The dimer–dimer interaction surface of the replication terminator protein of *Bacillus subtilis* and termination of DNA replication

(protein–protein interaction/contrahelicase activity)

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ABSTRACT The replication terminator protein (RTP) of *Bacillus subtilis* causes polar fork arrest at replication termini by sequence-specific interaction of two dimeric proteins with the terminus sequence. The crystal structure of the RTP protein has been solved, and the structure has already provided valuable clues regarding the structural basis of its function. However, it provides little information as to the surface of the protein involved in dimer–dimer interaction. Using site-directed mutagenesis, we have identified three sites on the protein that appear to mediate the dimer–dimer interaction. Crystallographic analysis of one of the mutant proteins (Y88F) showed that its structure is unaltered when compared to the wild-type protein. The locations of the three sites suggested a model for the dimer–dimer interaction that involves an association between two β -ribbon motifs. This model is supported by a fourth mutation that was predicted to disrupt the interaction and was shown to do so. Biochemical analyses of these mutants provide compelling evidence that cooperative protein–protein interaction between two dimers of RTP is essential to impose polar blocks to the elongation of both DNA and RNA chains.

Termination of DNA replication in most prokaryotic systems such as the *Escherichia coli* plasmid R6K (1–3), the Gram-negative bacterium *E. coli* (4, 5), and the Gram-positive bacterium *Bacillus subtilis* (6, 7) occurs at specific sequences called replication termini. The first step in this process is the sequence-specific arrest of the replication forks at the terminus, and it has been shown in all three systems that this is mediated by sequence-specific DNA-binding proteins called terminator proteins. The resulting protein–DNA complexes are able to arrest replication forks approaching from one direction but not the other. The mechanistic basis of this novel polarity involves specific inhibition of the replicative helicase—the so-called contrahelicase activity (8–17).

The replication terminator protein (RTP) of *B. subtilis* is a homodimer with a subunit molecular weight of 14,500 (7). The RTP–DNA complex comprises two RTP dimers bound tandemly and specifically to adjacent sites. One site (the core site) binds RTP tightly, but the second site (the auxiliary site) binds RTP weakly and requires a cooperative interaction with a prebound RTP at the core site. Variations on this scheme result in defective termination. Thus, the binding of two dimers of RTP to tandem core sites is unable to block fork movement (13), and a single dimer bound to one core sequence does not elicit replication termination either *in vivo* (7) or *in vitro* (13). Considered together, these observations suggest that the RTP–DNA complex has a precise architecture that involves specific protein–DNA and protein–protein interactions that are critical to the function of the replication terminus.

We have recently solved the crystal structure of the RTP dimer in the absence of DNA at 2.6 Å resolution (18). The

protein is mostly α -helical, and long C-terminal α -helices from each monomer interact to form an antiparallel coiled-coil dimerization domain. The dimer also contains a pair of two-fold related 3-strand β -sheets with associated β -ribbons. The structure of one monomer of RTP is shown in Fig. 1. Based on pertinent biochemical data, we have proposed that one RTP dimer binds two turns of DNA. In the model, the pair of α 3-helices make base-specific contacts in successive major grooves, the β -ribbons and the connecting loops interact with the peripheral minor grooves, and the flexible N-terminal arms wrap around the central minor groove (18). The crystal structure did not provide any clue as to the location of the dimer–dimer interaction surface, but computer graphics modeling exercises using two adjacent RTP–DNA model complexes suggest that it is close to the β -ribbon.

In this paper, we address the question of the location and structural nature of the dimer–dimer interaction surface by performing site-directed mutagenesis, purifying the mutant forms of the protein, and analyzing the biochemical properties of the mutant proteins. Initial results conclusively showed that tyrosine-88 plays a critical role in promoting dimer–dimer interaction. Substitution of the tyrosine by a phenylalanine completely abolishes dimer–dimer interaction, and fails to block *E. coli* DnaB helicase and replication fork progression. This result suggested that the dimer–dimer interaction involves strand β 3, and additional mutations in this region support this conclusion and suggest a plausible molecular model. These studies also conclusively prove that the cooperative interaction between RTP dimers is essential for the arrest of replication forks by RTP. The interaction is also essential for the recently discovered ability of RTP to block the elongation of RNA chains (20).

