Reverse gyrase is a unique type IA topoisomerase that is able to introduce positive supercoils into DNA in an ATP-dependent process. ATP is bound to the helicase-like domain of the enzyme that contains most of the conserved motifs found in helicases of the SF1 and SF2 superfamilies. In this paper, we have investigated the role of the conserved helicase motifs I, II, V, VI, and Q by generating mutants of the Thermotoga maritima reverse gyrase. We show that mutations in motifs I, II, V, and VI completely eliminate the supercoiling activity of reverse gyrase and that a mutation in the Q motif significantly reduces this activity. Further analysis revealed that for most mutants, the DNA binding and cleavage properties are not significantly changed compared with the wild type enzyme, whereas their ATPase activity is impaired. These results clearly show that the helicase motifs are tightly involved in the coupling of ATP hydrolysis to the topoisomerase activity. The zinc finger motif located at the N-terminal end of reverse gyrase was also mutated. Our results indicate that this motif plays an important role in DNA binding.

The majority of DNA processing including DNA replication, transcription, repair, and recombination requires resolution of topological problems linked to the separation of the two DNA strands. Topoisomerases and helicases are two classes of enzymes specialized for these processes. Helicases catalyze disruption of hydrogen bonds between the two strands (1). Topoisomerases catalyze the interconversion of topological isomers of DNA and are required for the resolution of torsional stress and the unlinking of topologically intertwined molecules (2, 3). Several examples show that helicases and topoisomerases can act in concert (4–6). As a model for this helicase/topoisomerase association, reverse gyrase is a unique enzyme composed of a N-terminal helicase-like domain and a C-terminal type IA topoisomerase domain, usually on a single polypeptide (7).

Reverse gyrase has the almost unique property of introducing positive supercoils into a covalently closed DNA at the expense of ATP (8, 9). This enzyme is only found in archael and bacterial hyperthermophiles (10, 11) and is the only known hyperthermophile-specific protein (12), indicating an important role for life at high temperature. Indeed it was shown that a lack of reverse gyrase leads to a significant reduction in the growth rate at a high temperature (13). Several functions related to maintenance of genome integrity have been proposed for reverse gyrase, including a role in rewriting of DNA strands after passage of a transcription complex or during migration of recombination junctions (15, 16). Reverse gyrase is able to anneal single-stranded DNA circles, also suggesting an action as a DNA renaturase (14). It was also proposed that reverse gyrase could act as a “resolvase” by removing abnormal DNA structures that impede replication and transcription processes (4). Lastly, it has been shown that reverse gyrase is recruited to DNA in vivo after UV irradiation (15) and that it plays a role in preventing double-stranded DNA breakage at high temperatures (16).

Sequence and structural analyses of the topoisomerase domain (7, 17) as well as biochemical data (18–20) indicate that reverse gyrase belongs to the type IA topoisomerases family. Similarly to these enzymes, reverse gyrase relaxes negatively supercoiled DNA and transiently cleaves single-stranded DNA, forming a 5′-phosphotyrosine covalent intermediate (20). However, whereas type IA topoisomerases do not require ATP to function (reviewed in Ref. 3), the positive DNA supercoiling activity of reverse gyrase is ATP-dependent (8, 18, 21). The ATP-binding site is located in the helicase domain, and it was proposed that ATP hydrolysis provides the energy required to drive the DNA strand passage performed by the topoisomerase domain (21). The helicase domain contains motifs I, Ia, II, III, V, VI, and Q found in helicases of the SF1 and SF2 superfamilies (7, 22, 23). Motif I and II (so called Walker motifs A and B) are mainly devoted to ATP binding and hydrolysis, whereas motifs V and VI in different enzymes can either be involved in nucleic acid binding, NTP hydrolysis, or both (for reviews see Refs 24 and 25). Motif III differs between SF1 and SF2 helicases, and its role is unclear. The Q motif was recently identified in DEAD box helicases. A close examination of reverse gyrase sequences allowed us to identify a highly conserved glutamine located upstream of motif I (see Fig. 1) that could correspond to this invariant residue. This motif may regulate ATP binding and hydrolysis (23). Among reverse gyrases, a highly conserved putative zinc finger motif (zinc motif) is located at the extreme N-terminal end of the helicase domain (22), but the function of this motif is presently unknown.

