Reverse gyrase reanneals denatured DNA and induces positive supercoils in DNA, an activity that is critical for life at very high temperatures. Positive supercoiling occurs by a poorly understood mechanism involving the coordination of a topoisomerase domain and a helicase-like domain. In the parasitic archaeon *Nanoarchaeum equitans*, these domains occur as separate subunits. We express the subunits, and characterize them both in isolation and as a heterodimer. Each subunit tightly associates and interacts with the other. The topoisomerase subunit enhances the catalytic specificity of the DNA-dependent ATPase activity of the helicase-like subunit, and the helicase-like subunit inhibits the relaxation activity of the topoisomerase subunit while promoting positive supercoiling. DNA binding preference for both single- and double-stranded DNA is partitioned between the subunits. Based on a sensitive topological shift assay, the binding preference of helicase-like subunit for underwound DNA is modulated by its binding with ATP cofactor. These results provide new insight into the mechanism of positive supercoil induction by reverse gyrase.

In the most inhospitable environments, life can survive and even thrive. A striking example of this adaptability is that of hyperthermophilic organisms, which have optimal growth conditions at temperatures of 80 °C or higher (1). Life at such extreme temperatures presents unique challenges, which include the propensity for double-stranded DNA to denature and a host of other potential genomic defects (reviewed in Refs. 2 and 3).

Hyperthermophiles, whether bacterial or archaeal, deal with the thermal instability of DNA by means of an enzyme called reverse gyrase (reviewed recently in Refs. 4, 5, and 6). Since its discovery in a hyperthermophilic archaeon (7), there is growing evidence suggesting a role of reverse gyrase in stabilizing genomes in hyperthermophiles. It appears to be the only gene specific to hyperthermophiles (8) and is present in all hyperthermophiles and in some thermophiles as well (9).

This unique function of reverse gyrase is further supported by genetic evidence. Although reverse gyrase is dispensable for growth of *Thermococcus kodakaraensis* below 65 °C, the strain without reverse gyrase showed retarded growth between 65–90 °C, and no growth at 93 °C (10), demonstrating the essential role of reverse gyrase in supporting life at extreme temperatures.

Reverse gyrase is a type IA topoisomerase and has a unique enzymatic activity in utilizing ATP hydrolysis to induce positive supercoils in DNA. It has not been established precisely how this enzyme can protect against DNA thermal instability. DNA positive supercoiling *per se* may not be the direct cause, because supercoiling in hyperthermophiles is highly variable ranging from positively supercoiled to relaxed or even negatively supercoiled (11). The biochemical activity of reverse gyrase as a renaturase for single-stranded DNA may have a critical role in maintaining genome stability at high temperature (12). In addition, reverse gyrase can promote DNA integrity through its role as a DNA chaperone to coat the damaged sites (13).

Reverse gyrase carries out directional strand transfer leading to an increase in linking number, rendering it capable of introducing positive supercoils to plasmid DNA and annealing complementary single-stranded circles. Although the biochemical mechanism for directional strand transfer remains unclear, the structural biological and biochemical studies on the enzyme provide important insight. Reverse gyrase is composed of a superfamily II helicase-like domain at its N-terminal half linked to a type IA topoisomerase domain (9, 14). The crystal structure of *Archaeoglobus fulgidus* reverse gyrase indicates that these domains are arranged back-to-back with the active site of each domain facing away from its counterpart (15, 16). When isolated recombinantly, the topoisomerase domain of *Sulfolobus acidocaldarius* reverse gyrase behaves like a type IA topoisomerase and weakly relaxes negatively supercoiled DNA but is unable to induce positive supercoils. The helicase-like domain hydrolyzes ATP but does not demonstrate helicase activity. Combining the separately expressed recombinant topoisomerase and helicase-like domains results in positive supercoiling, indicating that the domains are able to reconstitute reverse gyrase *in vitro*. Positive supercoiling does not occur when a noncognate topoisomerase or helicase is substituted for the respective domain (17). Thus, the helicase-like and topoisomerase domains of reverse gyrase are unique and can specifically cooperate to induce positive supercoils. One proposed mechanism for positive supercoiling is that a switch in the binding affinity of the helicase-like do-
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main depends on its bound nucleotide (18). This switch in DNA binding is intimately coupled with the strand passage activity of the topoisomerase domain, thus resulting in reannealing of single stranded DNA and increasing the linking number (4, 12).