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The *E. coli* JM 109 [F' *tra* D36, *lacI^q*, Δ (*lacZ*)M15, *pro* A⁺B⁺/e14[−] (*McrA*[−]), Δ (*lac-pro* AB), *thi*, *gyrA96*, (*Nal*^r), *endA*, *hsd* R17, (*r_k[−]m_k⁺*), *recA1* *relA1*, *supE44*] was used in all cloning. The *E. coli* strain HMS174[F[−] *recA*, *hsdR*, (*r_k[−]m_k⁺*) *rif*^r] (DE3) was used for preparation of cell extracts for the *in vitro* replication experiments, and the *E. coli* strain HMS 174 (DE3) pLysS was used for all the protein overproduction work. The plasmids pUC18BS3 and pUC19BS3 contained the replication terminator site of *B. subtilis* (called IRI or BS3) cloned between the *Eco*RI and the *Hind*III sites. The pUC19BS3 contained the BS3 site in the blocking orientation with respect to the origin, whereas pUC18BS3 had the BS3 site in the opposite, nonblocking orientation. For *in vitro* transcription experiments, two plasmids were used that contained the BS3 site at the *Eco*RI site of pET22b (Novagen) downstream from a T7 promoter in either the blocking (pET22bBS3) or nonblocking (pET22bBS3rev) orientations. For expression and overproduction of wild-type

FIG. 1. Ribbon diagram of the tertiary structure of a monomer of RTP. Note the locations of the four α -helices, the three-stranded β -sheet, the extended loop between strands $\beta 2$ and $\beta 3$, and the unstructured N-terminal arm. Helix $\alpha 4$ forms an antiparallel coiled-coil dimerization domain with its dimeric partner. Note that strand $\beta 3$ extends from residues 84 to 90. The diagram was made using the program MOLSCRIPT (19).

and the mutant forms of RTP, the *rtp* gene was cloned between the *Nde* I and *Bam*HI sites of the vector pET13a, and the proteins were overproduced under the influence of the strong T7 promoter.

Site-Directed Mutagenesis and Purification of Mutant Forms of RTP. The RTP mutants were generated by saturation oligonucleotide-directed mutagenesis using overlapping, degenerate oligonucleotides. The characterization of the library of mutants and other details is published elsewhere (41). RTP was purified as described (21). *E. coli* DnaB helicase was purified as described (15).

In Vitro Replication and Analysis of Replication Intermediates. Cell extracts were prepared and the reactions were carried out as described (22) using [α - ^{32} P]dATP and the pUC19BS3 and pUC18BS3 templates. The reactions were stopped with 5 mM EDTA and 0.1% SDS, followed by digestion with proteinase K (0.4 mg/ml) for 30 min at 50°C. The samples were denatured by boiling, chilled, and run in 4% polyacrylamide/6 M urea gel.

Helicase Assays and Gel Mobility-Shift Assays. These were carried out as described (15, 21).

X-Ray Crystallography of Mutant Y88F. The crystals of the mutant protein were grown under almost identical conditions as the crystals of the native protein (21) with minor changes [30 mg of protein per ml and 30% (wt/vol) PEG 2000 as precipitant]. The mutant crystals were isomorphous to those of the native (space group C2, $a = 76.7$ Å, $b = 52.7$ Å, $c = 70.4$ Å, $\beta = 90^\circ$). Diffraction data to 2.5 Å were collected and processed as described (18). The final R_{merge} for the 9870 unique reflections (27,940 total) was 3.6%. An atomic model incorporating the Y88F mutation was refined against the collected data with a 2 sigma cutoff using the program X-PLOR (23). The refinement consisted of 200 steps of Powell minimization, one round of simulated annealing using initial and final temperatures of 3000K and 300K, respectively, and a final 20 cycles of individual restrained *B*-factor refinement. The final structure was checked against $2F_o - F_c$, $F_o - F_c$, and $2F_o - F_c$ omit maps using the O program (24). The *R* factor of the final model was 21.5%, and the rms deviations on bond lengths and angles were 0.012 Å and 1.35°, respectively.