Here, we have examined the role of helicase motifs I, II, V, VI, Q, and zinc motif through a mutagenesis study of recombinant
reverse gyrase from *Thermotoga maritima*. As most archaeal reverse gyrase, the *T. maritima* enzyme is composed of a single polypeptide chain (1104 amino acids, 128 kDa) containing the helicase and zinc finger motifs present in reverse gyrase (26). The enzymatic properties of mutant proteins were analyzed by testing their positive DNA supercoiling activity, DNA binding and cleavage, and ATPase activity.

Our results clearly indicate that helicase motifs are intimately involved in the reverse gyrase activity, likely via their helicase ATP hydrolysis machinery. Mutations in motifs I, II, V, and VI abolished the reverse gyrase activity, whereas mutation in the Q motif significantly reduced it. The corresponding mutant proteins were strongly impaired in ATP hydrolysis, indicating the importance of these five motifs in ATP binding and hydrolysis. Mutagenesis of the zinc finger motif led to a mutant protein exhibiting a reduced positive supercoiling activity. We show for the first time that this motif is important for DNA binding.

**EXPERIMENTAL PROCEDURES**

*Cloning and Site-directed Mutagenesis*—A DNA fragment encoding the *T. maritima* reverse gyrase was amplified by polymerase chain reaction using genomic DNA as a template, *Pfu* DNA polymerase (Expand High Fidelity kit, Roche Applied Science), a forward primer containing a Ncol restriction site (5′-GGCATGCGATGGTGAATTCCTAAC-3′) and a reverse primer containing a Xhol restriction site (5′-GATCTCGCTGACGCCCCCTCCCATGAGATTTT-3′). PCR products were digested by Ncol and Xhol and cloned at the corresponding sites into the pET24d expression vector (Novagen). The resulting plasmid (pET24d-TmTopR) was verified by DNA sequencing and used as a template for site-directed mutagenesis. Seven reverse gyrase mutants were generated (see Fig. 1). Mutants of the putative active site tyrosine (Y851F), motifs I, Q, and zinc were obtained by using the QuikChange<sup>TM</sup> site-directed mutagenesis kit (Stratagene), following the manufacturer’s instructions. Mutants of motifs II, V, and VI were obtained by using the overlap extension strategy (27). All of the mutant constructs were sequenced to verify the presence of the desired mutations and the absence of additional mutations. The sequences of the oligonucleotides used in this study are available upon request.

*Expression and Purification of Recombinant Proteins*—The pET24d-TmTopR plasmid and derivatives permit the production of recombinant proteins with a C-terminal His tag. Wild type and mutant proteins were expressed in *E. coli* Rosetta (DE3) cells (Novagen) under the following conditions: LB medium was inoculated with a freshly transformed colony containing 35 μg/ml kanamycin and 35 μg/ml chloramphenicol and grown overnight at 37 °C. 30 ml of the preculture were added to 1.8 liters of the same medium and grown at 37 °C to an absorbance of 0.5. Induction was carried out by adding isopropyl-1-thio-β-D-galactopyranoside to the culture (final concentration, 1 mM). The cells were grown overnight at 24 °C and harvested by centrifugation. Approximately 4.5 g of cells were usually obtained (A<sub>600</sub> = 1.0–1.2). If necessary, the cell pellets were stored at −80 °C.