The hyperthermophilic archaeal parasite *Nanoarchaeum equitans* (19) does not have a single reverse gyrase gene. Instead, further analysis of its genome reveals two separate genes encoding the apparent topoisomerase IA and helicase-like domains of reverse gyrase (9, 20). This raises the possibility that what in other hyperthermophiles are two domains of a single peptide, in *N. equitans* are distinct subunits of a multi-protein complex. In this work, we separately express each protein and biochemically characterize them in isolation and in combination with each other. We establish that they form a heterodimer in solution, which is able to induce positive supercoils and is thus a functional reverse gyrase. The unique nature of the reverse gyrase of *N. equitans* allows analysis of each subunit in isolation while avoiding potential complications due to artificial separation of the subunits. We exploit this to dissect the contribution of each subunit/domain to positive supercoiling.

EXPERIMENTAL PROCEDURES

Sequence Alignment—Sequence alignments were performed using CLC Sequence Viewer (version 6.3) from CLC Bio A/S.

Expression and Purification—The culturing of *N. equitans* and isolation of its genomic DNA was described earlier (20). The topoisomerase subunit (NEQ318) was cloned from *N. equitans* genomic DNA by PCR amplification and placed in the pET-23b (Novagen) vector with a C-terminal hexahistidine tag. The resulting protein was expressed in *Escherichia coli* BL21-CodonPlus-RII (DE3, pLysS) cells (Stratagene) and grown in Luria Broth modified for a final NaCl concentration of 20 g/liter. Cells were induced with 1 mM isopropyl thiogalactopyranoside for 6 h at 30 °C. Pelleted cells were resuspended by Dounce homogenization in lysis buffer (1 mg/ml lysozyme, 20 mM Tris-HCl, pH 8.0, and 0.5 M NaCl). The lysate was incubated at 25 °C for 20 min and then sonicated. The soluble fraction was isolated by centrifugation at 12,000 *g* and set aside. The pelleted fraction was re-extracted according to the above procedure, and the resulting supernatant was loaded on a Bio-Rex 70 column (Bio-Rad) with buffer containing 15 mM NaPi, 10% glycerol. Fractions containing the topoisomerase subunit was washed with buffer containing 15 mM NaPi, 10% glycerol. Fractions containing the topoisomerase subunit were collected and labeled as fraction III. This was loaded on a hydroxyapatite column (Bio-Rad), which was washed with 0.1 M NaPi, and 0.5 M NaCl. Protein was manually eluted with buffer containing 0.5 M NaPi, and 0.5 M NaCl. Fractions containing the purified recombinant protein were pooled and labeled as fraction IV. Fraction IV was dialyzed overnight in 50% glycerol, 15 mM NaPi, 1 M NaCl, and 0.1 mM dithiothreitol and stored at −20 °C.

The cloning and expression of the helicase-like subunit (NEQ434) was the same as that of the topoisomerase subunit with the following differences. The helicase-like subunit was expressed in *E. coli* Rosetta (DE3, pLysS) cells (Novagen). Following lysis and isolation of the soluble fraction, fraction I was diluted 10-fold to a final salt concentration of 0.1 M NaCl. The column order was altered, with Bio-Rex 70 as the first column and the Ni²⁺ immobilized metal ion affinity chromatography column as the second. The Bio-Rex 70 column was washed with buffer containing 0.5 M NaCl and was eluted with a 0.5–1.5 M NaCl gradient. Coexpression of NEQ434 and NEQ318 in *E. coli* cells was accomplished by the pETDuet-1 vector (Novagen), and purification of the heterodimeric protein complex followed essentially the same protocols described for purifying NEQ318 protein.

Dimerization Assays—Gel filtration assays were conducted using a prepacked Superdex 200 Precision Column 2.3/30 (Amersham Biosciences), on an AKTA FPLC purifier system (Amersham Biosciences). To calibrate the column, the following proteins were used as column standards: thyroglobulin (with a Stokes radius of 86 Å, Sigma); β-galactosidase (69 Å, Boehringer Mannheim); type I horse spleen ferritin (63 Å, Sigma); lactate dehydrogenase (41 Å, Worthington); and bovine serum albumin (35 Å, Pentex). All protein samples were between 10 and 15 μg and in a buffer of 15 mM Tris-HCl and 0.5 M NaCl. The Stokes radii of the individual subunits and the reconstituted holoenzyme were calculated based on the characteristic retention patterns of the standard proteins.

Sedimentation-equilibrium analyses of NEQ434 and NEQ318 separately and in mixtures were performed on a Beckman XL-A ultracentrifuge by standard procedures. Samples with a range of concentrations from 1–10 μM were run at 6,000, 8,000, and 10,000 rpm and followed by absorbance at 275 nm. Best fit molecular weights were calculated by the Ideal I program from Beckman.