RESULTS

The Y88F Mutant Form of RTP Is Severely Impaired in Cooperative Dimer-Dimer Interaction at the Terminus. To

correlate the overall tertiary structure and the various structural features of RTP (Fig. 1) with its various biochemical activities, we performed saturation, site-directed mutagenesis of the *rtp* DNA using a series of overlapping, degenerate oligonucleotides. The details of mutagenesis and the extensive biochemical characterizations of the mutant forms of RTP is published elsewhere (41). A total of 64 point mutants were isolated, and we initially tested eight that are in the β -ribbon region that we predicted to mediate the dimer-dimer interaction. These eight mutants are E78G, G79V, K81M, Q83L, E84V, L87H, Y88F, and Q89L.

The assay for cooperativity is based on the observation that wild-type RTP fills the terminus in a stepwise fashion. At lower concentrations of protein, the core site is filled first with a RTP dimer. With increasing concentrations of the protein, the auxiliary site is filled by a second dimer (Fig. 2 *Top*) by

FIG. 2. Autoradiogram of an 8% nondenaturing polyacrylamide gel showing the binding of wild-type RTP and the mutant Y88F (YM88F) to a DNA probe containing the *B. subtilis* terminus BS3 (IRI). (*Top*) Model of two interacting dimers of RTP bound to the overlapping core and the auxiliary sites of the IRI (BS3) terminus. (*Middle*) Gel mobility-shift assay using a labeled 53-bp DNA fragment that contained the BS3 site. Lanes 1–14, mobility of the DNA band in the presence of 0, 15, 30, 60, 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 ng of wild-type RTP, respectively; f, free DNA; α , shift due to filling of the core site; β , shift caused by filling of both the core and the auxiliary sites. (*Bottom*) Gel mobility-shift caused by the Y88F (YM88F) protein. Lanes 1–14, shift in the presence of 0, 15, 30, 60, 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 ng of the mutant protein, respectively.

cooperative protein–protein interactions. The auxiliary site by itself showed little binding to a RTP dimer at very high protein/DNA ratios ($\approx 100:1$; data not shown). Normal binding of a mutant form of RTP to the core site, but failure to bind to the overlapping auxiliary site, should be diagnostic of a defect in cooperativity of interaction. We purified the wild-type and the mutant forms of RTP and incubated the proteins separately with a 53-bp, labeled DNA fragment containing the BS3 (IRI) site (21). The products were resolved on nondenaturing polyacrylamide gels. As expected, the wild-type RTP first filled the core site, shifting the free DNA (f) to position α . With increasing concentrations of the protein, the auxiliary site was also occupied as revealed by a second shift to position β (Fig. 2 *Middle*).

This pattern was also seen with all the mutants except one, Y88F. In sharp contrast, Y88F used in equivalent amounts filled the core site to yield the α shift but failed to fill the auxiliary site. A trace amount of the β shift was seen only at very high concentrations (Fig. 2 *Bottom*). We performed various additional experiments to verify that the core binding of Y88F was unaffected. DNase I footprinting experiments confirmed that the observed gel mobility shifts are specific and caused by occupation of the core site of BS3 (data not shown). We also performed gel-shift experiments with the isolated core fragment and found that the k_d of the wild-type protein was only 1.5 times higher than that of the mutant protein ($0.76 \pm 0.02 \times 10^{-11}$ M and $0.5 \pm 0.02 \times 10^{-11}$ M, respectively). Finally, we used an *in vivo* genetic assay to monitor binding of the proteins to the core site (25) and found no difference between the two types of RTP (data not shown).