The same purification procedure was followed for wild type and mutant enzymes. The cells were resuspended in 10 volumes (1 ml/g of cells) of buffer A (50 mM Tris·HCl, pH 7.5, 0.8 mM NaCl), lysed by sonication, and centrifuged at 15,000 × g for 15 min at 4 °C. The crude extract was then heated for 15 min at 75 °C and immediately centrifuged at 40,000 × g for 15 min at 4 °C. The supernatant was loaded onto a 1-ml nickel-Sepharose column (HisTrap HP; Amersham Biosciences) equilibrated with buffer A containing 5 mM imidazole. The column was washed with this buffer, and elution was performed at 250 mM imidazole in buffer A. Approximately 0.5–1 mg of protein was loaded onto a gel filtration column (Superdex S200 HR10/300; Amersham Biosciences) equilibrated with buffer A containing 0.1% Triton X-100. The wild type reverse gyrase and mutants all eluted as a single peak of 2 ml (retention volume, 11–12 ml). After the addition of glycerol (final concentration, 17%), the purified proteins were stored at −80 °C. Protein concentrations were determined by the method of Shaffner and Weissmann (28).

*Reverse Gyrase Assays*—Unless otherwise stated, the His-tagged proteins were incubated for 30 min at 80 °C in a 20-μl reaction mixture containing 50 mM Tris·HCl, pH 8.0, 0.5 mM dithiothreitol, 30 μg/ml bovine serum albumin, 10 mM MgCl<sub>2</sub>, 120 mM NaCl, 1 mM ATP (or another nucleotide), and 0.25 μg of negatively supercoiled DNA (pTZ18; Pharmacia). The reactions were stopped by the addition of 0.5% SDS, 10 mM EDTA, 5% glycerol, and 0.04% bromphenol blue. The reaction products were analyzed by one- or two-dimensional 1.2% agarose gel electrophoresis as described previously (19). 3 μg/ml chloroquine was added in the gel and the TEP buffer (36 mM Tris, 30 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM EDTA, pH 7.8) for the second dimension electrophoresis. The first electrophoresis was run for 3 h at 3.6 V/cm, and the second was run for 16 h at 1.2 V/cm. After chloroquine elimination (3 h in H<sub>2</sub>O), the gel was stained with 1 μg/ml ethidium bromide and photographed under UV light. Depending on the mutation introduced, the assays were repeated two to three times independently.

*Oligonucleotide Radiolabeling*—The 52-mer oligonucleotide 5′-TGATATTCATTACTTTATTCC(T)30 containing a preferred cleavage recognition sequence for reverse gyrase (29) was obtained from MWG Biotech. 1 pmol of this oligonucleotide was end-labeled with T<sub>4</sub> polynucleotide kinase (Biolabs) and [γ-<sup>32</sup>P]ATP (3000 Ci/mm mol) in a final volume of 10 μl as recommended by the manufacturer. After 30 min of incubation at 37 °C, the reaction was stopped by heating at 80 °C for 15 min. Unincorporated nucleotides were removed by using the Qiaquick nucleotide removal kit (Qiagen).

*Electrophoretic Mobility Shift and Oligonucleotide Cleavage Assays*—0.025 pmol of the 5′-end-labeled oligonucleotide was incubated with the recombinant proteins in buffer containing 50 mM Tris·HCl, pH 8.0, 0.5 mM dithiothreitol, 30 μg/ml bovine serum albumin, 10 mM MgCl<sub>2</sub>, and 40 mM NaCl for 30 min at indicated temperatures in a final volume of 20 μl. The reaction mixture was then divided into two and treated as follows: 2.5 μl of a 5× loading buffer (10 mM Tris, pH 7.5, 20% glycerol, 1 mM EDTA, 0.1 mg/ml bovine serum albumin, 1 mg/ml xylene cyanol) was added to 10 μl of the reaction, and 5 μl were loaded onto a 6% native polyacryl-
single-stranded φX174 DNA was replaced by double-stranded φX174 DNA. The results presented are the means of three independent experiments.