Supercoiling Assays—Reactions contained 0.45 μg of pUC19 DNA, 10 mM Tris-HCl, pH 8.0, 50 mM KCl, 10 mM MgCl₂, 0.1 mM EDTA, 50 μg/ml gelatin, 1 mM ATP, and the indicated concentrations of topoisomerase and helicase-like subunits in a total volume of 30 μl. A drop of mineral oil was added to cover the surface of the solution to prevent evaporation. Reactions were initiated by immersing the reaction tube in a water bath set at 80 °C, and terminated by adding EDTA and SDS to a final concentration of 10 mM and 0.1%, respectively. Sucrose and tracking dyes (bromphenol blue and xylene cyanol) were added to a concentration of 5% and 100 μg/ml, respectively. Time course reactions were carried out in separate aliquots for each time point, due to the difficulty of manipulating the reaction mixture at high temperature. Time
points used were 3.5, 7, 15, and 30 min. DNA products were analyzed by agarose gel electrophoresis in the presence of 30 μM chloroquine as outlined previously (21).

Topology Shift Assays—Topology shift assays were carried out under conditions similar to supercoiling assays with the following modifications. *Thermatoga maritima* topoisomerase I was purified and relaxation activity assayed as described previously (21) and was included at a concentration of 7 units per reaction. The helicase-like subunit only was used to allow observation of the activity of that subunit rather than the supercoiling of the reconstituted holoenzyme. Adenine cofactor was included at a concentration of 1 mM, as indicated.

**ATP Hydrolysis**—Reconstituted holoenzyme or helicase-like subunit was incubated at a concentration of 25 nM with 0.5 mM ATP (2.1 nM [γ-33P]ATP (PerkinElmer Life Sciences) at 80 °C in 10 mM Tris-HCl, pH 7.9, 50 mM KCl, 0.1 mM EDTA, 10 mM MgCl₂, 50 μg/ml BSA, and 1 mM dithiothreitol, in a total volume of 20 μl. To determine DNA dependence, reactions were done in the presence or absence of 56 μg/ml pBS/SC² single-stranded circular DNA. Reactions were covered with a drop of mineral oil to prevent evaporation. Time points were taken between 0 and 16 min, as indicated. Time courses were conducted and analyzed by thin layer chromatography as described previously (22).

To determine reaction velocity, reactions were done with 12 nM reconstituted holoenzyme or 427 nM helicase-like subunit and the indicated concentration of ATP (maintaining a constant concentration of 4.2 nM [γ-33P]ATP) in the presence of 50 μg/ml pBS/SC² single-stranded circular DNA. The timepoints used were 0, 5, 10, and 15 min or 0, 2.5, 5, and 10 min for the reconstituted holoenzyme, and 2.5, 5, 7.5, and 10 min for the helicase-like subunit. Velocity for each timecourse was determined by calculating the line of best fit, and at least three independent velocities were averaged together for each ATP concentration. Averaged velocities determined in the absence of enzyme were subtracted from enzymatic velocities as a background control.

**Electrophoretic Mobility Shift Assays**—For gel electrophoretic mobility shift assays, 0.1 μM enzyme as specified, or an equal volume of storage buffer, was incubated with 0.25 nM radiolabeled oligonucleotide substrate for 30 min at 80 °C in 10 mM Tris-HCl, pH 7.9, 50 mM KCl, 10 mM MgCl₂, and 50 μg/ml BSA in a total reaction volume of 20 μl. For assays examining nucleotide cofactor modulation of binding preference, 0.5 mM nucleotide cofactor (ADP, ATP, or AMP, as indicated) was included in the reaction mixture. After 30 min, 4 μl of non-denaturing loading mix was added to each reaction to a final concentration of 5% sucrose, 33 μg/ml bromphenol blue, and 33 μg/ml xylene cyanol. Reaction products were separated on an 89 mm Tris borate, 1 mM MgCl₂, 8% polyacrylamide gel by electrophoresis similar to the conditions described earlier (22) and subjected to phosphorimaging analysis.

Oligonucleotide substrates were prepared as described earlier (22). Sequences are included in supplemental Table 1.

**RESULTS**

**Identification of the Subunits of Reverse Gyrase in *N. equitans*—**Most of the reverse gyrases consist of a single peptide containing a type IA topoisomerase domain and a helicase-like domain. No such single gene is found in *N. equitans*, but genomic sequence analysis reveals two genes, *NEQ318* and *NEQ434*, whose protein products show significant homology to the topoisomerase and helicase-like domains of reverse gyrase, respectively (Fig. 1A). The protein encoded by *NEQ318* is 66% similar (47% identical) to the topoisomerase domain of *A. fulgidus* reverse gyrase. That encoded by *NEQ434* is 58% similar (35% identical) to the corresponding helicase-like domain. It is interesting to note that splitting genes into separately expressing proteins is not unusual in the *N. equitans* genome, and the organism possesses split genes for both a type IA topoisomerase unrelated to reverse gyrase (*NEQ045* and *NEQ324*) and a type II topoisomerase (*NEQ144* and *NEQ542*). Although it is plausible to expect that the products of *NEQ318* and *NEQ434* dimerize to form a reverse gyrase holoenzyme, direct experimental evidence is requisite to demonstrate that they are genes for reverse gyrase. We present in the following sections biochemical evidence that the purified recombinant proteins can combine to reconstitute reverse gyrase holoenzyme. Hereafter, the products of *NEQ318* and *NEQ434* will be referred to as the topoisomerase and helicase-like subunits of *N. equitans* reverse gyrase.

