BIOCHEMICAL AND KINETIC CHARACTERIZATION
OF THE DNA HELICASE AND EXONUCLEASE
ACTIVITIES OF WERNER SYNDROME PROTEIN

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Running Title: Biochemical and kinetic analyses of WRN catalytic functions
ABSTRACT

The WRN gene, defective in the premature aging and genome instability disorder Werner Syndrome, encodes a protein with DNA helicase and exonuclease activities. In this report, cofactor requirements for WRN catalytic activities were examined. WRN helicase performed optimally at an equimolar concentration (1 mM) of Mg$^{2+}$ and ATP with a K_m of 140 µM for the ATP-Mg$^{2+}$ complex. Initial rate of WRN helicase activity displayed a hyperbolic dependence on ATP-Mg$^{2+}$ concentration. Mn$^{2+}$ and Ni$^{2+}$ substituted for Mg$^{2+}$ as a cofactor for WRN helicase whereas Fe$^{2+}$ or Cu$^{2+}$ (10 µM) profoundly inhibited WRN unwinding in the presence of Mg$^{2+}$. Zn$^{2+}$ (100 µM) was preferred over Mg$^{2+}$ as a metal cofactor for WRN exonuclease activity and acts as a molecular switch, converting WRN from a helicase to an exonuclease. Zn$^{2+}$ strongly stimulated the exonuclease activity of a WRN exonuclease domain fragment, suggesting a Zn$^{2+}$ binding site in the WRN exonuclease domain.

A fluorometric assay was used to study WRN helicase kinetics. The initial rate of unwinding increased with WRN concentration, indicating that excess enzyme over DNA substrate improved the ability of WRN to unwind the DNA substrate. Under pre-steady state conditions, the burst amplitude revealed a 1:1 ratio between WRN and DNA substrate, suggesting an active monomeric form of the helicase. These are the first reported kinetic parameters of a human RecQ unwinding reaction based on real time measurements and provide mechanistic insights into WRN-catalyzed DNA unwinding.
INTRODUCTION

Werner Syndrome (WS) is a human autosomal recessive disorder that displays symptoms of premature aging and an increased incidence of cancer (1). Cellular phenotypes of WS include genomic instability (2-4), aberrant recombination (5-7), sensitivity to DNA damaging agents (8-11), and replication defects (12-14). The gene (WRN) defective in WS encodes a protein that belongs to the RecQ family of DNA helicases (15), which includes four other human helicases, including the genes defective in the chromosomal instability disorders Bloom syndrome (BLM) (16) and Rothmund-Thomson syndrome (RecQL4) (17). In addition to the conserved helicase motifs, WRN contains a region of similarity to the 3’ to 5’ exonuclease domain of E. coli DNA polymerase I and RNase D (18). In addition to its catalytic domains, WRN interacts with a number of proteins involved in DNA metabolism, suggesting important roles in cellular pathways of DNA replication, repair, and/or recombination (19;20).

It is generally believed that RecQ helicases play an important role in the maintenance of genome stability (21-23); however, the precise molecular and cellular functions of RecQ helicases are not well understood. Although the DNA substrate specificity of WRN helicase has been studied in some detail (24), the mechanism by which WRN catalyzes DNA unwinding is not known. WRN, like all other DNA helicases characterized to date, utilizes the energy from nucleotide hydrolysis to unwind double-stranded (ds) DNA (25-28). Although the nucleotide preference of WRN helicase and exonuclease activities has been examined (29;30), little is known about the optimal solution conditions for WRN catalytic activities. Recent work from the Kowalczykowski lab demonstrated that E. coli RecQ helicase activity is sensitive to the ratio of magnesium ion to ATP concentration with an optimal ratio of 0.8 and a free magnesium ion concentration of 50 µM (31). In addition, E. coli RecQ helicase activity displayed a sigmoidal dependence on ATP concentration, suggesting multiple interacting ATP sites (31). However, the assembly state of E. coli RecQ (32) and other RecQ helicases (33-37) remains open to debate.
Since little is known about the cofactor requirements for WRN helicase and exonuclease activities, we examined these parameters in this study. Evidence is presented that WRN helicase behaves similar to *E. coli* RecQ with respect to optimal Mg\(^{2+}\): ATP ratio for DNA unwinding, but displays distinct differences with respect to the effects of free Mg\(^{2+}\) ion and ATP concentrations on DNA unwinding activity. The ability of other divalent metals to substitute for magnesium in the WRN helicase reaction is metal-specific, and certain metal ions potently inhibited WRN helicase activity or stimulated WRN exonuclease activity. These results indicate that DNA metabolic processing by WRN helicase or exonuclease activities can be modulated by the availability of free metal ions.

To better understand the WRN helicase mechanism, we have utilized a fluorometric assay to monitor helicase-catalyzed unwinding of duplex DNA (38). This assay uses the principle of fluorescence resonance energy transfer (FRET) to observe the unwinding of duplex DNA in real time. A forked duplex DNA substrate, the preferred B-form DNA structure of WRN helicase (24), with fluorescein (F) covalently attached to the 3’ blunt end and hexachlorofluorescein (HF) attached to the 5’ blunt end of the duplex DNA molecule was employed for the kinetic analyses (Fig. 1). F is excited at a wavelength of 492 nm and emits a wavelength of 520 nm, which lies in the excitation spectrum of HF (Fig. 1A). FRET occurs between F (donor) and HF (acceptor) when both are in close proximity, during the duplex state of the substrate. However, upon helicase-catalyzed unwinding of the duplex and separation of the complementary strands, F and HF are no longer in close proximity, and the fluorescence emission from F excitation can be detected by a photosensor (Fig. 1B). This method of measuring helicase-catalyzed unwinding of duplex DNA using fluorescence stopped-flow instrumentation is valuable for kinetic analyses, where data is collected continuously throughout the reaction in real time.