RESULTS

Mutagenesis Strategy and Production of T. maritima Reverse Gyrase Mutants—The Archaeoglobus fulgidus reverse gyrase structure revealed the presence of two RecA like subdomains (H1 and H2) in the helicase domain (17). Within the helicase families both subdomains display conserved motifs that were subjected to mutagenesis (Fig. 1A). Motif Q found in the DEAD box helicases family may also be present in reverse gyrases. We therefore mutated Gln^{853} to Leu, because this residue could be the motif Q invariant glutamine in T. maritima reverse gyrase. Motifs I and II, from H1, are involved in ATP binding/hydrolysis in the SF1 and SF2 helicase families. To investigate the role of these motifs in reverse gyrase, we mutated the invariant lysine of motif I, GKT, to isoleucine and the two first aspartates of motif II, DDVD, to alanines. Motifs V and VI are located in the subdomain H2. Because the function of these motifs is unclear and seems to depend on the nature of the protein, we introduced mutations in both to evaluate their role in the overall enzyme activity (Fig. 1A). Motif V, RGVD, was changed to ALVA, and motif VI, GRSSR, was changed to LASSA. Finally, to analyze the role of the zinc finger motif conserved in the N-terminal part of the helicase domain of reverse gyrases, the first two cysteines of the motif CX_{2}CX_{14}CX_{2}, were mutated to alanines, and the putative active site tyrosine 851 (26) was replaced by a phenylalanine (Y851F mutant) to obtain an inactive form of the reverse gyrase. The wild type and seven mutant proteins were expressed in E. coli and purified to near homogeneity (Fig. 1B).

T. maritima Recombinant Reverse Gyrase Shares the Properties of Archaeal Reverse Gyrase—All of the reverse gyrase proteins used in this study are His-tagged at their C-terminal end, and we first examined the biochemical properties of the His-tagged wild type reverse gyrase from T. maritima. The results are presented on Fig. 2. We found that the supercoiling activity of the recombinant enzyme was dependent on temperature, as we reported for the natural reverse gyrase isolated from T. maritima cells (26). Reverse gyrase activity was monitored at 65 and 80 °C at different enzyme concentrations (Fig. 2A). At 65 °C a broad distribution of negatively and positively supercoiled DNA was produced, even at low enzyme concentration. At 80 °C, a more efficient positive supercoiling activity was observed. At this temperature, 0.01 pmol of enzyme was capable to transform 0.2 pmol of negatively supercoiled pTZ18 DNA into a ladder of positively supercoiled topoisomers (Fig. 2A). Comparison of reactions performed at 65 and 80 °C indi-
cated that temperature affects the overall activity of reverse gyrase (relaxation and positive supercoiling of DNA). The requirement for high temperature is common to all reverse gyrases. This is explained not only by the thermophilic nature of these enzymes but also by their preference for substrates that possess single-stranded regions, which are favored at high temperatures in negatively supercoiled DNA (21, 30, 31). We also verified that positive supercoiling produced by the recombinant *T. maritima* reverse gyrase was ATP-dependent. Indeed, we observed that without co-factor in the assay, the enzyme is totally inactive, even in relaxation (Fig. 2B). Replacement of ATP by the nonhydrolyzable ATP analog ADPNP3 led to a partial relaxation of negatively supercoiled pTZ18 DNA. No positive supercoiling was produced, attesting that the positive supercoiling activity of reverse gyrase is coupled to ATP hydrolysis (21, 32).

**All Mutations in Helicase Motifs Alter the Reverse Gyrase Activity**—The different mutant proteins were tested for their ability to produce positively supercoiled DNA (Fig. 3). As expected, we found that the Y851F mutant is totally inactive (Fig. 3A). Further analysis revealed that it was unable to perform the DNA cleavage reaction (see Fig. 6B), indicating that Tyr851 is likely involved in formation of the transient phospho-

*Fig. 2. Temperature and ATP dependence of positive supercoiling activity. A 0.2 pmol of pTZ18 DNA was incubated with increasing dilutions of enzyme in the presence of 1 μM ATP for 30 min at 65 or 80 °C, as indicated above the panels. Lanes 1–4 contained respectively 0.01 pmol, 5 fmol, 2.5 fmol, and 1.25 fmol of enzyme. The reaction products were visualized by one-dimensional agarose gel electrophoresis. Positive supercoiling activity was further analyzed by two-dimensional agarose gel electrophoresis (see “Experimental Procedures”); the left branch of the arch corresponds to negatively supercoiled topoisomers, and the right branch corresponds to positively supercoiled topoisomers. Fl, negatively supercoiled pTZ18 DNA. FII, nicked circular DNA present in the DNA preparations. C, control without enzyme. B, the assay using 0.01 pmol of reverse gyrase was performed without ATP or in the presence of 1 mM AMPNP as indicated above the panels.