**Expression and Purification of Subunits in Isolation**—The topoisomerase and helicase-like subunits of *N. equitans* reverse gyrase were expressed separately in *E. coli*. The C terminus of the topoisomerase subunit was fused with a hexahistidine tag and expressed in CodonPlus-RIL *E. coli* cells (Fig. 1B, lane 1). A three-step purification strategy was adopted. This consisted of nickel-nitrilotriacetic acid affinity chromatography (Fig. 1B, lane 2), Bio-Rex 70 cation exchange (Fig. 1B, lane 3), and hydroxyapatite chromatography (Fig. 1B, lane 4). The final enzyme was at >91% purity and stored in 50% glycerol, 15 mM sodium phosphate, pH 7.0, 1 mM NaCl, and 0.1 mM dithiothreitol at −20 °C.

The C terminus of the helicase-like subunit was also fused with a hexahistidine tag. The resulting recombinant gene was expressed in Rosetta *E. coli* cells (Fig. 1B, lane 5). A three-step purification strategy similar to that used for the topoisomerase subunit was employed, the primary difference being the reordering of columns used. Thus, the final strategy for purifying the helicase-like subunit was Bio-Rex 70 cation exchange (Fig. 1B, lane 6), followed by nickel-nitrilotriacetic acid affinity chromatography (Fig. 1B, lane 7), and lastly, hydroxyapatite chromatography (Fig. 1B, lane 8). The final enzyme was at >92% purity and stored in 50% glycerol, 15 mM sodium phosphate, pH 7.0, 1 mM NaCl, and 0.1 mM dithiothreitol at −20 °C.

**Topoisomerase and Helicase-like Subunits Dimerize to Form Reverse Gyrase**—To experimentally establish the predicted dimerization between the topoisomerase and helicase-like subunits to reconstitute reverse gyrase, size exclusion chromatography was conducted on each subunit and the *in vitro*
reconstituted holoenzyme. Each of the samples was eluted from the column as a single protein peak. Calculating the Stokes radius of each based on elution volume with respect to marker proteins leads to the following predicted radii: topoisomerase subunit, 35 Å; helicase-like subunit, 26 Å; and reconstituted holoenzyme, 48 Å (Fig. 2D). If the Stokes radius is instead calculated using the partition coefficient, the following radii are predicted: topoisomerase subunit, 35 Å; helicase-like subunit, 21 Å; and reconstituted holoenzyme, 49 Å (data not shown). The radii derived from each method of calculation are in reasonable agreement with each other and are consistent with the topoisomerase and helicase-like subunits forming a single globular mass when combined in solution.

To confirm that a heterodimer is formed by the two subunits in solution, sedimentation-equilibrium analysis was conducted on each subunit and the reconstituted holoenzyme. Sedimentation equilibrium experiments are based on the principles of thermodynamics, rather than hydrodynamics, and thus, the molecular weight determination is independent of any reference proteins. Analysis of such experiments resulted in a calculated molecular mass of 74 kDa for the helicase-like subunit (Fig. 2A), 89 kDa for the topoisomerase subunit (Fig. 2B), and 155 kDa for the reconstituted holoenzyme (Fig. 2C). These values are comparable with those predicted by the amino acid sequence (69, 82, and 151 kDa, respectively). Furthermore, the calculated mass of the reconstituted holoenzyme is consistent with the formation of a heterodimer from a topoisomerase subunit and a helicase-like subunit and suggests that the formation of this heterodimer is rapid and nearly complete given an equimolar concentration of the two subunits. Thus, two independent assays indicate that the topoisomerase and helicase-like subunits combine to form a heterodimeric holoenzyme, confirming predictions from sequence alignments. The consistent results from both methods also suggest that the predominant species in solution is the heterodimer. The concentrations of protein samples used in the sedimentation equilibrium analysis are in the 10⁻⁶ M range or less (Fig. 2 and data not shown), indicating a dimerization constant significantly lower than 10⁻⁶ M.

**Coordination between the Topoisomerase and Helicase-like Subunits: Supercoiling**—Positive supercoiling by reverse gyrase requires the active participation of each domain. The energy from ATP hydrolysis, occurring in the helicase-like domain, is coordinated with the strand passage activity mediated by the topoisomerase domain, to generate an increase in the linking number and thus positive supercoiling. To confirm that the holoenzyme reconstituted from separately expressed subunits retains such an activity, we assayed the supercoiling activity of the reconstituted holoenzyme.