We have applied FRET technology to study the kinetics of WRN helicase activity under pre-steady state conditions, in which the enzyme is saturated with an excess of DNA substrate (39). The results from pre-steady state kinetic studies were used to
determine the initial burst kinetics of DNA unwinding, enabling us to determine the rate of DNA unwinding by WRN under the assigned reaction conditions. Mechanistic information on the WRN helicase reaction was determined from these analyses and provides the first kinetic parameters of a human RecQ unwinding reaction based on real time measurements.

MATERIALS AND METHODS

**Proteins** Recombinant hexahistidine-tagged full-length WRN proteins (wild-type WRN or the exonuclease point mutant WRN-E84A) were overexpressed using a baculovirus/Sf9 insect system and purified as previously described (40). A recombinant hexahistidine-tagged truncated form of WRN (amino-terminal 368 amino acids, designated N-WRN) was overexpressed using the baculovirus/Sf9 insect system and purified as previously described (41). Purified recombinant UvrD and TraI helicas were kindly provided by Dr. Steve Matson (University of North Carolina at Chapel Hill). Purified recombinant BLM helicase (36) was kindly provided by Dr. Ian Hickson (Cancer Research UK Laboratories). Recombinant RECP1 helicase was overexpressed in insect cells using a baculovirus encoding recombinant human RECP1 kindly provided by Dr. Alessandro Vindigni (International Centre for Genetic Engineering and Biotechnology) and purified as described elsewhere (unpublished data).

**DNA Substrates** PAGE-purified oligonucleotides (FLAP26 and TSTEM25, (24)) were purchased from Midland Certified Reagent Co. FLAP26 and TSTEM25 with F and HF, respectively, were obtained from Proligo. DNA substrates were prepared as previously described (24) with the exception that DNA substrates used for fluorometric assays were not 5′-32P end-labeled.

**Fluorometric helicase kinetic assays** Fluorescence stopped-flow kinetic experiments were performed using Applied Photophysics SX.18MV Stopped-Flow Reaction Analyser. The instrument was equipped with a 150 W xenon lamp and the monochromator was set to a slit width of 1 mm. Fluorescein was excited at a wavelength of 492 nm and the fluorescence emission was monitored at wavelengths greater than 520
nm with the 51300 cut-on filter from Oriel Corporation. Experiments were carried out in the two syringe mode, where WRN and ATP were preincubated at 37°C in one syringe for one min while the DNA substrate was preincubated at 37°C in the second syringe. Each syringe contained 30 mM HEPES (pH 7.4), 5% glycerol, 40 mM KCl, the indicated concentration of MgCl₂, and 100 ng/ul bovine serum albumin (BSA). Concentrations of WRN, ATP, and DNA fork substrate in the syringe were double that of the indicated final concentration in the reaction. Equal volumes (60 µl) of sample from both syringes were mixed to initiate the reaction, which took place at 37°C. One thousand data points were collected from monitoring 20 µl of each kinetic time course reaction. For converting the output data from volts to percent unwinding, a time course with the same set up was performed, except with only the fluorescein oligonucleotide instead of the fluorescent forked duplex substrate. Data was then normalized by defining the voltage obtained with the fluorescein oligonucleotide as 100% unwinding.

**Radiometric helicase assays** Helicase assay reactions (20 µl) contained 30 mM HEPES (pH 7.4), 5% glycerol, 40 mM KCl, 100 ng/ul BSA, 0.8 nM DNA fork substrate, and the indicated concentrations of MgCl₂ and/or specified metal, ATP, and WRN. For time course assays imitating the fluorometric assay, WRN and ATP were preincubated in a tube together, while DNA was preincubated separately at 37°C for 1 min. Reactions were initiated by mixing the contents of both tubes together and 20 µl samples were quenched at the indicated times. For metal inhibition or substitution assays, the metal and WRN were preincubated on ice for 3 min before the initiation of a 15 min reaction at 37°C by adding DNA fork substrate. Reactions were quenched with 20 µl stop buffer (35 mM EDTA, 0.6% SDS, 25% glycerol, 0.04% bromophenol blue, 0.04% xylene cyanol) containing a 10-fold excess of unlabeled oligonucleotide of the same sequence as the labeled strand of the DNA fork substrate. The products of the helicase reactions were resolved on nondenaturing 12% polyacrylamide gels. Radiolabeled DNA species in polyacrylamide gels were visualized using a PhosphorImager and quantitated using the ImageQuant software (Molecular Dynamics). The percent helicase substrate unwound was calculated by the formula: percent unwinding = 100 x (P/(S + P)), where P is the product and S is the residual substrate. The values of P and S have been corrected after
subtracting background values in controls having no enzyme and heat-denatured substrate, respectively. Helicase data represent the mean of at least three independent experiments with mean ± standard deviation (SD) shown by error bars.

**Radiometric exonuclease assays** Exonuclease assay reactions (20 µl) contained the same components as in radiometric helicase assays, except that Zn²⁺ was substituted for Mg²⁺ where indicated. The metal was preincubated with WRN for 3 min on ice before initiating the 15 min reaction at 37°C with radiolabeled fork substrate. Each reaction was quenched with 10 µl stop buffer (80% formamide, 0.5X TBE, 0.1% xylene cyanol, 0.1% bromophenol blue) and heated at 95°C for 10 min. Products were resolved on denaturing 14% polyacrylamide gels and visualized by a PhosphorImager.

**RESULTS**

To better understand the catalytic functions of the Werner syndrome protein and how they may be important in DNA metabolism, we have investigated the cofactor requirements for WRN helicase and exonuclease activities. In addition, we have examined the kinetics of WRN-catalyzed DNA unwinding to better understand its helicase mechanism.

**Fluorescence Resonance Energy Transfer (FRET) for measuring WRN helicase unwinding of duplex DNA**

To begin to understand the mechanism of duplex DNA unwinding catalyzed by the WRN helicase, we have performed kinetic analyses of the unwinding reaction using a fluorometric helicase assay and a more traditional gel-based assay that uses a radiolabeled DNA substrate. The advantage of the fluorometric assay is that helicase activity can be measured in real time, enabling one to determine kinetic parameters that rely on fast kinetic analysis in the millisecond scale. The conventional gel-based assay enabled us to conveniently determine optimal divalent cation and ATP concentrations for WRN helicase activity using smaller amounts of enzyme and to substantiate our helicase data from fluorescence assays to verify that we were, in fact, measuring DNA unwinding.
Combining these approaches has enabled us to investigate the kinetics of WRN helicase activity under optimized reaction conditions.