**Fig. 3. Comparison of supercoiling activities of wild type and mutant reverse gyrase.** A, 0.2 pmol of pTZ18 DNA was incubated with 0.01 pmol of enzyme for 30 min at 80 °C in the presence of 1 μM ATP in a standard mixture. The reaction products were analyzed by two-dimensional gel electrophoresis as described in legend to Fig. 2. B, 0.2 pmol of pTZ18 DNA was incubated as in A with increasing dilutions of enzyme. Lanes 1–4 contained respectively 0.01 pmol, 5 fmol, 2.5 fmol, and 1.25 fmol of enzyme, and the reaction products were visualized by one-dimensional agarase gel electrophoresis. C, the assays were performed as in A, in the presence of three ATP concentrations. For each panel, the ATP concentration and nature of the mutation are indicated. WT, wild type; Zn, zinc finger motif.

3 The abbreviations used are: ADPNP, adenylylimidodiphosphate; ss, single-stranded; ATPγS, adenosine 5′-O-(thiotriphosphate).
Analysis of DNA Binding and Cleavage Properties of Wild Type T. maritima Reverse Gyrase—We analyzed the DNA binding and cleavage properties of the wild type enzyme to access the first two steps of the topoisomerization reaction. Previous results indicated that reverse gyrase preferably binds ssDNA (30, 31). We therefore used a 52-mer single-stranded oligonucleotide as substrate (Fig. 5A). This is composed of a T30-mer linked to a 22-mer fragment corresponding to the viral SSV1 DNA sequence that contains a preferred cleavage site for the *Sulfolobus shibatae* reverse gyrase (CATT↓ACTT) (29). The ability of the wild type reverse gyrase to bind and cleave this oligonucleotide was tested at 37, 65, and 80 °C (Fig. 5, B and C). The assay was performed at different reverse gyrase/DNA ratios (1, 4, or 16), and products were analyzed on a native polyacrylamide gel. As shown in Fig. 5B, the binding of reverse gyrase to the oligonucleotide is similar at 37 and 65 °C. As the amount of reverse gyrase increased, protein-DNA complexes that migrate with different electrophoretic mobilities were produced. At 80 °C, complex formation was reduced, indicating that reverse gyrase does not require high temperature for DNA binding, contrary to what we observed for the supercoiling activity (Fig. 2).

To analyze the cleavage step, half of the reaction products from the mobility shift experiments was loaded onto a denaturing 18% urea-polyacrylamide gel, after the addition of 1% SDS (final concentration) to the reaction mixture. The addition of SDS allows the release of the 5′ end-labeled fragments generated during cleavage. The results (Fig. 5C) show that the preferred site for cleavage generates a 24-nucleotide product at all temperatures, even at low protein concentration where only the CI complex is observed (Fig. 5B). At 37 and 65 °C for a protein-DNA ratio of 16, where the CI and CII complexes were formed (Fig. 5B), *T. maritima* reverse gyrase released two cleavage products, corresponding to fragments of 11 and 24 nucleotides (Fig. 5C). A minor cleavage site at position 19 was also observed. The CI and CII complexes observed (Fig. 5B) may therefore correspond respectively to one or two molecules of reverse gyrase bound to one oligonucleotide molecule.

All of the cleavage sites identified display a cytosine in position −4 (Fig. 5A). This preference was also shown for other reverse gyrase and type IA topoisomerases (29, 33) and confirms the close relation that exists between bacterial and archaeal reverse gyrase.

