. This conclusion was supported by mapping the cleavage sites by RG on the A1 and A2 strands of HJ, which showed major sites located 3-8 bases from the strand crossing; importantly, exactly the same cleavage sites were also found on ss DNA (data not shown).
shown). Taken together, these results suggest that cleavage is a secondary event occurring on ss produced in the reaction and is not involved in HJ processing. We thus conclude that HJ unwinding by RG occurs via a mechanism distinct from that used by resolvases.

**Specificity of the unwinding reaction**

Previously, we have obtained the two domains of RG in isolation (21) and have shown that the N-terminal domain is a DNA-dependent ATPase, whereas the C-terminal domain behaves like a canonical Type IA DNA topoisomerase; both domains show the ability to bind ss, ds and mixed substrates, as predicted from the presence of two putative Zn-fingers motives (see Fig.5A). When the two isolated domains are combined in the reaction, they make specific physical interaction and induce ATP-dependent positive supercoiling of DNA molecules (21). We tested the ability of the isolated domains to induce HJ unwinding (Fig. 5B); whereas the N-terminal domain was completely inactive, the C-terminal module was unable to unwind the HJ, but produced a small amount of cleavage product. This latter is likely due to cleavage of the ss DNA present in the HJ preparation, an activity typical of type IA topoisomerases. In order to test the specificity of the HJ unwinding activity, we cloned and purified a his-tagged version of *E. coli* Topo3, a Type IA DNA topoisomerase homologous to the C-terminal domain of reverse gyrase (24); the enzyme was suitable for our purposes since it was active in DNA relaxation experiments at 55 °C (Supplementary Fig. 4S, D-E). When used under the same conditions and high P/DNA ratio, Topo3 produced a small amount of shorter products similar to those observed for RG and its C-terminal domain, likely due to ss cleavage (Fig. 5C); however, it was unable to process HJ. In contrast, when the two domains of RG were combined together, HJ processing activity was restored (Fig 5B). Taken together, these results suggest that the HJ unwinding activity is specific for reverse gyrase, requires both domains and is not a general property of type IA DNA topoisomerases.

**Reverse gyrase unwinds different branched structures**

Due to its 4-nucleotide homologous core, our HJ may shift between two extreme conformations (see Fig 7C), and a number of studies revealed that changes in HJ structure may facilitate its recognition and resolution by junction resolving enzymes (6, 26). RG was able to process with similar efficiency a completely heterologous, “immobile” HJ (IM-HJ) identical to HJ except for the central core (Tab SI), suggesting that the reaction is not specific for the mobile HJ or its AT-rich core sequence (Fig 6A). Previous studies failed to demonstrate true helicase activity for RG or its N-terminal domain (18, 21). However, when incubated under the same conditions reported above for HJ and IM-HJ, and using high P/DNA ratio (>20), RG efficiently unwound the Cy3-Cy5 labelled fork substrate in an ATP-independent reaction (Fig 6B). In addition, the protein unwound with similar efficiency the single-fluorophore labelled forks formed by oligonucleotides A1+A4 and A2+A3, respectively (Supplementary Fig 1S, data not shown). Under the same conditions, RG was not able to unwind a 40-bp fully ds substrate (Fig 6C), thus suggesting that the reaction requires the presence of a ss-ds junction.

**Reverse gyrase distorts HJ structure upon binding**

Many HJ processing proteins show binding selectivity or higher affinity for four-way structures. Reverse gyrase binds ds, ss and mixed DNA substrates with increasing affinity and certain degree of cooperativity (21, 27, 28). In order to correlate ATP-independent unwinding activity with binding affinity, we performed EMSA experiments (Fig 7A). The efficiency of binding of RG for HJ was similar to that for ds oligonucleotides and significantly lower than that for a fork, which is the preferred substrate (21).

Many HJ-binding proteins cause structural distortion of HJs, which stabilizes HJs in their open form facilitating unfolding of the junction (6, 26). To determine if RG might induce modification of the HJ structure, we used the permanganate probing technique (Fig 7B). The experiment was performed at 37 °C in order to prevent unwinding of the junction and assess the effect of the protein binding. In the presence of RG, high reactivity to permanganate was observed specifically at the level of two thymines, located 4 bases apart at the centre of the oligonucleotide A2 and thus falling exactly at the point of strand crossing in the two possible HJ conformations (Fig 7C). This experiment indicated that RG distorts HJ structure by disrupting base pairing specifically at its core, suggesting that HJ processing might be facilitated by decrease of duplex DNA stability brought about by contacts between RG and DNA, starting from the point of strand crossing.