The DNA substrate that we elected to use for these studies was based on our previous work (24) that defined the minimal length requirements for the 5' and 3' single-stranded (ss) DNA tails of forked duplex substrates required by WRN for efficient unwinding. Based on these results, we examined WRN helicase activity on an optimal forked DNA substrate with a 19 bp duplex flanked by tail lengths of 25 nucleotides (3') and 26 nucleotides (5'). As shown in Fig. 1, fluorescein (F) was positioned on the 3' blunt end, whereas hexachlorofluorescein (HF) was positioned on the 5' blunt end. Since the 3' to 5' exonuclease activity of WRN can attack the 3' blunt end of a forked duplex DNA molecule (42), it was a concern to optimize WRN helicase activity while minimizing or obstructing its exonuclease activity. Under conditions of DNA unwinding (presence of ATP), the DNA oligonucleotides of the helicase substrate lacking the F and HF moieties, were not appreciably degraded by WRN exonuclease activity as judged by quantitation of DNA products resolved on denaturing gels (24). However, it was possible that a minimal amount of fluorescent signal may have been attributed to a small population of DNA helicase substrates whose fluorescein molecules had been removed by WRN exonuclease activity. Denaturing gel analysis of WRN reaction products from a radiolabeled helicase substrate possessing the F and HF moieties demonstrated that WRN exonuclease activity was completely blocked from attacking the DNA substrate, even in the absence of ATP (data not shown). From these results, we were able to conclude that the F/HF conjugates on the blunt end of the helicase substrate effectively blocked WRN exonuclease activity. Hence, we did not have to be concerned about the interference of any fluorescent signal attributed to the incision of the F label on the 3' blunt end by WRN exonuclease activity.

In order to substantiate the fluorescence-based helicase assay, we collected and compared data from fluorometric and radiometric assays. This was accomplished by performing WRN helicase reactions under identical conditions using either assay and then comparing the results from fluorescence measurements with those from native gel
analysis of radiolabeled products obtained by chemical quench at specific time points throughout the 200 sec time course. For the radiometric assay, the fluorescent DNA substrate (with covalently attached F and HF moieties) was 5’-32P end-labeled. Throughout the fluorescence-based time course, we observed the output voltage signal increase with time, which was presumably due to the increase in the number of excited F molecules which were no longer in close proximity to HF upon unwinding of the duplex substrate by WRN. The fluorescence data was normalized by defining the voltage obtained with only the F-labeled single-stranded oligonucleotide as 100% unwinding of the substrate (43). This enabled us to convert the voltage output data to percent unwinding. The results from these parallel kinetic experiments using 0.8 nM DNA substrate and 12 nM WRN are shown in Fig. 2A. Helicase data points from the radiometric assay (large filled circles) were overlayed on the normalized fluorometric helicase data and found to agree well. This comparison substantiated the fluorometric assay for measuring WRN helicase activity.

Next, we examined WRN helicase activity using the fluorometric assay at a fixed concentration of forked duplex DNA substrate (0.8 nM) and a range of WRN protein concentrations. At each WRN concentration, fluorescence data was obtained for a time course of 200 sec. For each WRN protein concentration, the fluorescent signal (voltage) increased with respect to time. At lower WRN concentrations (0.6 and 3 nM), output voltage continued to increase throughout the time course whereas a signal plateau was approached by 100 sec in reactions containing higher WRN concentrations (16 or 32 nM). The fluorescent traces from reactions containing 16 and 32 nM WRN were superimposable, suggesting that the DNA substrate was saturated with WRN enzyme at these concentrations. The data was normalized and converted to percent unwinding with respect to time, as shown in Fig. 2B. Initial rates of unwinding were determined for each time course and are listed in Table I. As WRN protein concentration increased, initial rates of unwinding also increased. However, this increase in initial rates was seen only up to the saturation point (i.e. 16 nM WRN).

Optimal ATP-Mg\(^{2+}\) concentration for WRN helicase activity
Before initiating a kinetic analysis using the fluorometric assay, we were interested in optimizing the reaction conditions for WRN helicase activity, which had not been done previously. Since the ATP-Mg$^{2+}$ complex is the source of chemical energy for helicase-catalyzed unwinding, and therefore a critical component of the helicase reaction, we focused our efforts to determine optimal concentrations of Mg$^{2+}$ and ATP for WRN helicase activity. Initially, we sought to determine an estimated ratio between ATP and Mg$^{2+}$ where the greatest initial rate of WRN unwinding would be detected and then to determine the optimal ATP-Mg$^{2+}$ concentration. For these purposes, we used the radiometric assay because significantly smaller reaction volumes can be used compared to the fluorometric assay. The optimal Mg$^{2+}$: ATP ratio was determined by maintaining ATP at a fixed concentration of 2 mM and titrating in various concentrations of MgCl$_2$. A 20 sec time course was performed at each MgCl$_2$ concentration and the initial rates of unwinding were plotted as a function of the [Mg$^{2+}$]: [ATP] ratio (Fig. 3). WRN helicase activity increased fairly linearly with Mg$^{2+}$: ATP ratios up to 1; greater Mg$^{2+}$: ATP ratios of up to 4 did not significantly further increase or decrease WRN unwinding. A Mg$^{2+}$: ATP ratio of 1 was determined to be optimal for WRN helicase activity. This was confirmed by carrying out the same set of experiments using 5 mM ATP, which gave a similar profile as that obtained with 2 mM ATP (Fig. 3). Each set of data was best fit to a sigmoidal curve, which displayed the optimum at a Mg$^{2+}$: ATP ratio of 1.