**FIGURE 1. Sequence and purification of the subunits of** N. equitans reverse gyrase. A, the amino acid sequences of the protein products of N. equitans genes 318 and 434 were aligned with that of A. fulgidus reverse gyrase. Identical residues are indicated by an asterisk, and similar residues are indicated by a period. The N-terminal helicase domain of A. fulgidus reverse gyrase is 35% identical (58% similar) to NEQ434. The C-terminal topoisomerase domain is 47% identical (66% similar) to NEQ318. B, gel outlining the three-step purification of NEQ318 and NEQ434. Lanes 1 and 5 are the soluble portions of the respective whole cell lysates (fraction I). Lanes 2 and 6 are the pooled peak fractions of the first columns employed (fraction II), which are a Ni²⁺ immobilized metal ion affinity chromatography column for NEQ318 and a Bio-Rex 70 column for NEQ434. Lanes 3 and 7 are the pooled peak fractions of the second columns employed (fraction III), which are a Bio-Rex 70 column for NEQ318 and a Ni²⁺ immobilized metal ion affinity chromatography column for NEQ434. Lanes 4 and 8 are the dialyzed pooled peak fractions from a hydroxyapatite column (fraction IV).
percoiling activity of the topoisomerase domain and its complex with the helicase-like domain. Time courses were conducted by incubating negatively supercoiled plasmid DNA with the topoisomerase subunit and varying amounts of the helicase-like subunit. The DNA products were analyzed by agarose gel electrophoresis in the presence of chloroquine (Fig. 3). With such gel electrophoretic analysis, DNA species with positive/negative supercoiling or in the relaxed state can be distinguished. Densitometric analysis of these species at the 3.5- and 30-min time points indicates that the initial time point is generally reflective of the early stages of the reaction (supplemental Fig. 1). In the absence of helicase-like subunit, the DNA is quickly relaxed by the topoisomerase subunit, with the reaction nearly complete at the first time point taken at 3.5 min (Fig. 3, lanes 1–4). At a helicase-like to topoisomerase subunit ratio of 0.5:1, relaxed and positively supercoiled DNA species are observed (Fig. 3, lanes 5–8). Some positively supercoiled species are observed at the initial time point, indicating rapid positive supercoiling and confirming that the heterodimeric reconstituted holoenzyme is an active reverse gyrase. Negatively supercoiled species are also present, unlike reactions done in the absence of the helicase-like subunit (Fig. 3, compare lanes 1 and 5). Thus, the helicase-like subunit reduces the relaxation activity of the topoisomerase subunit while allowing positive supercoiling activity. At a helicase-like to topoisomerase subunit ratio of 1:1 (Fig. 3, lanes 8–12), rapid positive supercoiling is observed; relaxed species are not observed, but a significant amount of negatively supercoiled species are observed. The retention of nega-
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tive supercoils and absence of relaxed species confirms the trend seen at the 0.5:1 ratio. Because the relaxed DNA is an intermediate formed while positively supercoiling the negatively supercoiled substrate, the absence of relaxed species suggests that the helicase-like subunit sequesters the topoisomerase subunit, causing it to switch from mere relaxation to processive positive supercoiling activity. The DNA binding affinity of reverse gyrase is enhanced, comparing with that of the topoisomerase subunit. The two subunits can thus undergo multiple strand passage events per DNA binding event, and this occurs even under high salt conditions that should favor DNA dissociation (data not shown). Supercoiling rates from helicase-like to topoisomerase subunit ratios greater than 1:1 (Fig. 3, lanes 13–20) are not significantly different from those at 1:1. This confirms that the dimeric interaction between the two subunits is tight and that they do not readily dissociate from each other. Therefore, the topoisomerase subunit of N. equitans reverse gyrase, when isolated, acts as a topoisomerase capable of relaxing supercoiled DNA. When the topoisomerase subunit is incubated with the helicase-like subunit, supercoiler reaction activity is diminished. The two subunits dimerize tightly from those at 1:1. This confirms that the dimeric interaction between the two subunits is tight and that they do not readily dissociate from each other.

Coordination between Topoisomerase and Helicase-like Subunits, ATP Hydrolysis—The helicase-like subunit acts to switch the function of the topoisomerase subunit from relaxing supercoiled DNA to positive supercoiling induction. Further examination of the nature of the contribution of the helicase-like subunit to positive supercoiling requires analysis of its activity in isolation and in the presence of the topoisomerase subunit. The ability to unwind DNA has never been directly observed for the reverse gyrase helicase-like domain. However, other studies have shown it to hydrolyze ATP in a DNA-dependent manner. This, then, provides a convenient means of examining the effect of the topoisomerase subunit on the helicase-like subunit.