**DISCUSSION**

The uniqueness of reverse gyrase phylogenetic distribution, structure and activity has been repeatedly underlined (16, 15). We have described another activity of reverse gyrase, the ability to unwind substrates containing helical junctions independent of the ATPase and DNA cleavage activity. Unwinding requires high temperature, both domains of reverse gyrase, the presence of either a crossover or a ss-ds junction in the substrate, and saturating amounts of protein; upon binding, reverse gyrase distorts the HJ
specifically at is crossing point inducing local base un-pairing. The *E. coli* Topo3 enzyme could not substitute for reverse gyrase in this reaction, suggesting that it is not a general property of Type IA topoisomerases. The experiments presented here imply a non-catalytic activity of reverse gyrase, in which structural recognition, distortion and junction unwinding are all tightly connected. We suggest that RG works as a branched structure-specific helix destabilizing protein: due to its peculiar structure and fluctuation between stacked (open) and unstacked form, the HJ core is prone to distortion by reverse gyrase, which induces base melting or stabilizes “breathing” regions. These may in turn facilitate binding of other DNA molecules and possibly further distortion followed by strand destabilization. On the fork, the ss-ds junction acts analogously to the HJ core triggering the reaction. In this model, the energy required to unwind DNA is provided by protein-DNA or protein–protein interactions instead of ATP hydrolysis.

It was previously reported that the stoichiometric binding of reverse gyrase to an open circular DNA causes a decrease of the DNA linking number (after nick closure by a thermophilic ligase); this result was interpreted as local duplex unwinding induced by reverse gyrase binding (29). Interestingly, the activity was shown to be ATP- and DNA cleavage independent, required high P/DNA ratio and the presence of both domains. Although the biological meaning of reverse gyrase-induced plasmid linking number decrease was not addressed, this activity is reminiscent of the junction unwinding described here, since at high temperature plasmids may expose ss-ds junctions.

The analysis of the 3D structure of reverse gyrase suggested the presence of a number of predicted DNA binding regions spanning both N- and C-terminal domains, including Zn fingers and extended regions of positive potential (30). The result that the two separate RG domains are not effective in HJ processing might suggest that cooperation among these binding sites might be required for this reaction.

The fact that HJ and fork unwinding is independent of the two reverse gyrase catalytic activities might seem unsound; however, both DNA topoisomerases and helicases have been reported to function independently of their catalytic activities under specific circumstances. For instance, human Topo IIIα (a Type IA DNA topoisomerase) stimulates HJ unwinding by the RecQ homolog BLM, yet the catalytic activity of Topo IIIα is dispensable for the enhancement of this reaction (31). Furthermore, ATPase deficient alleles of the yeast helicase gene *SGS1* are functional in limiting crossovers induced by a site-specific double-strand break, a function likely requiring the ability to process HJ intermediates (32).

The high amounts of reverse gyrase needed for efficient HJ processing might question the physiological significance of this activity; however, we have shown that in *S. solfataricus* reverse gyrase is rather abundant and there is enough intracellular protein to deal with at least 100 branched structures/cell. Although ATP-independent unwinding of HJ has not been reported so far, several ss-DNA binding proteins, including human RPA and *S. solfataricus* SSB show the ability to destabilize duplex DNA in an ATP-independent manner requiring P/DNA ratios ranging from 10 (RPA) to 125 (SSB) (33, 34). High P/DNA ratios are also required for the enzymatic resolution of HJ by the resolvase Hjc (6). In addition, so-called nucleic acids chaperones, such as rotavirus NSP2 and the HIV NS2 proteins, are also able to destabilize DNA-RNA or RNA-RNA duplexes; in these cases, 100-500 fold molar excess of protein relative to the nucleic acid strand are typically needed to observe optimal activity (35, 36); both classes of proteins work by coating the nucleic acid strand, rather than through a catalytic mechanism. Interestingly, reverse gyrase has been previously suggested to have nucleic acids “chaperone” activity, which was ATP-independent (37).

The junction melting property of reverse gyrase might be involved in one or more important cell processes. It may obviously participate in HJ resolution during recombination or restart of arrested replication forks, but also remove cruciform structures, thus assisting replication or helicase/translocase tracking along DNA. In addition, since reverse gyrase is able to bind RNA (A. Valenti, unpublished results), it might also remove secondary structures in DNA-RNA hybrids. Interestingly, cruciform extrusion is induced DNA supercoiling (38), thus, based on its *in vitro* activities, reverse gyrase might both generate (changing the DNA supercoiling) and resolve four-way structures (through the ATP-independent activity described here), possibly regulating four-way structure formation during DNA transactions.
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FOOTNOTES