The Y88F RTP Has a Severe Defect in Contrahelicase Activity. We have previously shown that the wild-type RTP of *B. subtilis* is a polar contrahelicase of *E. coli* DnaB helicase (12). We tested this activity of the Y88F mutant in the following way. Two duplexed helicase substrates were prepared by annealing 57-bp labeled oligonucleotides containing each strand of the BS3 sequence to the complementary strand that was present in recombinant single-stranded circular M13 DNAs. The M13mp19BS3 substrate had the terminus sequence in the blocking orientation, and the M13mp18BS3 substrate had the same sequence in the reverse orientation. A fixed amount of the substrate and *E. coli* DnaB was incubated with increasing amounts of the wild-type and mutant terminator proteins, and the release of free oligonucleotide as a result of DNA unwinding was measured after fractionation of the reaction products in nondenaturing polyacrylamide gels. The results of the experiments with wild-type and mutant RTPs and the mp19BS3 and the mp18BS3 substrates are shown in Fig. 3*A Top* and *Bottom*, respectively. As expected, the normal RTP impeded the DNA unwinding by *E. coli* DnaB on the mp19BS3 substrate but not on the mp18BS3 substrate (compare Fig. 3*A Top*, lanes 2–8 with the corresponding lanes in Fig. 3*A Bottom*). In contrast, the Y88F protein was unable to impede DNA unwinding either on the mp19BS3 substrate (Fig. 3*A Top*, lanes 10–15) or on the mp18BS3 substrate (Fig. 3*A Bottom*).

The Y88F Protein Fails to Arrest Replication Forks *in Vitro*. We constructed two DNA templates, one with the terminus in the blocking orientation with respect to a unidirectional replication origin (pUC19BS3) and one with the sequence in the reverse orientation (pUC18BS3). The two DNA templates were replicated *in vitro* in cell-free extracts of *E. coli* in the presence of [α - ^{32} P]dATP. The denatured reaction products were resolved on denaturing polyacrylamide gels. The wild-type RTP arrested forks and generated a diagnostic ≈ 415 -nt-long DNA band with the blocking template (Fig. 4, lanes 2–5). The replication of the nonblocking template did not generate such a band, confirming the well known polarity of fork arrest (lanes 11 and 12). In contrast, the Y88F protein did not generate significant amounts of the ≈ 415 -nt band. Only at the

FIG. 3. Assays for ability of normal and mutant RTP to impede *E. coli* DnaB-catalyzed DNA unwinding. (A) (Top) Lane 1, 10 fmol of mp19BS3 duplexed helicase substrate; lane 2, substrate with no RTP but with 100 ng of *E. coli* DnaB; lanes 3–8, same as lane 2 but with 20-, 40-, 60-, 80-, 100-, and 120-fold molar excess of normal RTP over substrate (60 ng of RTP = 1 pmol). Lanes 9–15, equivalent to lane 1 and lanes 3–8 except that equivalent amounts of the mutant protein Y88F (YM88F) were used. (Bottom) Lanes 1–15, controls for Top using the substrate mp18BS3 in which the terminus sequence is in the reverse orientation. Autoradiograms are of 8% nondenaturing polyacrylamide gels. (B) Quantitations of the released oligonucleotides shown in A using a PhosphorImager.

highest concentrations of the protein was a faint ≈ 415 -nt band generated (compare lanes 6–9 with the lanes 2–5). In the absence of RTP, *in vitro* replication did not generate the diagnostic 415-nt band (lanes 1 and 10). Thus, consistent with the loss of contrahelicase activity, the Y88F mutant also loses the ability to arrest replication forks *in vitro*.

The Crystal Structure of Mutant Y88F. To directly visualize the structural consequences of the Y88F mutation, the crystal structure of the mutant protein was determined to 2.5 Å resolution. Surprisingly, when the α -carbon backbones of the wild-type and mutant proteins are compared, there are no significant differences (Fig. 5). The protein backbones of the two structures superimpose with an rms deviation of 0.352 Å, and the orientations of the tyrosine and phenylalanine side chains are almost identical.