We next performed experiments to determine the optimal concentration of ATP-Mg$^{2+}$ for WRN helicase activity. Twenty sec time courses were performed for a series of WRN helicase reactions in which the ATP-Mg$^{2+}$ complex, maintained at a 1:1 ratio, was increased. As shown in Fig. 4, initial rates of WRN helicase activity increased with an initial hyperbolic regime, followed by a peak regime, and then a significant decrease in initial rate after 1 mM ATP-Mg$^{2+}$. Applying a Michaelis-Menton kinetic analysis to the initial hyperbolic regime of the plot shown in Fig. 4 yielded a $K_m$ value of 140 µM ATP for the WRN helicase reaction when ATP and Mg were in equimolar concentrations. The optimum concentration of ATP-Mg$^{2+}$ for WRN helicase activity, determined to be 1 mM, was used for subsequent WRN helicase studies.
Metal ion effects on WRN helicase activity

The WRN helicase reaction is dependent on nucleoside triphosphate hydrolysis; however, little or no information is available on the divalent metal cofactor requirements of WRN. Hence, we were interested in determining whether other metal ions could substitute for Mg\(^{2+}\) in the WRN unwinding reaction. Mg\(^{2+}\) (1 mM) was substituted with 1 mM of the following metal ions: lead Pb (II), arsenic As (III), iron Fe (II), cadmium Cd (II), cobalt Co (II), nickel Ni (II), copper Cu (II), chromium Cr (II), zinc Zn (II), and manganese Mn (II). The results demonstrate that Ni\(^{2+}\) and Mn\(^{2+}\) could substitute for Mg\(^{2+}\) in the WRN helicase reaction (Fig. 5A, lanes 8 and 12 compared to lane 2). Mg\(^{2+}\) and Mn\(^{2+}\) served as a better cofactor for WRN helicase activity compared to Ni\(^{2+}\) since the extent of unwinding in the 15 min reaction was less in the presence of Ni\(^{2+}\) compared to Mg\(^{2+}\) or Mn\(^{2+}\). Substitution of Mg\(^{2+}\) with Zn\(^{2+}\) in the WRN helicase reaction resulted in the appearance of two novel radiolabeled species resolved on native gels that were suggestive of WRN exonuclease activity (Fig. 5A, lane 11). To address this possibility, we performed radiometric helicase assays with the forked duplex substrate using an exonuclease deficient mutant WRN protein (WRN-E84A, designated X-WRN), under reaction conditions identical to those used for wild-type WRN protein. Fig 5B shows that Zn\(^{2+}\) was unable to substitute for Mg\(^{2+}\) in the X-WRN helicase reaction (lane 3). The anomalously migrating products from wild-type WRN helicase reactions (Fig. 5A, lane 11) were not evident in the reactions with X-WRN, suggesting that Zn\(^{2+}\) was unable to substitute for Mg\(^{2+}\) in the helicase reaction and that the products of the wild-type WRN reaction in the presence of Zn\(^{2+}\) were presumably the result of exonuclease activity only. These results suggest that Zn\(^{2+}\) and Mg\(^{2+}\) may differentially affect WRN exonuclease activity, a topic that was further investigated (see below).

Adverse effects of metals on DNA metabolic processes such as the fidelity of DNA synthesis (44) may contribute to the toxicity of certain environmental metal compounds. Evidence suggests that environmental metals are co-mutagenic by inhibiting DNA repair (45;46). Since WRN is suggested to have a role in DNA repair and replication, we sought to evaluate the effects of metal ions on WRN by examining WRN helicase activity in the presence of various metals at low micromolar concentrations in
reactions that also contained a physiological concentration of Mg$^{2+}$ (1 mM). First, we tested each metal at a final concentration of 10 µM and found that Fe$^{2+}$ and Cu$^{2+}$ very effectively inhibited WRN unwinding (Fig. 6, lanes 5 and 9). We next titrated increasing concentrations of Fe$^{2+}$ and Cu$^{2+}$ to determine if inhibition was dose-dependent. Cu$^{2+}$ showed a dramatic inhibition of WRN helicase activity at 10 µM, but did not significantly inhibit at lower concentrations (Fig. 7, open circles). Fe$^{2+}$ inhibited WRN helicase activity to a greater extent with increasing concentrations of metal starting at 2.5 µM where unwinding was reduced by 38% compared to the control (no Fe$^{2+}$) reaction (Fig. 7, closed circles). At the highest level tested (10 µM), Fe$^{2+}$ and Cu$^{2+}$ each resulted in an inhibition of ~90%. WRN helicase reactions were also performed in the presence of 2 mM Mg$^{2+}$ and 10 µM metal to determine if Mg$^{2+}$ concentration had any effects on the inhibition by any of the metals tested; however, similar results were obtained in the presence of 1 or 2 mM MgCl$_2$ (data not shown).

