The Zinc Finger Motif of *Escherichia coli* RecQ Is Implicated in Both DNA Binding and Protein Folding*

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The RecQ family of DNA helicases has been shown to be important for the maintenance of genomic integrity. Mutations in human RecQ genes lead to genomic instability and cancer. Several RecQ family of helicases contain a putative zinc finger motif of the C₄ type at the C terminus that has been identified in the crystalline structure of RecQ helicase from *Escherichia coli*. To better understand the role of this motif in helicase from *E. coli*, we constructed a series of single mutations altering the conserved cysteines as well as other highly conserved residues. All of the resulting mutant proteins exhibited a high level of susceptibility to degradation, making functional analysis impossible. In contrast, a double mutant protein in which both cysteine residues Cys³⁹⁷ and Cys⁴⁰⁰ in the zinc finger motif were replaced by asparagine residues was purified to homogeneity. Slight local conformational changes were detected, but the rest of the mutant protein has a well defined tertiary structure. Furthermore, the mutant enzyme displayed ATP binding affinity similar to the wild-type enzyme but was severely impaired in DNA binding and in subsequent ATPase and helicase activities. These results revealed that the zinc finger binding motif is involved in maintaining the integrity of the whole protein as well as DNA binding. We also showed that the zinc atom is not essential to enzymatic activity.

The transient formation of single-stranded DNA (ssDNA) intermediate is essential to all aspects of DNA metabolism including DNA replication, recombination, and repair. The unwinding and separation of the individual strands of double-stranded DNA (dsDNA) is catalyzed by a class of specialized enzymes known as DNA helicases (1, 2). These enzymes function as molecular motors that use the energy released from the hydrolysis of ATP to unwind and translocate along DNA in a sequential fashion (3–5). These ubiquitous enzymes have been identified in all living organisms from virus to human. It appears that they evolved from a common ancestor (6).

The RecQ helicase family is critical to the maintenance of genomic stability in prokaryotes and eukaryotes (7). Mutations of RecQ genes can lead to genomic instability and several human diseases including the Bloom and Werner syndromes (8). Recently, it has been shown that the tumor suppressor BRCA1-associated protein, BACH1, which shares homologies with other members of the RecQ family, possesses ATPase and helicase activities (9). The mutant BACH1 participates directly in breast and ovarian cancer development (9). The RecQ helicase from *Escherichia coli* is the prototype helicase of this family (10) and has been shown to initiate homologous recombination as well as suppress illegitimate recombination (11, 12).

The RecQ helicase family members contain a helicase domain characterized by the presence of seven so-called “helicase” motifs necessary for using energy derived from ATP binding and hydrolysis to unwind DNA (13, 14). Sequence analyses revealed that all of the RecQ helicases contain a C-terminal extension that can be further divided into two domains (Fig. 1A); the HRDC domain (helicase and RNase D C-terminal), which functions as an auxiliary DNA-binding domain (15, 16), and the RecQ C-terminal domain that contains a conserved CX₆CX₄CDXC motif (in which X is any amino acid) among the RecQ family of helicases (Fig. 1A) of which the function is still not clear. Recently, the three-dimensional structure of a C-terminal truncated form of RecQ helicase has revealed that the enzyme folds into four subdomains, two of which combine to form the helicase region, whereas the others form zinc binding (Fig. 1B) and winged-helix motifs (17). The zinc atom is bound by four conserved cysteine residues located at a platform composed of α-helices 17 and 18. The cysteine residue Cys³⁸⁰ (labeled as C1) is located at the beginning of the α-helix 17. Cys⁴⁰⁰ (labeled as C3) and Cys⁴⁰³ (labeled as C4) are at the beginning and the middle of the α-helix 18, respectively, whereas Cys³⁹⁷ (labeled as C2) is located in the loop linking the two helices (Fig. 1B). In addition, the zinc finger motif may be further stabilized by three hydrogen bonds formed among the conserved residues of phenylalanine (Phe³⁷⁴), arginine (Arg³⁸¹), and asparagine (Asp¹⁰¹) (Fig. 1B). Previous studies have established that the zinc finger domains and other metal-binding protein domains are involved in diverse functions including protein-DNA interactions, protein folding, and protein-protein interactions (18). To elucidate the roles played by the zinc atom and the zinc finger motif in RecQ helicase, mutant RecQ molecules were engineered by site-directed mutagenesis within the zinc finger motif. Biochemical characterizations of these mutants showed that the zinc binding motif is essential to efficient