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Fig. 1. Gel-FRET assay for HJ processing by RG. A) Principle of the assay. HJ was assembled by annealing the A1-A4 oligonucleotides shown in Tab. 1. The 5’ end of strand A2 and the 3’ end of strand A1 were labelled with Cy5 and Cy3 fluorophores, respectively. The cartoon shows HJ and predicted junction unfolding products. B) Cy5/Cy3 labelled HJ (20 nM) was incubated at 55°C for 30 min. without (lane 1) or with increasing concentrations of RG as indicated (lanes 2-5). Samples were run and the same gel was scanned under different excitation/emission conditions as described under Experimental procedures section. The panels show the images obtained for Cy3, Cy5, FRET and the merge of all three images, respectively. Reaction substrate and products (forks, ss and cleavage products) are indicated. The complicated band pattern seen in the merge panel is due to different rates of migration of the two fluorophore-labelled oligonucleotides with each other and with respect to the unlabelled ones. The gel is representative of twenty independent experiments obtained with three different protein preparations.

Fig. 2. HJ processing by nRG. A) Gel-FRET assay for HJ processing by native RG from *S. solfataricus* (nRG). HJ (20 nM) was incubated for 30’ at 55°C without (lane 1) or with increasing concentrations of nRG as indicated (lanes 2-5). Gel electrophoresis and analysis were as described in the legend to Fig. 1B. The panels show the images obtained for Cy3, Cy5 and FRET, respectively. The gel is representative of three independent experiments. B) Quantification of reverse gyrase in cell extract. Soluble *S. solfataricus* cell extracts (lanes 1-5) and purified RG (lanes 6, 7) were probed with the anti-TopR1 polyclonal antibody (19) used at 1:10000 dilution. This antibody recognises both recombinant TopR1 and TopR2 (A. Valenti, data not shown), the two in *S. solfataricus* reverse gyrase isoforms. The intensity of the bands, determined using the VersaDoc instrument (BioRad) and expressed as arbitrary units, is reported above the amount of protein loaded in each lane. The intensity of reverse gyrase band in lane 5 (16 μg of cell extract) corresponds roughly to that obtained with 0.06 μg of purified protein (lane 6), thus reverse gyrase accounts for about 0.37% of total soluble protein.

Fig. 3. Fluorescence measurements of HJ. A) Q-HJ was assembled by annealing the A3-A4-A8-A9 oligonucleotides shown in Tab. 1. The 3’ end of strand A8 and 5’ end of strand A9 were labelled with TAMRA fluorophore and the BHQ2 quencher, respectively. The cartoon shows the principle of the assay. B) Fluorescence emission spectra were recorded as described in the Experimental procedures section. In each reaction, 0.1 μM of Q-HJ was used. Plots of the change in fluorescence intensity on the Y-axis (arbitrary units), in the absence (dashed lines) or presence of 4.0 μM RG (black lines) are shown. The inset shows the effect of increasing RG concentrations on the fluorescence intensity at 580 nm (Y-axis); the plateau was reached between 2.0 and 4.0 μM.

Fig. 4. RG catalytic activities are dispensable for HJ processing. A) HJ (20 nM) was incubated at 55°C (lanes 1-3) or 37°C (lanes 4-6) for 30 min. without (lanes 1 and 4) or with (lanes 2, 3, 5 and 6) RG at 0.8 μM; in lanes 2 and 5, 5 mM of ATP was added. Only the scans from CY5 emission is shown. The gel is representative of three independent experiments. B) Schematic diagram of RG showing the two domains, the point mutations in the ATP binding site of the N-terminal domain and in the catalytic tyrosine of the C-terminal domain and the position of the two putative zinc finger motives for DNA binding (19). C, D) Gel-FRET assays. HJ (20 nM) was incubated under standard conditions without (lane 1) or with (lanes 2) reverse gyrase mutants, as indicated. All proteins were used at 0.8 μM; only the scans from CY3 emission are shown. The gels are representative of four independent experiments performed with two independent mutant proteins preparations.

Fig. 5. Isolated RG domains are inactive in HJ processing. A) Schematic diagram showing the RG N-terminal and C-terminal domains with the two putative zinc finger motives for DNA binding are indicated. B) Gel-FRET assays. HJ (20 nM) was incubated under standard conditions without (lanes 1) or with (lanes 2) reverse gyrase mutants, as indicated. All proteins were used at 0.8 μM; only the scans from Cy3 emission are shown. The gels are representative of three independent experiments. C) HJ (20 nM) was incubated under standard conditions without (lane 1) or with (lane 2) *E. coli* Topo3 (0.8 μM). The gels are representative of three independent experiments performed.