To address if the inhibition of helicase activity by Fe$^{2+}$ or Cu$^{2+}$ was specific to WRN, we tested the effects of these metals on UvrD (6 nM) and TraI (5 nM), two well characterized DNA helicases with opposing polarities of unwinding that do not belong to the RecQ family (47;48). For either helicase, neither Fe$^{2+}$ nor Cu$^{2+}$ inhibited DNA unwinding under reaction conditions identical to those used for WRN assays (data not shown). Since WRN helicase activity was potently inhibited by Fe$^{2+}$ or Cu$^{2+}$, we speculated that other RecQ helicases might also be negatively affected by these metal ions. To address this, we tested the effects of Fe$^{2+}$ or Cu$^{2+}$ (10 µM) on the unwinding activity catalyzed by human BLM (5 nM) or RECQ1 (20 nM) helicases. As shown in Fig. 8A, Fe$^{2+}$ inhibited BLM helicase activity by 90% (lane 3) whereas Cu$^{2+}$ completely inhibited BLM unwinding (lane 4). RECQ1 unwinding activity was inhibited ~80% by Fe$^{2+}$ and Cu$^{2+}$ (Fig. 8B, lanes 3 and 4). These results suggest that the Fe$^{2+}$ or Cu$^{2+}$ inhibition of WRN helicase activity may be specific to RecQ helicases. In support of the idea that the metal inhibition of WRN helicase activity was mediated by protein inactivation, Fe$^{2+}$ adversely affected WRN unwinding activity when the metal was preincubated with the WRN protein in the absence of DNA substrate, but did not have as great a negative effect when the metal was preincubated with the DNA substrate prior to
the initiation of the helicase reaction with the addition of WRN enzyme (data not shown). A possible mechanism for the metal inhibition of WRN helicase activity was suggested by the identification of a Zn$^{2+}$ binding domain in a C-terminal domain of *E. coli* RecQ that is conserved by sequence homology in WRN (49). It was conceivable that the competing metal ion displaced the putative Zn$^{2+}$ ion bound to WRN, thereby impairing the structure of WRN protein and its helicase activity. Simultaneous or sequential preincubation of WRN with exogenous Zn$^{2+}$ (10 µM) and the inhibiting metal (i.e. 10 µM of Fe$^{2+}$ or Cu$^{2+}$) did not reverse the inhibition in reactions containing of 1 mM ATP-Mg$^{2+}$ (data not shown). In simultaneous preincubation, equimolar concentrations of Fe$^{2+}$ or Cu$^{2+}$ and Zn$^{2+}$ were preincubated with WRN at the same time for 3 min. In sequential preincubation, Zn$^{2+}$ was preincubated with WRN for 3 min prior to a further 3 min preincubation after the addition of Fe$^{2+}$ or Cu$^{2+}$. Reactions were then initiated with the addition of DNA substrate. Since no reversal of the Fe$^{2+}$/Cu$^{2+}$ inhibition by Zn$^{2+}$ was observed, these results suggest that WRN may have a higher affinity for Fe$^{2+}$ or Cu$^{2+}$ than the proposed Zn$^{2+}$ ion or that competition between Fe$^{2+}$ or Cu$^{2+}$ with Zn$^{2+}$ was not responsible for the helicase inhibition. Other mechanisms such as metal-induced protein aggregation (50) may be responsible for the inactivation of WRN helicase activity.

**Pre-steady state kinetic analysis of WRN helicase activity**

Having established the optimal concentration of ATP and Mg$^{2+}$ for the WRN helicase reaction, we began a kinetic analysis using the fluorometric assay. We were interested in determining the burst kinetics for WRN-catalyzed unwinding under conditions where we could assert that each WRN molecule was active in unwinding DNA substrate. This could be done under pre-steady state conditions, which can be defined as having excess substrate concentration over enzyme concentration (39). Under these conditions, it can be asserted that each enzyme is engaged by substrate. Our primary interest was to determine the initial burst kinetics that could be attributed to the first round of products formed by WRN. Pre-steady state conditions were established by using 75 nM DNA substrate and 4, 8, or 16 nM WRN with an optimal ATP-Mg$^{2+}$ concentration of 1 mM. A 20 sec time course was carried out using the fluorescence-based assay. To address the question of whether there was reannealing of the unwound
strands of the helicase substrate, we carried out a fluorometric assay where we included 75 nM of the oligonucleotide containing the F moiety and 75 nM of the oligonucleotide containing the HF moiety. We observed for quenching of the fluorescent signal in the first 20 sec of the reaction, which would correlate to the annealing of the two complementary oligonucleotides, and saw that there was little to no annealing of the complementary strands (data not shown).

As shown in Fig. 9, the resulting curve showed biphasic kinetics. The first phase is referred to as the pre-steady state phase, whereas the second phase is referred to as the steady state phase. Here, we were interested in analyzing the pre-steady state phase, which gives the initial burst of products. For each level of WRN, the pre-steady state phase achieved an amplitude at a concentration of unwound DNA which was very close to the concentration of WRN enzyme in the reaction (i.e. 4, 8, or 16 nM). The WRN:DNA substrate ratio of 1:1 suggests that a 19 bp duplex DNA substrate is unwound by a single WRN molecule under these conditions. Furthermore, the burst rate can be defined as the slope of the initial linear regime of the pre-steady state phase. Taking the slope of this regime gives the initial pre-steady state rate, which was much faster from the rate obtained from doing the same analysis with the steady state regime. Rates and amplitudes are tabulated in Table II.

**Effects of Zn\textsuperscript{2+} and Mg\textsuperscript{2+} on WRN Exonuclease Activity**

The unique products of reactions containing WRN, ATP, Zn\textsuperscript{2+} and the forked duplex substrate that were detected on native gels (Fig. 5A, lane 11) suggested that WRN catalytic function is altered by Zn\textsuperscript{2+} compared to Mg\textsuperscript{2+}. Since Zn\textsuperscript{2+} did not serve as a cofactor for the WRN helicase reaction, we wanted to better understand the proposed effect of Zn\textsuperscript{2+} on WRN exonuclease activity. To address this, the products of WRN reactions containing 1 mM ATP and increasing concentrations of Zn\textsuperscript{2+} up to 1 mM were examined on denaturing polyacrylamide gels. A Zn\textsuperscript{2+} dose-dependent increase in WRN exonuclease activity was observed (Fig. 10A). WRN exonuclease activity was detectable at 100 µM Zn\textsuperscript{2+} concentration (Fig. 10A, lane 5), but little to no degradation was observed at 10 or 50 µM Zn\textsuperscript{2+} (Fig. 10A, lanes 3 and 4). At 1000 µM Zn\textsuperscript{2+}, WRN
excised up to ~16 nt of the 19 bp forked duplex substrate (lane 7). In contrast, at 1000 
µM Mg\textsuperscript{2+}, WRN excised only a small fraction of the DNA substrate, removing only a 
single nt (lane 8). The low level of WRN exonuclease activity in the presence of Mg\textsuperscript{2+} 
and ATP can be explained by the robust unwinding of the forked duplex DNA substrate 
by WRN (Fig. 5A, lane 2), resulting in intact ssDNA that is not efficiently acted upon by 
WRN exonuclease when only Mg\textsuperscript{2+} and/or ATP is present (34;53). Collectively, these 
results demonstrate that in contrast to Mg\textsuperscript{2+}, Zn\textsuperscript{2+} dramatically stimulated WRN 
exonuclease activity on the forked duplex DNA substrate in the presence of ATP, but 
failed to serve as a cofactor for WRN catalyzed DNA unwinding.