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1 The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; HRDC, helicase and RNase D C-terminal; FRET, fluorescence resonance energy transfer; PAR, 4-(2-pyridylazo)resorcinol disodium salt; mantATP, 2′(3′)-O-(N-methylanthraniloyl)adenosine 5′-triphosphate; DTT, dithiothreitol; CD, circular dichroism.
DNA binding and stabilization of the three-dimensional structure of RecQ molecules.

MATERIALS AND METHODS

{\[1^{25}P\]ATP was obtained from Amersham Biosciences, 4-2-Pyridyl- 
averoborocinol disodium salt (PAR), EDTA, 2-mercaptoethanol, ATP, 
and o-chymotrypsin were obtained from Sigma. Chelex® 100 resin was 
purchased from Bio-Rad. The N-methylnthraniloyl derivatives of ad-
ene nucleotides were synthesized according to Hiratsuka (19) and 
peroxidase Edinburgh (20). The non-tubulin binding of RecQ helicase was 
captured, and anion-exchange chromatography as described previously 
(20). Mutations were made by a two-step polymerase chain reaction 
method (21). The outside primers were QFN (5'-GAGCTACTCTTCGTCATCGCCATCAACATG-3') and QMF2 (5'-GCAGGAGCTTCGAATCGTTTCGTCATCGCCATCAACATG-3'). The primers used to introduce mutations were QMF1 (5'-GGAGCTACTCTTCGTCATCGCCATCAACATG-3') and QMF2 (5'-GCAGGAGCTTCGAATCGTTTCGTCATCGCCATCAACATG-3'). The outside primers were QFN (5'-GAGCTACTCTTCGTCATCGCCATCAACATG-3') and QMF2 (5'-GCAGGAGCTTCGAATCGTTTCGTCATCGCCATCAACATG-3').