Further Mapping of the Dimer–Dimer Interaction Surface. The close similarity of the Y88F and wild-type RTP structures strongly suggests that the partially exposed tyrosine-88 is not important to the dimer structure but is critical at the dimer–dimer interface. To directly test this, several additional mutations were made to residues close to tyrosine 88 that should also be at or near the interface. The following mutations were constructed; Q72A, I73G, V75G, K77A, and V85G. Also made

FIG. 4. Autoradiogram of a 4% polyacrylamide/6 M urea gel showing reaction products of *in vitro* replication of DNA substrates from a unidirectional origin. Two substrates were used that contain the BS3 terminus in the functional (pUC19BS3) and the reverse orientation (pUC18BS3) with respect to the origin. M, molecular size markers. Lanes 1–5, products of replication of the pUC19BS3 template (2 pmol) in the presence of 0, 1, 2, 5, and 8 pmol of wild-type RTP, respectively. Note the termination band of ≈ 415 nt (arrowhead). Lanes 6–9, same as lanes 2–5 but wild-type RTP was replaced with equivalent amounts of Y88F mutant protein. Lanes 10–12, products of replication of the pUC18BS3 template (2 pmol) in the presence of 0, 2, and 5 pmol of wild-type RTP, respectively. Lanes 13 and 14, same as lanes 11 and 12 except equivalent amounts of mutant protein were used.

was the double mutation EK78/81AA. Finally, mutation G34R, which is also close to tyrosine-88, was available from random mutagenesis. When all of these mutants were tested with the complete spectrum of assays described above, G34R also inhibited the dimer–dimer interaction to the same extent as Y88F, and V85G inhibited between 90% and 95% when compared to Y88F (data not shown). All the rest were wild type.

DISCUSSION

Fig. 6A is a magnified view of the β -sheet region of RTP that shows the locations of the residues that appear to be involved and not involved in the dimer–dimer interaction. It is immediately obvious that β -strand β_3 is crucial to the interaction, whereas β -strand β_2 is not. To develop a possible model for the interaction, two RTP molecules were docked onto the entire BS3 sequence by computer graphics. The proteins were centered on the pseudo-twofold axes of the core and auxiliary

FIG. 5. Comparison of crystal structures of wild-type RTP and mutant Y88F (YM88F). For clarity, only the α -carbon backbones of the two molecules are shown. Wild type is shown as a thick line and Y88F is the thinner line. Superposition was performed using the O program (24).

FIG. 6. Model of the dimer–dimer interaction for RTP protein of *B. subtilis*. (A) β -Sheet region of RTP showing residues that affect (ball-and-stick) and that do not affect (thin lines) dimer–dimer interaction. For clarity, only the former are labeled. (B) Relative positions of two RTP dimers on the complete IRI terminus. On the undistorted DNA, the dimers are separated azimuthally by $\approx 180^\circ$. They can be brought into contact across the major groove by unwinding of the DNA. (C) Model for the dimer–dimer surface adapted from A. Note that the four residues from each dimer that appear to mediate this interaction are in close proximity. This model should not be considered as an accurate representation of the actual interface. For example, the flexible loop connecting strands β_2 and β_3 and that contains leucine-82 is likely to adopt a defined conformation that is impossible to predict. The figure was produced with the MOLSCRIPT program (19).

cognate sequences (Fig. 6B). A simple way to bring together the β_3 strands from adjacent molecules is to unwind the DNA at the dimer–dimer junction by some 180° , and the two strands would then meet across the major groove. Such a model results in two predictions. First, strand β_2 would be in close contact with the DNA backbone, whereas strand β_3 would not. We have recently analyzed the RTP–DNA interaction by site-directed mutagenesis and shown that this is indeed true (41). Second, the binding of each RTP to its cognate DNA would induce a distortion of the nucleic acid. Such a distortion is commonly found in protein–DNA complexes (26) and we have preliminary evidence that RTP does indeed bend DNA (unpublished results). It should also be noted that DNA bending is a feature of a recently published model of the RTP–DNA complex (27).