Mutations in the Zinc Finger Motif Change the Properties of DNA Binding and Cleavage of Reverse Gyrase—The binding and cleavage properties of the recombinant reverse gyrase mutants were analyzed at 65 °C (Fig. 6). The Y851F mutant binds the 52-mer oligonucleotide with the same efficiency as the wild type enzyme (Fig. 6A), although it is unable to cleave the oligonucleotide (Fig. 6B). This shows that in cleavage-proficient proteins the cleaved fragments remain tightly associated in the complexes, as was reported for type IA topoisomerases (34). Mutants of motifs I, II, and VI present the same efficiency as the wild type to bind (Fig. 6A) and cleave (Fig. 6B) the substrate, indicating that these motifs are not directly involved in the DNA binding and cleavage step of catalysis.

Analysis of the binding properties of motif V and Q mutants showed that they are slightly affected for CI complex formation (Fig. 6A). At low protein concentration they generated 32 and 51%, respectively, of CI complex as compared with wild type, and 76 and 85%, respectively, at the intermediate protein con-
Helicase-like Domain of T. maritima Reverse Gyrase

FIGURE 6. Binding and cleavage of single-stranded DNA by reverse gyrase mutants. The 52-mer oligonucleotide substrate is described in the legend to Fig. 5. A, electrophoresis mobility shift assay. The assays were conducted at 65 °C as described in the legend to Fig. 5. The amount of protein added in the reaction mixture was 0.025, 0.1, and 0.4 pmol for each protein set. The amount of complexes formed was quantified for each reaction (free DNA + bound DNA = 100%) and then normalized with complexes formed by wild type taken as 100%. B, analysis of the corresponding cleavage products. Half of the reaction mixtures corresponding to 0.4 pmol of protein in the assays of Fig. 6A were loaded on an 18% urea-polyacrylamide gel. M: 8–32 nucleotide ladder 5′ end-labeled. C, control without enzyme. WT, wild type; Zn, zinc finger motif.

concentration. No significant difference was observed at the highest protein concentration between these mutants and the wild type protein and resulted in the same cleavage profile as that observed for wild type (Fig. 6B).

Of all mutants, the zinc motif mutant is the most affected for DNA binding (Fig. 6A). At low and intermediate protein concentration, 10 and 16%, respectively, of CI complex were formed as compared with wild type. At the highest protein concentration, the amount of CI and CII complexes formed by the zinc mutant were comparable with the amount of complexes formed by wild type at the intermediate concentration, strongly suggesting that the zinc motif mutant has a decreased affinity for DNA. The zinc motif mutant retained some cleavage efficiency but was only able to produce the 24 nucleotides fragment (Fig. 6B), corresponding to the preferred cleavage site, a property consistent with a lower affinity for DNA. The zinc motif located at the N terminus of the helicase domain of reverse gyrase may therefore play an important role in DNA binding.

ATPase Activity of Wild Type and Mutant Reverse Gyrases—On the basis of the supercoiling and binding results, it appeared important to examine the ATPase activity of the reverse gyrase mutants compared with the wild type enzyme. We first determined the characteristics of the ATPase activity of the recombinant wild type reverse gyrase from T. maritima. Phosphate release was measured at 37, 65, and 80 °C in the presence of single-stranded φX174 DNA at different reverse gyrase concentrations. The results show an optimal ATPase activity at 65 °C and a less efficient ATPase activity at 80 °C (Fig. 7A), reinforcing the idea that the strong temperature dependence of the positive supercoiling reaction is likely due to the need for reverse gyrase to bind denatured regions of the plasmid (30, 31).

As previously described for archaeal reverse gyrases (21, 31), the ATPase activity of T. maritima reverse gyrase is strongly stimulated by ssDNA (Fig. 7B). Indeed, when double-stranded DNA replaced ssDNA in the reaction mixture, the ability of T. maritima reverse gyrase to hydrolyze ATP was significantly reduced to a level just above that in the absence of DNA. This preference for ssDNA in the ATPase activation is shared by most DNA helicases (24, 35). We then compared the ATPase activity of the reverse gyrase mutants with that of the wild type enzyme at 65 °C (Fig. 7C). Mutations in motifs I, II, V, and VI totally abolished the ATPase activity, and mutation in motif Q severely impaired this activity, showing that these motifs are involved in ATP binding or hydrolysis. One could expect that the only mutant altered in DNA binding would also be affected in ATP hydrolysis, because this process depends on DNA. However, we observed no change in ATPase activity for the zinc motif mutant. The larger DNA to protein ratio engaged in this experiment is indicated on the figure. For mutants I, II, V, and VI, the amount of Pi released was similar to that of the control without protein, and the different curves could not be distinguished.
activity. In any case, this result strongly suggests that the zinc finger motif is not involved in ATP hydrolysis, although it is important for binding to DNA.