To that end, ATP hydrolysis was assayed for the helicase-like subunit in isolation and in the context of the reconstituted holoenzyme. ATP hydrolysis, regardless of context, required the presence of DNA (Fig. 4A). The holoenzyme was found to hydrolyze ATP at a significantly faster rate than the helicase-like subunit alone. The enzymological basis for the rate enhancement was further dissected by determining the Michaelis-Menten parameters, $k_{cat}$, $K_m$, and catalytic specificity $k_{cat}/K_m$. Kinetic analysis of ATP hydrolysis found the helicase-like subunit to have a catalytic specificity of 16 mol$^{-1}$ s$^{-1}$ (Fig. 4B). The reconstituted holoenzyme, on the other hand, has a catalytic specificity of 105 mol$^{-1}$ s$^{-1}$, due to both a 2.6-fold increase in $k_{cat}$ and a 2.6-fold decrease in $K_m$ of the enzyme for ATP. This indicates that the topoisomerase subunit significantly improves the ability of the helicase-like subunit to hydrolyze ATP. Because the effect is seen in both $k_{cat}$ and $K_m$, the activity enhancement is due to an increase in both the catalytic efficiency of ATP hydrolysis and binding affinity for ATP.

DNA Binding Preferences of Each Subunit and Reconstituted Holoenzyme—To further dissect the contribution made by each subunit to supercoiling, the DNA binding preference of each individual subunit and the reconstituted holoenzyme were examined. Electrophoretic mobility shift assays were conducted with single-stranded and double-stranded oligonucleotide substrates at 80 °C (with the double-stranded substrates being designed to have a melting temperature of

FIGURE 4. The helicase-like subunit ATPase activity is stimulated by the topoisomerase subunit. A, time courses of ATP hydrolysis by the helicase-like subunit (squares, dashed lines) and the reconstituted reverse gyrase (circles, solid lines) in the presence or absence of DNA (filled and open symbols, respectively). Background ATP hydrolysis (in the absence of enzyme) is indicated by X. Hydrolysis in the absence of DNA is by either enzyme is not significantly above background levels. In the presence of DNA, reconstituted reverse gyrase is more active than the helicase-like subunit alone. B, measurement of the velocity of ATP hydrolysis by the helicase-like subunit (open squares) and the reconstituted reverse gyrase (filled squares) as a function of ATP concentrations. The Michaelis-Menten parameters determined from these data are: $k_{cat} = 7.99$ mmol ATP hydrolyzed/(mmol enzyme × s), $K_m = 76.4$ μM for the holoenzyme; and $k_{cat} = 3.12$ mmol ATP hydrolyzed/(mmol enzyme × s), $K_m = 197$ μM for the helicase-like subunit. The reconstituted holoenzyme has a higher maximal velocity and a lower $K_m$, indicating that it has an improved catalytic specificity over the helicase-like subunit alone. Error bars indicate S.D. Each data point represents at least three independent trials.
90 °C. The reconstituted holoenzyme showed a slight preference for double-stranded DNA over single-stranded DNA (Fig. 5A, quantified in Fig. 5B). The helicase-like subunit exhibited a preference for double-stranded DNA, whereas the topoisomerase subunit preferred single-stranded DNA. Similar trends in DNA binding affinities were observed with lower enzyme/DNA ratios or when data were converted to $K_D$ measurements (data not shown). Neither subunit bound DNA as well as the reconstituted holoenzyme, which is consistent with the tight coupling and coordination between these two subunits. The biochemical function of reverse gyrase as a DNA renaturase suggests that it must have a dual binding affinity for both single- and double-stranded DNA. The separation of substrate preference between subunits indicates a similar segregation in the reconstituted holoenzyme. This has important implications for the mechanism of inducing positive supercoils.

Helicase activity has not been observed for either the reconstituted enzyme or the isolated helicase-like subunit (data not shown). This is consistent with other published results (17) and confirms that although the helicase-like subunit demonstrates DNA-dependent ATP hydrolysis, it does not have strand translocation or separation activities. It is possible that ATP hydrolysis is employed in positive supercoiling to modulate DNA substrate affinity. To test this, electrophoretic mobility shift assays were conducted for the helicase-like subunit and the reconstituted holoenzyme, which both single-and double-stranded DNA substrates, varying the nucleotide cofactor (no cofactor; ADP; ATP; and the nonhydrolyzable ATP analog, AMPPNP). Varying nucleotide cofactor did not significantly alter binding affinity for either the helicase-like subunit or the reconstituted holoenzyme (supplemental Fig. 2). If the phosphorylation state of the nucleotide cofactor has any effect on substrate affinities, it is possible that they are too small or too transient to be detected by electrophoretic mobility shift assay.