Expression of Wild-type Enzyme and Construction of the Zinc Finger 
Mutant of RecQ Helicase—The E. coli RecQ helicase containing a N-
terminal His tag was expressed in E. coli and purified by nickel-
chelating and anion-exchange chromatography as described previously 
(22). The outside primers were QFN (5'-GAGCTACTCTTCGTCATCGCCATCAACATG-3') and QMF2 (5'-GCAGGAGCTTCGAATCGTTTCGTCATCGCCATCAACATG-3'). The primers used to introduce mutations were QMF1 (5'-GGAGCTACTCTTCGTCATCGCCATCAACATG-3') and QMF2 (5'-GCAGGAGCTTCGAATCGTTTCGTCATCGCCATCAACATG-3'). The primers used to introduce mutations were QMF1 (5'-GGAGCTACTCTTCGTCATCGCCATCAACATG-3') and QMF2 (5'-GCAGGAGCTTCGAATCGTTTCGTCATCGCCATCAACATG-3'). The primers used to introduce mutations were QMF1 (5'-GGAGCTACTCTTCGTCATCGCCATCAACATG-3') and QMF2 (5'-GCAGGAGCTTCGAATCGTTTCGTCATCGCCATCAACATG-3'). The primers used to introduce mutations were QMF1 (5'-GGAGCTACTCTTCGTCATCGCCATCAACATG-3') and QMF2 (5'-GCAGGAGCTTCGAATCGTTTCGTCATCGCCATCAACATG-3'). The primers used to introduce mutations were QMF1 (5'-GGAGCTACTCTTCGTCATCGCCATCAACATG-3') and QMF2 (5'-GCAGGAGCTTCGAATCGTTTCGTCATCGCCATCAACATG-3'). The primers used to introduce mutations were QMF1 (5'-GGAGCTACTCTTCGTCATCGCCATCAACATG-3') and QMF2 (5'-GCAGGAGCTTCGAATCGTTTCGTCATCGCCATCAACATG-3'). The primers used to introduce mutations were QMF1 (5'-GGAGCTACTCTTCGTCATCGCCATCAACATG-3') and QMF2 (5'-GCAGGAGCTTCGAATCGTTTCGTCATCGCCATCAACATG-3'). The primers used to introduce mutations were QMF1 (5'-GGAGCTACTCTTCGTCATCGCCATCAACATG-3') and QMF2 (5'-GCAGGAGCTTCGAATCGTTTCGTCATCGCCATCAACATG-3'). The primers used to introduce mutations were QMF1 (5'-GGAGCTACTCTTCGTCATCGCCATCAACATG-3') and QMF2 (5'-GCAGGAGCTTCGAATCGTTTCGTCATCGCCATCAACATG-3'). The primers used to introduce mutations were QMF1 (5'-GGAGCTACTCTTCGTCATCGCCATCAACATG-3') and QMF2 (5'-GCAGGAGCTTCGAATCGTTTCGTCATCGCCATCAACATG-3'). The primers used to introduce mutations were QMF1 (5'-GGAGCTACTCTTCGTCATCGCCATCAACATG-3') and QMF2 (5'-GCAGGAGCTTCGAATCGTTTCGTCATCGCCATCAACATG-3'). The primers used to introduce mutations were QMF1 (5'-GGAGCTACTCTTCGTCATCGCCATCAACATG-3') and QMF2 (5'-GCAGGAGCTTCGAATCGTTTCGTCATCGCCATCAACATG-3'). The primers used to introduce mutations were QMF1 (5'-GGAGCTACTCTTCGTCATCGCCATCAACATG-3') and QMF2 (5'-GCAGGAGCTTCGAATCGTTTCGTCATCGCCATCAACATG-3').
denatured HindIII-cut pGEM-7Zf linear DNA (3 kb) or ssDNA (60-mer) at the indicated concentration of ATP. The reactions were initiated by the addition of RecQ helicase into 100 μl of reaction mixture and stopped by pipetting 80 μl of aliquots from the reaction mixture every 30 s into a hydrochloric solution of ammonium molybdate. The liberated radioactive 32Pi was extracted with a solution of 2-butanol-benzene-acetone-ammonium molybdate (750:750:15:1) saturated with water. An aliquot of the organic phase was counted in 6 ml of Aquasol.

The competitive inhibition constant, Ki, for mantATP was determined by measuring the ATPase rate as a function of mantATP concentration and fit to Equation 7,

\[
v = \frac{[\text{ATP}]_0 \cdot k_{\text{cat}}}{[\text{ATP}] + K_{\text{m}}^{\text{ATP}} (1 + \frac{[\text{mantATP}]}{K_i})}
\]  
(Eq. 7)

where v is the initial ATPase rate, \( k_{\text{cat}} \) is the catalytic constant, and \( K_{\text{m}}^{\text{ATP}} \) is the \( K_m \) for ATP.

**Helicase Assay**—An unwinding assay was performed using the Beacon 2000 fluorescence polarization instrument (26). An appropriate quantity of fluorescein-labeled duplex oligonucleotide was added to the helicase-unwinding buffer (150 μl of total volumes) in a temperature-controlled cuvette. The anisotropy was measured successively until it stabilized. The data were fit to the exponential equation: \( A = A_0 \exp(-k_{\text{obs}} t) \), where A is the anisotrop...
The residues Arg^{381} and Asp^{401} were thus replaced by asparagine and alanine, respectively. We found that all of these mutants displayed a high level of proteolysis and could not be purified to homogeneity, making functional studies impossible.