We were next interested in comparing the abilities of Zn\textsuperscript{2+} and Mg\textsuperscript{2+} to serve as 
cofactors for WRN exonuclease activity under conditions where WRN-catalyzed DNA 
unwinding does not occur (absence of ATP). At the lowest concentrations of either Zn\textsuperscript{2+} 
or Mg\textsuperscript{2+} (10, 50 µM), little to no WRN exonuclease activity was detectable (Fig. 10B, 
lanes 3-6). At 100 µM Zn\textsuperscript{2+}, WRN exonuclease efficiently excised the forked duplex 
substrate, removing up to ~17 nt from the 3’ blunt end (lane 7). In contrast, little to no 
exonucleolytic digestion by WRN was observed in reactions containing 100 µM Mg\textsuperscript{2+} 
(lane 8). At higher concentrations of divalent cation (500, 1000 µM Zn\textsuperscript{2+} or Mg\textsuperscript{2+}), 
digestion of the DNA substrate was reproducibly slightly greater in the presence of Mg\textsuperscript{2+} 
compared to Zn\textsuperscript{2+} (lanes 9 to 12). To confirm that the exonuclease activity stimulated by 
Zn\textsuperscript{2+} was intrinsic to the WRN protein, we tested the effect of Zn\textsuperscript{2+} on an exonuclease 
defective WRN mutant protein (X-WRN). X-WRN did not have exonuclease activity in 
the presence of Mg\textsuperscript{2+} (Fig. 10C, lanes 3 and 6), whereas minimal degradation was 
observed for X-WRN in the presence of Zn\textsuperscript{2+} (Fig. 10C, lanes 4 and 7). We conclude 
from these analyses that Zn\textsuperscript{2+} is clearly preferred as a metal cofactor for WRN 
exonuclease activity at a low concentration of metal (100 µM).

The sequence homology of the N-terminal region of WRN, harboring the 
exonuclease domain, with a region of *E. coli* DNA polymerase I that catalyzes 3’ to 5’ 
exonuclease activity (18) suggests that the two proteins may have a similar mechanism 
for the exonuclease reaction. Structural data from *E. coli* DNA polymerase I crystals
indicated two metal binding sites in the protein (54). A two metal ion mechanism for the 3’ to 5’ editing exonuclease reaction of DNA polymerase I was proposed with Zn\(^{2+}\) bound to site A and Mg\(^{2+}\) bound to site B (54). Based on this work, we hypothesized that the stimulation of WRN exonuclease activity by Zn\(^{2+}\) might be attributed to a Zn\(^{2+}\) binding domain in the N-terminal exonuclease domain of WRN as opposed to interaction of Zn\(^{2+}\) with a putative Zn\(^{2+}\) binding domain in the C-terminal region of WRN protein (49). Therefore, we tested the ability of Zn\(^{2+}\) to serve as a cofactor for the exonuclease activity of N-WRN, a recombinant protein fragment that harbors the exonuclease domain of WRN but lacks the helicase and C-terminal domains. At a low concentration of divalent cation (100 µM), Zn\(^{2+}\) strongly stimulated the exonuclease activity of N-WRN, resulting in nearly complete digestion of the top strand of the forked duplex DNA substrate (Fig. 10D, lane 4). The extent of degradation by N-WRN in the presence of 100 µM Zn\(^{2+}\) was remarkable as evident by the appearance of ssDNA chains ranging from 14-22 nt. Thus N-WRN, in the presence of Zn\(^{2+}\), exonucleolytically removed from the 3’ blunt end the entire portion of the DNA strand that was annealed to its complementary strand (19 nt), and extended the degradation into the ssDNA region of the 5’ noncomplementary tail of the forked duplex substrate. In contrast, N-WRN exonuclease activity on the forked duplex DNA substrate was not detectable in the presence of 100 µM Mg\(^{2+}\) (Fig. 10D, lane 3). As observed for the full-length WRN protein (Fig. 10A, lane 7), N-WRN also exonucleolytically degraded the forked duplex substrate in the presence of 1 mM ATP and 1000 µM Zn\(^{2+}\) (Fig. 10D, lane 7), but the extent of degradation was not as great as that observed for N-WRN in the presence of 100 µM Zn\(^{2+}\) when ATP was absent. Although Mg\(^{2+}\) can serve as a cofactor for N-WRN exonuclease activity, a greater concentration (4 or 5 mM) is required for significant exonuclease activity (41;53).

Under physiological conditions, both ATP and Mg\(^{2+}\) are present in the cell. We were therefore curious to learn if WRN exonuclease activity might be enhanced by the presence of Zn\(^{2+}\) on a forked DNA substrate that WRN efficiently unwinds when ATP-Mg\(^{2+}\) (1 mM) is available (Fig. 4 and Fig. 5A, lane 2). As shown in Fig. 10E, Zn\(^{2+}\) stimulated WRN exonuclease activity in a dose-dependent fashion up to 500 µM Zn\(^{2+}\).
At 500 µM Zn\textsuperscript{2+}, WRN exonuclease activity effectively removed up to ~10 nt from the 3' blunt terminus of the forked duplex substrate; in addition, removal of up to ~16 nt of the 19 bp duplex region was evident to a lesser extent (lane 6). At this concentration of Zn\textsuperscript{2+}, WRN helicase activity was inhibited (Fig. 10E, inset). Thus, the additional presence of Zn\textsuperscript{2+} in the WRN reaction mixture converts WRN from a DNA helicase mode to an exonuclease mode.