To detect subtle changes due to nucleotide cofactor, a topology shift assay was employed. In this assay, reactions with negatively supercoiled plasmid DNA were done in the presence of $T. maritima$ topoisomerase I, which relaxes the plasmid unless an induced topological shift interferes. The extent of the induced shift correlates precisely with variation in the extent of plasmid relaxation. The effect of helicase-like subunit binding to plasmid DNA was thus assayed in the presence of ADP, ATP, AMPPNP, or the absence of nucleotide cofactor. Shifts were evident in the absence of cofactor, or in the presence of AMPPNP, indicating that under these conditions the helicase-like subunit alters the topology of DNA. These shifts are not seen in the presence of ADP or ATP (which is readily hydrolyzed to form ADP), indicating that ATP hydrolysis modulates the interaction of the helicase-like subunit with DNA. The DNA species generated without any helicase-like domain (lanes 1–4) mark the positions for relaxed species under these reaction conditions. Increasing amounts of helicase-like domain generate DNA species with more negative supercoils (compare lanes 9 and 12 with lanes 5 and 8). We have also run the identical samples in an agarose gel with chloroquine to confirm that the DNA species with higher mobility shown here are more negatively supercoiled (not shown).

![FIGURE 5. DNA substrate preference is partitioned between subunits. A, a representative electrophoretic mobility shift assay of each subunit and reconstituted reverse gyrase with single-stranded and double-stranded oligonucleotide substrates (lanes 1–4 and lanes 5–8, respectively). Reverse gyrase binds either substrate well (lanes 4 and 8). The topoisomerase subunit strongly prefers single-stranded (ssDNA) substrates (lanes 2 and 6), and the helicase-like subunit strongly prefers double-stranded substrates (lanes 3 and 7). B, quantification of electrophoretic mobility shift assays. Error bars indicate S.D. (n = 3).](image)

![FIGURE 6. Nucleotide cofactor modulates helicase-like subunit binding. Topology shift assays were done with $T. maritima$ topoisomerase I and the helicase-like subunit in the presence of ADP, ATP, AMPPNP, or the absence of nucleotide cofactor. Shifts were evident in the absence of cofactor, or in the presence of AMPPNP, indicating that under these conditions the helicase-like subunit alters the topology of DNA. These shifts are not seen in the presence of ADP or ATP (which is readily hydrolyzed to form ADP), indicating that ATP hydrolysis modulates the interaction of the helicase-like subunit with DNA. The DNA species generated without any helicase-like domain (lanes 1–4) mark the positions for relaxed species under these reaction conditions. Increasing amounts of helicase-like domain generate DNA species with more negative supercoils (compare lanes 9 and 12 with lanes 5 and 8). We have also run the identical samples in an agarose gel with chloroquine to confirm that the DNA species with higher mobility shown here are more negatively supercoiled (not shown).](image)
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Species generated without helicase-like subunit (lanes 1–4), it is apparent from the topological shift experiments that the binding of the subunit with ADP as a cofactor does not result in DNA unwinding. The difference in topological shift between substrates bound in the presence of AMPPNP and ADP (Fig. 6, lanes 12 and 10) indicates that there is a modulation in binding with the underwound DNA, resulting from ATP binding and hydrolysis.

**DISCUSSION**

We have established that the genes NEQ318 and NEQ434, found in N. equitans, encode the topoisomerase and helicase-like subunits, respectively, of a heterodimeric reverse gyrase. Most reverse gyrases are single polypeptide chains with the helicase-like domain in the N-terminal half and topoisomerase domain in the C-terminal half. Previously, the only known exception was the reverse gyrase from Methanopyrus kandleri. It is a heterodimer with part of the topoisomerase domain inserted into the helicase-like domain (23). However, the reverse gyrase from N. equitans is the only one encoded by separate genes that cleanly split the helicase-like domain from the topoisomerase domain. We showed here that the purified recombinant subunits associate in a 1:1 ratio and work together to induce positive supercoils. The topoisomerase subunit is able to act on its own to relax negative supercoils. Similarly, the helicase-like subunit in the absence of the topoisomerase subunit acts as a DNA-dependent ATPase, though it does not actually unwind DNA. Characterizing these activities as functions of the respective isolated subunit and of the reconstituted holoenzyme has allowed insight into the mechanism of positive supercoiling by reverse gyrase.