To disturb mildly the zinc finger motif, we decided to simultaneously substitute C2 and C3 with asparagine. This residue was chosen, because its side chain could conceivably act as ligand to zinc ion (27). Moreover, the single mutation with the selected RecQ enzymes were first engineered with single alanine or serine substitution at the position of each of the four cysteines.

### Table I

| Helicase                          | [Zn^{2+}] / [protein] | Stokes radius | Catalysis \( k_{cat}(ATPase) \) | DNA binding \( K_d^{ssDNA} \) | DNA binding \( K_d^{dsDNA} \) | ATP binding \( K_d^{(mantATP)} \) |
|-----------------------------------|-----------------------|---------------|---------------------------------|-------------------------------|-------------------------------|---------------------------------|
| Wild type                         | 0.98 ± 0.11           | 35.2          | 36.1 ± 1.1                      | 52 ± 1.2                      | 81 ± 1.1                      | 43.6 ± 1.1                      |
| Mutant (C397N/C400N)              | 0.02 ± 0.015          | 35.6          | 0                               | ND                            | ND                            | 56.8 ± 1.3                      |
| Zinc-depleted helicase             | 0.28 ± 0.05           | 34.5          | 34.6 ± 1.2                      | 65 ± 2.1                      | 98 ± 1.3                      | 47.6 ± 0.9                      |

\( ^a \) Determined from Fig. 4.
\( ^b \) Determined from Fig. 5B. ND, non-detectable, i.e., no DNA binding detected up to 3 \( \mu \)M protein.

\( ^c \) Determined from Fig. 3D.

Rationale for Site-directed Mutagenesis of the Zinc Finger Motif of RecQ Helicase—Site-directed mutagenesis was used to explore the functional significance of the putative zinc finger motif of the RecQ helicase. As shown in Fig. 1, six residues (Cys^{380}, Arg^{382}, Cys^{397}, Cys^{400}, Asp^{401}, and Cys^{403}) within this region are totally conserved among RecQ family members. Mutant RecQ enzymes were first engineered with single alanine or serine substitution at the position of each of the four cysteines. In addition, a careful analysis of the three-dimensional structure of the enzyme revealed that the conformation of the zinc finger motif is obviously stabilized through three hydrogen bonds among the highly conserved residues, Arg^{381}, Asp^{401}, and Phe^{374} (Fig. 1). These interactions may contribute to the relative positioning of the helices \( \alpha_{16} \) and \( \alpha_{15} \) in the zinc finger motif (Fig. 1B). The residues Arg^{381} and Asp^{401} were thereby replaced by asparagine and alanine, respectively. We found that all of these mutants displayed a high level of proteolysis and could not be purified to homogeneity, making functional studies impossible.

To assess whether zinc ion is required for RecQ helicase function, zinc ion was extracted from wild-type enzyme by extensive dialysis against the dialysis solution (20 mM Tris-HCl at pH 7.9, 150 mM NaCl, 1 mM DTT, 5% glycerol) containing 15 mM EDTA. The obtained zinc-extracted wild-type helicase was termed zinc-depleted RecQ helicase. Therefore, three preparations of enzymes were used for the following studies: the wild-type helicase; the double mutant helicase; and the zinc-depleted helicase.
Structural Characterization of the Mutant Protein—It is well established that metal ions have important effects on secondary structure formation. We were wondering whether the purified double mutant RecQ helicase (C397N/C400N) has a normal structure. We first performed CD studies to check the effect of mutation on the secondary structure of the protein. The replacement of both cysteines with asparagines leads to a subtle modification in the secondary structure of RecQ helicase as judged from the CD spectra (Fig. 2A), suggesting that the double mutation induced slightly a local conformational change. In contrast, no significant CD spectra change was observed with the zinc-demetalated RecQ helicase. We next performed the limited proteolysis experiments on wild-type, mutant, and zinc-demetalated helicases under the same experimental conditions. Fig. 2B shows that both mutant and zinc-demetalated proteins display similar proteolysis-resistant patterns as the wild-type RecQ helicase, suggesting that the three proteins assume similar structures. These results have been further confirmed by size-exclusion chromatographic studies. Because both ultracentrifugation analyses and three-dimensional structure studies have shown that RecQ helicase takes a globular shape (17, 20), the apparent molecular masses of wild-type, double mutant, and zinc-demetalated helicases were used to estimate their Stokes radii, thus determining their overall spherical shapes. As shown in Table I, these helicases have similar Stokes radii. Taken together, these results indicate that these enzymes (wild-type, double mutant, and zinc-demetalated helicases) possess similar three-dimensional structures.