**DISCUSSION**

We present in this paper the first report on the significance of helicase motifs and zinc finger motif present in the N-terminal part of a reverse gyrase (see Fig. 1). Several mutant proteins of *T. maritima* reverse gyrase were produced and tested for their ability to supercoil DNA, to bind and cleave DNA, and to hydrolyze ATP. A main conclusion from this mutational analysis is that all of the motifs that we studied are important for DNA supercoiling, attesting to the tight cooperation between the helicase and topoisomerase domains in the reverse gyrase activity.

We first investigated some properties of the recombinant wild type reverse gyrase from *T. maritima*. Like other reverse gyrases studied, the *T. maritima* enzyme positively supercoils DNA in an ATP-dependent manner. In the presence of a non-hydrolyzable ATP analog, the enzyme is only able to partially relax negatively supercoiled DNA. Introduction of positive supercoils is strictly dependent on ATP hydrolysis, although some positive supercoiling activity can be observed in the presence of any of the four NTPs. A recent study showed that reverse gyrase was also able to hydrolyze ATPγS to promote positive supercoiling of DNA (32). In the absence of ATP, *T. maritima* reverse gyrase cleaves DNA and shows a strong preference for cytosine in the position −4 of the cleavage site, as observed for other type IA topoisomerases (29, 33). Moreover, *T. maritima* reverse gyrase possesses an intrinsic ATPase activity that is strongly stimulated in the presence of ssDNA.

Our mutational analysis revealed that the zinc finger motif present in the N-terminal part of the helicase domain is important for DNA binding. This motif is found in all reverse gyrases (22), and its sequence, $CX_2CX_2CX_2C$, is similar to the consensus sequence of GATA transcription factors (36), but its role was unknown up to now. Here we show that replacement of the two first cysteines with alanines in the zinc motif of *T. maritima* reverse gyrase leads to a reduction in positive supercoiling activity compared with the wild type enzyme. A more detailed examination of the reaction revealed that the binding of single-stranded oligonucleotide to the mutant is significantly reduced, whereas its ATPase activity is unchanged. These results show that this zinc finger motif constitutes a DNA-binding site in the helicase domain.

The structural analysis of *A. fulgidus* reverse gyrase revealed the presence of two RecA-like subdomains (H1 and H2) in the helicase domain (17) with the signature helicase motifs located at the interface of the subdomains. Motifs I, II, and Q are located in subdomain H1 and motifs V and VI in subdomain H2. Motifs I and II are known to be important for ATP binding/hydrolysis in SF1 and SF2 helicases (24). It should be noted that the Asp-Glu residues conserved in motif II of SF1 and SF2 helicases were changed to Asp-Asp in all of the reverse gyrases (22), making these enzymes a new subgroup of the SF2 family. The ADPNP reverse gyrase co-crystal structure revealed that the conserved lysine of motif I interacts with the $\beta$ and $\gamma$ phosphates of ADPNP, and the two conserved aspartic acids of motif II interact with Mg$^{2+}$ bound to the nucleotide (17). Consistent with the structural data, our results provide evidence that these two motifs are required for ATP binding/hydrolysis. Indeed, mutations of the lysine in motif I to isoleucine and of the two first aspartates in motif II to alanine completely abolished the ATPase activity of *T. maritima* reverse gyrase. Whereas the mutant proteins were able to bind and cleave DNA like the wild type enzyme, they were unable to relax and supercoil DNA. The loss of relaxation activity suggests that mutations in motifs I or II impair ATP binding to the enzyme.