Fig. 6. Substrates of ATP-independent unwinding. A) Processing of immobile HJ: IM-HJ (20 nM) was produced by annealing the A1, A2, A5 and A6 oligonucleotides shown in Table 1 and was incubated at 55°C for 30’ without (lane 1) or with increasing concentrations of RG, as indicated (lanes 2-4). The panels show the images obtained for Cy3 and Cy5, respectively. The gel is representative of two independent experiments. B) ATP-independent unwinding of a fork substrate. Gel-FRET assay with fork substrate (oligos A1+A2 in Tab. S1, 20 nM) and RG (0.8 μM). Incubation was for 30’ min. at 55°C. The panels show the images obtained for Cy3, Cy5 and FRET, respectively. The gel is representative of four independent experiments. C) RG does not unwind a ds
substrate. Gel-FRET assay was performed with the 40-bp ds substrate (oligos A2+A7 in Tab 1, 20 nM) incubated without (lane 1) or with RG (0.8 μM; lane 2) at 55 °C for 30 min. Lane 3, denatured ds. Only the scan from Cy5 emission is shown. The gel is representative of two independent experiments.

**Fig. 7. Binding of RG to HJ distorts its structure.** A) RG binding to HJ was analyzed by EMSA. Increasing concentrations of RG (60, 120, 240, 480 nM) were incubated at 37°C with the following 32P-labelled substrates (20 nM): HJ, 40-bp ds (oligo A2+A7 in Tab S1), fork (oligos A1+A2 in Tab. S1), and 80-bp ds (Valenti et al, 2008). The graph shows the quantification of results expressed as percentage of shifted DNA versus the P/DNA ratio. For each DNA ligand, the fraction of shifted DNA versus the amount of protein used is plotted. Binding assays were performed in triplicate and the results were averaged. Values are the mean ± SE of three independent experiments. B) HJ probing by potassium permanganate. 32P-labelled-HJ (20 nM) was incubated with RG at 0.6 μM (lanes 1 and 3) or without protein (lanes 2 and 4) at 37 °C for 30 min; after incubation, samples were reacted with KMnO4 followed by piperidine cleavage (lanes 1 and 2) or directly incubated with piperidine (lane 3); lanes 5 and 6 show molecular weight markers of 22 nt and 18 nt, respectively. Controls were mock-treated exactly as samples. The gel is representative of three independent experiments. C) The two extreme conformations of HJ with its 4-base homologous core (underlined). Arrows indicate the thymines specifically cleaved by piperidine after KMnO4 treatment in the presence of RG.
Tab. 1 Oligonucleotides used in this work. Sequences and 5’/3’ modifications are indicated.

| name | Sequence (5’-3’) | Mod. 5’ | Mod. 3’ |
|------|------------------|---------|---------|
| A1   | GCCGTGATCACCAATGCAGATTGACGAA CTTTGCCCACGT | /       | CY3     |
| A2   | GACGTGGGCAAAGGTTGTCATCAATGGACT GACAGCTGCATGG | CY5     | /       |
| A3   | GCCATGCAGCTGTCACTCCATTTGTCACTG CTAAGGCCTACTGC | /       | /       |
| A4   | GCCAGTAGGCCTAGCATGACAAATCTGCA TTGGTGATCACGG | /       | /       |
| A5   | GCCATGCAGCTGTCACTCCAGCTCATG CTAAGGCCTACTGC | /       | /       |
| A6   | GCCAGTAGGCCTAGCATGACGCCTCTGCA TTGGTGATCACGG | /       | /       |
| A7   | GCCATGCAGCTGTCACTCCATTTGACGAA CCTTGCCCACGT | /       | /       |
| A8   | GCCGTGATCACCAATGCAGATTGACGAA CTTTGCCCACGT | /       | TAMRA   |
| A9   | GACGTGGGCAAAGGTTGTCATCAATGGACT GACAGCTGCATGG | BHQ2    | /       |
| Topo 5’ | ATAAAGGATCCCTATGCGGTTGTTATTGCC | /       | /       |
| Topo 3’ | ATATCTCGAGCGCTATCGCCCGCTTCC GAC | /       | /       |
Fig. 3

A

LOW FLUORESCENCE

HIGH FLUORESCENCE

B

Normalized Fluorescence Intensity

Wavelength (nm)

Ex = 550 nm
Max Em = 580 nm
Fig. 7

A

![Graph showing the effect of RG (μM) on the shifted DNA (%).

B

| KMnO₄ | Piperidine | RG   | M22 | M18 |
|-------|------------|------|-----|-----|
| +     | +          | -    |     |     |
| +     | +          | +    |     |     |
| +     | -          | +    |     |     |
|       | -          | -    | M22 | M18 |

C

Diagram showing the interaction between multiple elements labeled A1, A2, A3, A4, 23, 19, ATG, CAAT, etc.

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The archaeal topoisomerase reverse gyrase is a helix-destabilizing protein that unwinds four-way DNA junctions

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