Zinc Finger Motif Is Not Required for ATP Binding—We next studied the binding of ATP to the double mutant using a fluorescent nucleotide analogue (Fig. 3A, mantATP). The spectral properties of the mant fluorophore are ideally suited for monitoring nucleotide binding by FRET from the intrinsic tryptophan fluorescence of RecQ to the mant fluorophore bound at the ATP-binding site. The comparison between the fluorescence excitation and emission spectra of RecQ and those of mantATP showed that the emission spectrum of RecQ overlaps with the excitation spectrum of mantATP (Fig. 3B), indicating the possibility of FRET.

Fig. 3. Binding equilibrium of mantATP to wild-type and mutant RecQ helicases. A, structure of mantATP. B, the overlap of the RecQ emission spectrum ($\lambda_{em} = 280$ nm) and the mantATP excitation spectrum ($\lambda_{ex} = 440$ nm). The excitation wavelength for the mantATP emission spectrum is $\lambda_{ex} = 345$ nm. C, determination of the competitive inhibition constant ($K_i$) of mantATP. The ATPase reaction was initiated by adding $2 \mu M$ ATP to a mixture of $10 \mu M$ RecQ protein, $6 \mu M$ ssDNA (nucleotide, 60-mer), and an increasing concentration of mantATP. The initial ATPase rate was measured, plotted versus inhibition concentration, and fitted to Equation 7 using $K_i = 250 \mu M$ (determined from Fig. 4A). D, changes in fluorescence intensity when $0.5 \mu M$ wild-type (squares), double mutant (closed circles), and zinc-demetalated (open circles) helicases were titrated with mantATP. The inset shows the titration curves obtained with low concentrations of mantATP. Solid lines represent the best fit of the data to Equations 5 and 6.
Although the three-dimensional structure of RecQ helicase has shown that the enzyme has only one ATP-binding site, we want to first determine whether mantATP binds to the same ATP-binding site of RecQ. For this purpose, the competitive inhibition constant, \( K_i \), for mantATP was determined by measuring the ATPase rate of the helicase as a function of mantATP concentration (Fig. 3C). The data were fit to a competitive inhibition equation with a \( K_i \) of 85 \( \mu M \), indicating that mantATP binds competitively to the ATP-binding site. The apparent \( K_d \) values for the wild-type, double mutant, and zinc-demetalated helicases were measured using standard fluorimetric titration methods. From the titration curves as shown in Fig. 3D, the apparent \( K_d \) values determined are 43.6 \( \mu M \) for the wild-type helicase, 56.8 \( \mu M \) for the double mutant helicase, and 47.6 \( \mu M \) for the zinc-demetalated helicase, revealing that neither the zinc finger nor zinc ion is essential to ATP binding. This study sheds light not only on ATP binding but also on folding of the mutant protein. The fact that the double mutant protein binds ATP normally indicates that the overall three-dimensional structure of the double mutant was not altered.

It is also interesting to note that the apparent mantATP binding constant determined in this study (\( K_i = 43.6 \mu M \)) is lower than that of ATP determined from bulk ATPase (250 \( \mu M \), Fig. 4A) and helicase assays (200 \( \mu M \)) (28). However, the constant is close to the value determined from a single molecule assay (50 \( \mu M \)). The discrepancy among these values may be due to different experimental approaches and different experimental conditions. Regardless, the FRET method used in this study for determining the ATP binding constant is a more direct approach compared with that through the measurement of enzymatic activities.