The effect of Zn\textsuperscript{2+} on WRN exonuclease activity was also examined when Mg\textsuperscript{2+} (1 mM), but not ATP, was present. As shown in Fig. 10F, 10 and 50 µM Zn\textsuperscript{2+} did not stimulate WRN exonuclease activity (lanes 3 and 4 compared to lane 2); however, the presence of 100 µM Zn\textsuperscript{2+} in reaction mixtures that also contained 1 mM Mg\textsuperscript{2+} resulted in a significantly greater extent of exonucleolytic digestion, although a greater amount of the intact 45-mer was reproducibly observed compared to WRN reactions containing 0, 10, or 50 µM Zn\textsuperscript{2+} (lane 5). At 500 and 1000 µM Zn\textsuperscript{2+}, WRN exonuclease activity was inhibited (lanes 6 and 7). These results suggest that 100 µM Zn\textsuperscript{2+} is optimal for WRN exonuclease activity, under these conditions.

**DISCUSSION**

Although significant progress has been made in determining the DNA substrate specificity of WRN, only limited information is available on the reaction conditions suitable for optimal WRN helicase or exonuclease activities. Since the Mg\textsuperscript{2+}: ATP ratio was reported to have a significant effect on *E. coli* RecQ helicase activity (31), we examined their importance for WRN-catalyzed DNA unwinding. We determined that optimal WRN helicase activity is displayed when ATP and Mg\textsuperscript{2+} are in equimolar concentrations of 1 mM. The $K_m$ for the ATP-Mg\textsuperscript{2+} complex in the WRN helicase reaction was determined to be 140 µM. Under slightly different reaction conditions that included a fixed MgCl\textsubscript{2} concentration of 4 mM, the Loeb lab reported a WRN $K_m$ value of 51 µM with respect to ATP concentration for DNA unwinding (30), suggesting that WRN binds ATP with slightly reduced affinity in the presence of equimolar ATP and Mg\textsuperscript{2+} compared to excess free Mg\textsuperscript{2+}. Furthermore, we have demonstrated here that up to a four-fold excess of Mg\textsuperscript{2+} over ATP did not significantly affect the initial rate of WRN
helicase activity compared to equimolar concentration of ATP and Mg\(^{2+}\). *E. coli* RecQ helicase activity was found to achieve an optimum at a Mg\(^{2+}\): ATP ratio of 0.8 (31); however, unlike WRN, RecQ helicase activity decreased significantly at a Mg\(^{2+}\): ATP ratio of 1 to 1.2 using 1 or 3 mM ATP, respectively. This difference suggests that WRN unwinding is not nearly as sensitive to free Mg\(^{2+}\) ion concentration as observed for its bacterial prototype. The sensitivity of other eukaryotic RecQ helicases to free Mg\(^{2+}\) ion concentration remains to be determined.

From metal substitution studies, we determined that Mn\(^{2+}\) and Ni\(^{2+}\) could replace Mg\(^{2+}\) as a cofactor for WRN helicase activity; however, Ni\(^{2+}\) was less effective than either Mg\(^{2+}\) or Mn\(^{2+}\). Mn\(^{2+}\) was also found to serve as a cofactor for Sgs1 helicase (55) and RecQ ATPase (56) reactions. An interesting result was obtained when Zn\(^{2+}\) was used to replace Mg\(^{2+}\) in the WRN enzymatic reaction. Native polyacrylamide gel analysis of WRN helicase products revealed two novel migrating bands, one slower and one faster than the labeled single-stranded oligonucleotide released by heat-denaturation. Denaturing gel analysis of the products demonstrated that WRN 3’ to 5’ exonuclease activity degraded the labeled strand of the forked duplex DNA substrate by acting from the blunt end of the 19 bp forked duplex substrate. Further analysis demonstrated that Zn\(^{2+}\) is preferred over Mg\(^{2+}\) for WRN exonuclease activity in the absence of ATP. A Zn\(^{2+}\) binding domain in the exonuclease domain of WRN was suggested by the ability of Zn\(^{2+}\) to stimulate the exonuclease activity of the WRN exonuclease domain fragment (N-WRN). The tentative assignment of a Zn\(^{2+}\) binding domain in the WRN exonuclease domain is supported by the structure of the sequence-related DNA polymerase I 3’ to 5’ exonuclease domain (54). Although no crystallographic data of the WRN exonuclease domain is currently available, it seems reasonable that a Zn\(^{2+}\) binding domain will be identified and WRN exonuclease activity may function by a two metal ion mechanism as proposed for DNA polymerase I (54). Importantly, under conditions suitable for WRN-catalyzed DNA unwinding, Zn\(^{2+}\) acts as a molecular switch to convert WRN from DNA helicase mode to exonuclease mode. Regulation of WRN catalytic activities by metal ion availability may dramatically impact the cellular functions of WRN in pathways of DNA metabolism. The DNA replication phenotypes of WS cells (12-14) suggest that WRN
has a role in DNA synthesis. WRN preferentially digests DNA with a single 3’ terminal mismatch (29), suggesting that the 3’ to 5’ exonuclease activity of WRN may remove a mismatched nucleotide that was incorporated by a DNA polymerase. The results presented here indicate that Zn$^{2+}$ is an important metal cofactor for WRN exonuclease activity. The importance of Zn$^{2+}$ mobilization on WRN exonucleolytic digestion of DNA replication and repair intermediates is currently under investigation.