Positive supercoiling is not a function of the simple combination of the separate activities of each subunit. Each subunit alters the activity of its counterpart when associated. In the case of the ATP hydrolysis activity of the helicase-like subunit, the association of topoisomerase domain results in an enhancement in catalytic specificity by increasing substrate affinity and turnover rate. The supercoil relaxation activity of the topoisomerase subunit, on the other hand, is reduced by the presence of the helicase-like subunit. Along with this partial inhibition of supercoil relaxation activity by the topoisomerase subunit, there is also a concomitant increase in the processivity of the positive supercoiling. The holoenzyme does not rapidly relax the population of negatively supercoiled DNA and then induce positive supercoils. Instead, it relaxes and positively supercoils a given DNA molecule in a single binding event, before dissociating and working on the next negatively supercoiled molecule. As a result, incomplete reactions contain both negatively and positively supercoiled DNA, but little (if any) relaxed DNA. Although processive positive supercoiling is an efficient means for reverse gyrase reactions, such a mode of reaction may not be universally adopted by all reverse gyrases. For example, the kinetic analysis of the supercoiling reaction by A. fulgidus reverse gyrase demonstrates an apparent accumulation of relaxed DNA intermediates, suggesting a much lower processivity (21).

DNA binding studies shed further light on the mechanism of positive supercoiling. DNA substrate preference is partitioned between subunits, with the topoisomerase subunit binding single-stranded DNA and the helicase-like subunit binding double-stranded DNA. To reanneal single strands to form a duplex and to induce positive supercoils, it is likely that reverse gyrase binds at the junction between double- and single-stranded DNA. It is well established that type IA topoisomerases prefer single stranded DNA for binding (24). Although the biochemical analysis of the helicase-like domain is still at its early stage, it probably has a basal affinity for the double-stranded DNA, which can be modulated by its binding to ATP/ADP. Using electrophoretic mobility shift assays to monitor DNA binding affinities by the helicase-like domain, we could not detect any effect of ATP/ADP on protein binding to double- versus single-stranded DNA. Because these binding assays are not sensitive to small or transient changes in DNA binding affinities, we used topology shift assays to detect any such changes. Our results indicated that either in the absence of any cofactor or in the presence of AMPPNP, the helicase-like domain can induce a slight DNA unwinding, implicating a relative preference of binding to underwound DNA under such conditions.

DNA gyrase and reverse gyrase are unique among topoisomerases in that they can mediate directional strand passage to induce either an increase (reverse gyrase) or decrease (gyrase) in linking number. The mechanistic basis for gyrase to reduce linking number or to generate negative supercoils is better understood (reviewed in Ref. 25). The right-handed wrapping of the DNA gate segment by gyrase provides a clear topological basis for vectorial strand passage. In contrast, the mechanistic basis for the positive supercoiling action by reverse gyrase remains to be fully elucidated. There are several lines of evidence that suggest a plausible mechanism. Reverse gyrase has an important biochemical function as a DNA reanneaturase (12). This is supported by two lines of experiments. The first is that the enzyme can readily reanneal two single-stranded DNA circles with complementary base sequences. The second is that reverse gyrase can more efficiently generate positive supercoils in a DNA substrate containing a permanent single-stranded bubble. For the bubble substrate, the continuous (but futile) action of reannealing leads to a higher level of positive supercoiling. DNA reannealing is proposed to start with the targeting of reverse gyrase to the underwound or single-stranded region (21), and this preferential binding is induced by the association of helicase-like domain with a specific nucleotide (e.g. ATP). The subsequent nucleotide switch (e.g. from ATP to ADP) can induce a binding preference of the helicase-like domain for rewound or double-stranded DNA, thus promoting the re annealing of DNA helix. Such a topological shift can be facilitated and fixed through the strand passage action of the topoisomerase domain. The key element in this mechanistic proposal, DNA binding preference shift as modulated by the nucleotide switch, has also been described by del Toro Duany et al. (18). The specific details remain to be fully elucidated. The data presented here suggest that the AMPPNP (or ATP) bound form has a slight preference for underwound DNA, relative to the ADP bound form. Earlier work with the A. fulgidus enzyme demonstrated that in the presence of AMPPNP, highly negatively super-
coiled DNA products can be generated (21). This was interpreted to indicate that reverse gyrase when bound with AMP-PNP can associate with underwound DNA and thus entrap negative supercoils. However, there could be variations to the theme. Using fluorescence anisotropy to monitor DNA binding at 37 °C, the recombinantly engineered helicase-like domain from *T. maritima* reverse gyrase was shown to have a binding affinity for single-stranded DNA regardless of the cofactors (18). The relative preference of single-stranded DNA is reduced in the presence of ADP when compared with the conditions without cofactor or with ATP analog. On the other hand, the engineered recombinant helicase-like domain from the *Sulfolobus solfataricus* enzyme showed no nucleotide-modulated shift in DNA binding affinity by mobility shift assays (26), similar to our observations reported here. The complicated difference in the binding preferences observed in these experiments could be due to methodology for monitoring binding reactions or to the use of reverse gyrases from different species. Despite this, the essential feature of the proposed nucleotide switch mechanism through which DNA structures could be modified appears to be retained by all reverse gyrases. Further experimentation will be needed to dissect the biochemical mechanism of this unique DNA machine.

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