The Zinc Finger Motif Is Important for DNA-dependent ATPase and Helicase Activities—Previous studies have shown that the ATPase activity of wild-type RecQ is greatly stimulated by ssDNA and, to a less extent, by dsDNA (10). To test the role of the zinc finger motif in ATP hydrolysis, the ATPase activities of the double mutant RecQ and zinc-demetalated helicases were compared with the wild-type enzyme in the presence of ssDNA (60-mer). The mutant protein was severely compromised in DNA-dependent ATPase activities, exhibiting no detectable ATPase activities when compared with wild-type and zinc-demetalated helicases (Fig. 4A and Table I). Similar results were obtained with 3-kb linear plasmid DNA (results not shown).

The above observations demonstrate that the integrity of the zinc finger motif of RecQ is important for ATPase activity. We reasoned that the helicase activity should also be affected when the zinc binding motif is altered. As expected, although the wild-type enzyme unwinds the duplex DNA substrate completely within 5 min, no helicase activity was detectable under the same conditions for the double mutant protein (Fig. 4B) and zinc-demetalated helicases (Fig. 4A and Table I). These results demonstrate that the zinc finger motif is needed for both ATPase and helicase activities.

The Zinc Finger Motif Is Required for Stable DNA Binding—To further understand the molecular basis of the observed decrease in ATPase and helicase activities for the double mutant protein, the effect of the mutation on DNA binding was first investigated using the gel mobility shift assay under the best experimental condition that we determined previously (25). Whereas a shift of 3 kb of DNA was observed as wild-type and zinc-demetalated helicase concentrations increases (Fig. 5A, lanes 2–5), no protein-DNA complexes were observed under the same condition for mutant enzyme (Fig. 5A, lanes 6 and 7), suggesting that the DNA binding ability of mutant protein is completely compromised. However, it is still possible that the mutant protein displays weak DNA binding activities that cannot be detected by the electrophoretic mobility shift assay method due to the physical separation of free DNA from the protein. Therefore, we measured the DNA binding activities of both wild-type and mutant protein under equilibrium conditions using fluorescence anisotropy assays as performed previously (24). Fig. 5B shows that both wild-type and zinc-demetalated helicases display a high affinity for the 5'-fluorescein-labeled ssDNA. The apparent \( K_d \) values of both wild-type and zinc-demetalated helicases determined from this experiment are very close to each other (Table I). In contrast, no DNA binding activity is detectable for the mutant helicase, even at a high protein concentration (3 \( \mu M \)). Similar results were obtained with dsDNA (Fig. 5B, inset, and Table I). Together, these results demonstrate that the zinc finger motif plays an essential role in DNA binding.

Role of the Zinc Atom—The zinc ion may play roles both in...
structure and in enzymatic catalysis of RecQ. To better define the role of zinc atom, we performed three kinds of experiments. We first compared the properties of both wild-type and zinc-demetalated RecQ helicases. For this purpose, the zinc-demetalated RecQ helicase was obtained by EDTA extraction protocol (17) and analyzed in parallel with wild-type and mutant enzymes. As can be seen from Figs. 2–5 and Table I, the zinc-demetalated RecQ helicase displays very similar properties with wild-type helicase in terms of the full tertiary structure of holoenzyme, DNA binding, ATPase, and helicase activities. We next investigated the effect of the zinc atom on ATPase activity of both wild-type and zinc-demetalated RecQ helicases with increasing zinc concentrations. The results revealed that the zinc atom does not significantly influence the activities of both wild-type and zinc-demetalated RecQ helicases (Fig. 6A). Finally, the stabilities of the wild-type and zinc-demetalated helicases were assessed by measuring ATPase activity at different temperatures ranging from 25 to 54 °C. Fig. 6B shows that both enzymes display similar thermostability except at 54 °C where the zinc-demetalated enzyme displays a modest decrease in $K_{cat}$ and an increase in $K_m$. The similar thermostabilities of both enzymes indicate that zinc ion is not essential to protein stability. Taken together, these observations indicate that the zinc atom is not absolutely required for enzymatic catalysis, DNA binding, or stabilization of the protein conformation.