The role of motifs V and VI appears more variable among the different helicases of the SF1 and SF2 superfamilies. Their function is unclear and seems to be dependent on the nature of the enzymes studied. It was shown that mutations in motif V and motif VI can either alter the nucleic acid binding, NTP hydrolysis, or both (24, 25). Mutations in motif V or VI of *T. maritima* reverse gyrase led to mutant proteins unable to relax or positively supercoil DNA. Their DNA binding and cleavage abilities were unaffected, although they lost the ability to hydrolyze ATP. The sequences RGXD and GRXXR present in these two motifs are highly conserved among reverse gyrases (22). Our results show that motifs V and VI are essential for supercoiling activity of reverse gyrase through their involvement in ATP binding/hydrolysis. The co-crystal structure of *A. fulgidus* reverse gyrase with ADPNP (17) has shown that motifs V and VI are located in the H2 subdomain, which does not contact the nucleotide. In fact, it was proposed that upon ssDNA binding, H2 would move toward H1 to form the ATP hydrolysis pocket. The strong stimulation of ATPase activity by ssDNA observed with *T. maritima* (this paper) and *A. fulgidus* (21) reverse gyrases supports this hypothesis.

A detailed examination of reverse gyrase sequences allowed us to identify a highly conserved glutamine located upstream of motif I (Fig. 1) that could correspond to the invariant residue found in the Q motif of the DEAD box helicases (23, 37). A close analysis of the *A. fulgidus* reverse gyrase-ADPNP complex (17, 2) has shown that glutamine 61, corresponding to the Q motif, interacts with the N-7 of the adenine ring. The same interaction is observed in the co-crystal structure of the ATPase domain of *Saccharomyces cerevisiae* translation initiation factor 4A with ADP (38). To investigate the role of this glutamine in reverse gyrase activity, we analyzed the properties of the corresponding *T. maritima* reverse gyrase Q motif mutant. Replacement of glutamine 83 by leucine reduced the supercoiling activity of the enzyme, which appeared dependent on the ATP concentration. The ATPase activity of the mutant was also impaired, indicating the important role of this glutamine in the control of ATP binding/hydrolysis. Interestingly, the mutation introduced changed the nucleotide selectivity because CTP appeared to be a co-factor as efficient as ATP, likely meaning that the glutamine of the Q motif is involved in recognition of the adenine ring.

In summary, we have shown that the role of helicase motifs present in the N-terminal part of the *T. maritima* reverse gyrase is intimately linked to the supercoiling activity of the enzyme. Motifs I, II, V, VI, and Q are mainly involved in ATP binding and/or hydrolysis, whereas the zinc motif is important for DNA binding. However, the precise role of ATP in the reverse gyrase
mechanism still has to be solved. It was shown that reverse gyrase does not possess the strand displacement activity characteristic of true helicases (39). The same was observed for other proteins (E. coli Mfd, human Rad54, and human RecQ4) that possess conserved helicase motifs but have no helicase activity (40–42). In the absence of ATP, reverse gyrase is unable to relax and positively supercoil DNA. In the presence of the nonhydrolyzable ATP analog ADP•P, it is only able to relax negatively supercoiled DNA, yet with a very low efficiency (this work and Ref. 21). ADP also allows relaxation of negative supercoils (32), whereas a very low level of positive supercoiling was reported (43). Therefore, ATP hydrolysis is not essential for the relaxation step, although binding of the nucleotide to the enzyme is required, suggesting that conformational changes occur when the nucleotide is bound. However, ATP hydrolysis is essential to generate positive supercoils in DNA, a reaction that is energetically unfavorable. It was proposed that nucleotide hydrolysis would force reverse gyrase to perform controlled strand passage toward increasing linking number, via conformational changes of the protein (17). More recent studies (43) suggested that reverse gyrase has a structure-specific affinity for single- or double-stranded DNA depending on its association with ATP or ADP and that this distinct affinity would control the directionality of DNA supercoiling. Our results suggest that the conformational changes induced by ATP binding are required to perform the DNA strand passage step. However, many questions remain open, and further work will be necessary to elucidate the exact role of ATP hydrolysis in DNA positive supercoiling.

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