The model also predicts that the loop connecting strands β_2 and β_3 would make contact with the body of the adjacent dimer. Such an interaction would explain the inhibitory effect

of the G34R mutation, which is located in the relevant area of the molecule. It would also explain the curious leucine-82, which is exposed at the end of the loop since it could mediate the interaction by a hydrophobic contact. This was directly tested by constructing the mutant L82S, which replaces the exposed hydrophobic moiety with a small polar group. As predicted by the model, this mutant elicits no dimer-dimer interaction when tested by gel-shift assays (data not shown). A diagrammatic representation of the dimer-dimer interface is shown in Fig. 6C. That the dimer-dimer interface is restricted to this region of RTP is supported by mutations in other regions of the protein that have been tested. The only mutation that reduces the interaction (by $\approx 50\%$) is R6G in the N-terminal arm. We have shown that this arm is crucial for DNA binding (41) and the effect may therefore be indirect. It should also be noted that the mutant Y88F is far more soluble than the wild-type protein (as revealed in the crystallization experiments), which supports a reduced level of protein-protein interaction in solution.

Unlike the replication termination protein Ter (tus) of *E. coli*, which functions as a monomer of molecular mass of 36,000 (28, 29), RTP of *B. subtilis* is a dimer with a subunit molecular mass of 14,500 (7). The interaction between two RTP dimers bound to the overlapping core and the auxiliary sites (7, 12, 13) has mechanistic significance in at least three ways. First, since the structure of the RTP dimer is symmetrical (18), it raises the question as to how such a molecule is able to generate functional asymmetry in the form of polar or unidirectional arrest of the replication fork. We have postulated that the interaction of two dimers hides one of the two putative helicase-blocking surfaces at the terminus. Since the contact points of the dimer binding to the auxiliary site are only a subset of that established by the dimer with the core sequence (13, 30), the nonblocking end of the RTP-DNA complex is potentially different from that of the blocking end. The structural asymmetry thus generated could provide the basis for the functional polarity. Second, the cooperativity of interaction may generate the requisite binding strength for DNA that a single dimer bound to a core site could not adequately provide. It has been shown that a single dimer bound to a core is incapable of arresting replicative helicases and replication forks *in vivo* (7) and *in vitro* (13). Finally, the tight association of the two overlapping dimers at the terminus predicted by our model would explain how the replication fork approaching from the nonblocking direction could displace the proteins (7). The fork would displace the weakly bound dimer at the auxiliary site, which in turn would peel off its partner.

We have recently discovered that the replication terminator proteins of both *E. coli* and *B. subtilis* can block RNA chain elongation catalyzed by several prokaryotic RNA polymerases in a polar mode (20). We tested the ability of all the mutants described in this paper to block RNA transcription at the BS3 site and showed that only those that are defective in dimer-dimer interactions are not able to arrest RNA chain elongation (data not presented). This clearly demonstrates that the cooperative dimer-dimer interaction is also essential for this blocking process. However, it should be noted that the structural requirements for blocking DNA chains are not identical to those needed to block RNA chains. We have recently analyzed a mutant form of RTP that binds DNA almost normally, that blocks RNA chain elongation, but that can no longer block replication progression *in vitro* (unpublished data).

Our model for the dimer-dimer interaction of RTP involves a novel association between two peripheral β -ribbons. This is radically different from other known modes of protein tetramerization such as the bundles of four α -helices seen in the lac repressor (31, 32) and the p53 tumor suppressor protein (33). However, there have been examples of β -rib-

bon-mediated protein-protein interactions in other systems. The most notable is seen in GroES, where seven subunits each donate a projecting β -ribbon to form a 14-strand cap-like structure (34). Ribosomal protein L14 also has a projecting β -ribbon that forms an important crystal contact with a symmetry-related β -ribbon and that may interact in a similar fashion with a second protein in the ribosome (35). Finally, it has been suggested that HU proteins tandemly bound along the DNA may interact through an extended ribbon-like DNA-binding arm (36). Mutational studies of λ and P22 repressor have revealed a common primary sequence motif that seems to be the determinant of dimer-dimer interaction (37), but this sequence motif is not present in RTP (38). Finally, it is interesting to note that the replication initiator protein π of plasmid R6K loops DNA by dimer-dimer interaction, and mutants that affect the looping process are located at or near a region that has a potential leucine zipper (39, 40).

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