DISCUSSION

The three-dimensional structure of the E. coli RecQ helicase (17) reveals the existence of a zinc binding motif. In this study, the importance of this motif to RecQ helicase function was highlighted by the loss-of-function mutations at highly conserved residues within the zinc finger motif by site-directed mutagenesis. Consistent with the notion that zinc fingers are structural modules of a major ubiquitous class of DNA binding motifs (29), our data illustrated that a mutation at the zinc finger, which drastically reduces zinc binding, abrogates DNA binding to the helicase and leads to decreased ATPase and helicases activities. Furthermore, this motif is also crucial for the integrity of the whole protein.

The Zinc Finger Motif Is Essential to DNA Binding—The most striking observation in this report is that alterations of
the zinc finger motif by site-directed mutagenesis lead to a complete loss of RecQ-DNA binding ability. In view of the fact that RecQ helicase is a DNA-stimulated ATPase and an ATP-dependent helicase, the impairment of DNA binding should be the primary cause of the observed defects in ATPase and helicase activities. These observations indicate that the zinc finger motif is essential to DNA binding. Alternatively, the replacement of amino acids in the zinc finger motif leads to an overall conformational change of the helicase, which resulted in the observed defects in helicase functions. Consistent with this possibility, all of the single-point mutations were rapidly degraded, making functional analysis impossible. However, a careful characterization of the double mutant helicase indicated that the protein undergoes a subtle conformational change rather than a radical modification of the overall protein architecture. First, both wild-type and mutant enzymes displayed very similar limited proteolysis pattern. Second, the size-exclusion chromatography analysis indicated that both wild-type and mutant helicases have similar Stokes radii, suggesting that these proteins fold in overall similar fashions. Third, the fact that the mutant protein possesses almost the same affinity for mantATP binding as wild-type helicase suggests that the tertiary structure of the mutant protein is not dramatically altered. These results clearly demonstrate that a local subtle conformational change in the zinc finger motif abrogates DNA binding capability, and as a consequence, the ATPase and helicase activities were abolished. This interpretation gains support from CD experiments where the small reduction in α-helix may be attributed to the altered structure of the zinc finger motif because of the replacement of two cysteine amino acids with asparagines. The zinc finger motif thus has a dominant effect in DNA binding. We hypothesize that some conserved positively charged residues (Arg286 and Lys380) and solvent-exposed hydrophobic residues (Phe374 and Phe389) harbored by this zinc motif could be directly involved in the recognition and binding of DNA.

For other helicases without zinc finger motif within the molecules, such as PrcA, Rep, UvrD, and HCV (hepatitis C virus) helicases, their DNA binding surfaces are composed primarily of motifs Ia, IV, and V. Several lines of experimental evidence suggest that the same situation may be held in the RecQ family of helicases (13). It is also interesting to note that most RecQ family of helicases harbor a HRDC domain, which possesses DNA binding activities (16). Because the double mutant that we studied appears to keep its overall three-dimensional structure and because the helicase domain and HRDC domain could still bind DNA, the observation that DNA binding activity is completely compromised is not expected. How can we reconcile the observation that alterations of the zinc finger motif lead to the RecQ helicase losing its DNA binding activity almost completely? One of the answers to this question lies in the fact that, for RecQ helicase, the zinc finger motif plays an essential role in DNA binding, whereas both the helicase and HRDC domains may function as auxiliary DNA-binding domains. Without zinc finger motif, both helicase and HRDC domains display very low affinities for DNA binding. Indeed, in work to be published elsewhere, it has been shown that the isolated helicase domain and HRDC domain fragment proteins display very low affinity for ssDNA and dsDNA. It is likely that when DNA is bound to zinc finger motif, the helicase domain plays an essential role in unwinding activity, whereas HRDC domain may direct DNA binding specificity. Thus, it is tempting to speculate that the coordination among the zinc finger motif, the helicase domain, and HRDC will determine the DNA substrate specificity, the kinetics, and the processivity of the helicase. Here again, these analyses further enforce the notion that the zinc finger motif plays an essential role in DNA binding.

The Zinc Finger Motif Plays an Important Role in the Tertiary Structural Stability—The results from this study and the three-dimensional structural analysis reveal that the zinc finger motif is stabilized by four cysteine residues. It is important to note that altering one of the four conserved cysteine residues leads to highly unstable proteins. These results showed that the zinc finger motif is unexpectedly involved in the integrity of the whole enzyme. It is possible that, in addition to its DNA binding function, the zinc finger motif also functions in the folding cascade, linking the domains to each other to stabilize the protein tertiary structure. In addition to the four highly conserved cysteine residues, the zinc finger motif could be further stabilized through very important interactions among α-helices 16, 17, and 18. It appears that the hydrogen bonds between Phe374 and Arg381 and between Arg381 and Asp401 play an important role in stabilizing the zinc finger motif and the whole protein structure. Consistent with this postulation, the mutation of Arg381 or Asp401 leads to the enzyme becoming very sensitive to protease degradation. Similar results were observed with the Bloom syndrome protein (30). The replacement of Asp1064 (which is located at a position equivalent to that of Asp401 of RecQ helicase) with Ala substantially reduced DNA binding and helicase activities, whereas the protein displayed essentially the same activities as the wild-type enzyme when Asp1064 was replaced by Asn. This phenomenon is probably the result of the properties of the side chain of the replacing residues. Asn probably could still possibly establish hydrogen bond with Arg1037, thus stabilizing the protein structure, whereas

3 J. L. Liu and X. G. Xi, unpublished observation.
discovered, RecQ enzymes without perfect folding of the zinc enzyme to this small subdomain. Although much remains to be selected a complex mechanism to link all of the functions of the binding and folding of the whole enzyme. Thus, evolution has motif in the RecQ helicase plays important roles both in DNA binding and zinc-finger motif have a quite relevant role in determining the full development of secondary structure in metalloproteins, whereas demetalated proteins still have well defined tertiary structures (31).

Biological Relevance of the Cysteine Cluster to the RecQ Helicase Family—Amino acid sequence alignment of several RecQ helicases revealed that the cysteine residues involved in the formation of zinc finger in E. coli helicases are conserved among the RecQ family of helicases (Fig. 1). Available evidence indicates that mutations of the cysteine residues in the zinc finger motif in most RecQ family of helicases can have dramatic effects on the enzymatic activities. Two disease-causing Bloom missense mutations map to Cys1036 and Cys1055, respectively (32, 33), among four conserved cysteine residues in the zinc finger motif. In vitro analysis shows that these mutations abolish BLM ATPase and helicase activities (34). In vitro studies of the RecQ core of the Bloom syndrome protein have shown that when three of the four cysteines in the zinc finger motif were mutated, respectively, the resulting mutants were very unstable. Furthermore, even mutations of residues near the conserved cysteine residues such as R1038A and D1064A prevented the modified enzymes from binding DNA and caused them to lose ATPase and helicase activities (29). It has also been shown that this region is essential to in vitro function of the yeast RecQ homologue Sgs1 (35, 36).

Sequence comparisons among the DNA helicases suggest that the zinc finger motif appears to be unique to the RecQ helicase family. Both the sequence analysis and three-dimensional structural studies showed that other DNA helicases, such as T7 gene 4 helicase (37), RepA (39), and hepatitis C virus NS3 helicases (40), do not use the zinc finger motif for DNA binding. Whether the use of zinc finger motif for DNA binding is a unique feature of RecQ family of helicases is currently being investigated in our laboratory. In summary, the present study reveals that the zinc finger motif in the RecQ helicase plays important roles both in DNA binding and folding of the whole enzyme. Thus, evolution has selected a complex mechanism to link all of the functions of the enzyme to this small subdomain. Although much remains to be discovered, RecQ enzymes without perfect folding of the zinc finger motif would indeed be unable to ensure the helicase functions.

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