Environmental metals can exert their co-mutagenic effects by their inactivation of DNA repair processes (57). Inhibition of a mutation avoidance system (mismatch repair) in yeast cells or human cell extracts by an environmentally relevant concentration of a metal ion, as opposed to direct DNA damage by the mutagen, was recently suggested to be the primary mechanism for genomic destabilization (46); however, the cellular protein targets remain to be identified in that system. Evidence suggests that WRN helicase activity is important in the metabolism of DNA structures associated with a blocked replication fork (40) or homologous recombination (7). The proposed role of WRN in DNA repair related processes that might be affected by metal ions led us to a second line of inquiry concerning metal effects on WRN helicase activity. Certain metals might adversely affect WRN function by inhibiting WRN helicase activity despite the co-presence of a physiological metal cofactor such as Mg$^{2+}$. To address this question, we examined the effects of a low concentration (10µM) of various environmental metals on WRN helicase activity in the presence of 1 mM Mg$^{2+}$. Under these conditions, we observed substantial inhibition of WRN helicase activity by Fe$^{2+}$ and Cu$^{2+}$ whereas the other metal ions tested had no effect. Inhibition of WRN helicase activity by Fe$^{2+}$ was dose-dependent whereas significant helicase inhibition by Cu$^{2+}$ was observed only at the highest concentration tested, 10µM. Neither Fe$^{2+}$ nor Cu$^{2+}$ inhibited the DNA unwinding reactions catalyzed by TraI or UvrD, but significant inhibition was observed for human BLM and RECQ1 helicases, suggesting that the inhibition may be specific to RecQ DNA helicases. Collectively, these results suggest that Fe$^{2+}$ or Cu$^{2+}$ potently inhibit the WRN helicase and the inhibition is not mediated by a non-specific DNA-metal interaction. Furthermore, our results from the in vitro WRN catalytic reactions with metal ions lend support to the idea that certain environmental metals may affect WRN
function by altering either the helicase or exonuclease activity of the enzyme and that perturbation of WRN catalytic behavior may be a molecular mechanism for metal toxicity by the alteration of DNA damage response pathways involving WRN. This does not exclude the possibility that certain metals (e.g., Zn\(^{2+}\)) serve as important cofactors for WRN in DNA processing events that selectively require a single WRN catalytic activity.

Several models for DNA unwinding catalyzed by a helicase invoke either monomeric or oligomeric protein structures as the functional entity (reviewed in (25;26;27). Since very little is known about the mechanism of DNA unwinding by any of the eukaryotic RecQ helicases characterized so far, the RecQ protein assembly state has been of considerable interest. Electron microscopic studies have provided evidence that BLM forms oligomeric ring-like structures in solution (36); however, the assembly state of BLM that is important for DNA unwinding activity is open to debate, since recent evidence indicated that a catalytically active, truncated form of BLM was monomeric (35). Glycerol gradient and gel filtration analysis of human RECQ1 helicase suggested that the protein exists as a dimer in solution (33). A minimal exonuclease domain fragment of WRN was shown to exist in a trimer-hexamer equilibrium in the absence of DNA when examined by gel filtration chromatography and atomic force microscopy, and the hexamer form is stabilized by the presence of DNA or PCNA (37). In another study, full length WRN was proposed to exist as a trimer based on data from gel filtration chromatography analysis of the purified recombinant protein (34). Up to this point, however, there has been no evidence addressing the assembly state of the WRN helicase actively unwinding dsDNA.

To begin to understand the mechanism of DNA unwinding by WRN, we investigated the kinetics of the WRN helicase reaction under several different conditions using fluorometric or radiometric based helicase assays. One approach was to examine the kinetics of DNA unwinding as a function of ATP or protein concentration to determine if there was cooperative interaction between helicase subunits for DNA unwinding. The *E. coli* RecQ helicase activity was found to display a sigmoidal dependence on the ATP concentration, suggesting multiple interacting ATP binding sites
to mediate duplex DNA unwinding (31). However, another study reported that RecQ exists as a monomer in solution and that both DNA unwinding activity and ATPase activity were independent of RecQ concentration (32). Our results from studies of initial rates (0-20 sec) of helicase activity show that WRN unwinding of dsDNA displays a hyperbolic dependence on ATP-Mg\textsuperscript{2+} concentration through an ATP-Mg\textsuperscript{2+} range of 0.1 – 0.6 mM. These results suggest that WRN helicase activity on the 19 bp forked duplex substrate is not cooperative with respect to ATP concentration under our reaction conditions.

An alternative approach to addressing the assembly state of an actively unwinding helicase is to examine the stoichiometry of the unwinding reaction, i.e., the number of helicase molecules required to unwind a single duplex DNA substrate. To accomplish this, we utilized a fluorometric stopped-flow assay to measure the kinetics of WRN-catalyzed DNA unwinding. Under optimal pre-steady state conditions, with excess DNA substrate over enzyme, we examined the burst kinetics of DNA unwinding by WRN. Analysis of the pre-steady state regime indicated that the burst phase amplitude (unwound DNA substrate molecules, (nM)) was very close to the WRN protein concentration (WRN (nM)). This 1:1 ratio value provides an estimate of the stoichiometry for the number of WRN protein molecules that unwind each 19 bp forked duplex DNA substrate. We conclude from the kinetic analysis that WRN can function as a monomer to unwind the duplex DNA substrate. We determined a much faster unwinding rate in the pre-steady state regime, followed by a much slower rate in the steady state regime under the conditions of the assay for each level of WRN tested. Interestingly, although the unwinding rate (nM bp unwound / sec) increased with protein concentration, it did not do so proportionally. Meaning, the specific activity in the pre-steady state (unwinding rate divided by WRN concentration = bp unwound / sec / WRN) was not the same for the three levels of WRN tested. We speculate this may be the result of many random events of WRN dissociating from a partially unwound duplex DNA, and then having to reinitiate unwinding of a “new” duplex DNA, ultimately slowing down the rate of unwinding.
The unique requirement for the single-stranded DNA binding protein RPA to stimulate WRN-catalyzed unwinding of long DNA duplexes has been reported (58). Future studies can be directed towards pre-steady state analysis of WRN helicase activity to determine if RPA might serve as a loading factor or processivity factor for the WRN DNA unwinding reaction. The RPA interaction may stabilize the WRN reaction, leading to a constant specific activity term in the pre-steady state phase.

ACKNOWLEDGEMENTS
We wish to thank Dr. Ian Hickson (Cancer Research UK Laboratories) for generously providing purified recombinant BLM protein, Dr. Steve Matson (University of North Carolina at Chapel Hill) for purified UvrD and TraI helicases, Dr. Alessandro Vindigni (International Centre for Genetic Engineering and Biotechnology) for the RECQ1 baculovirus. We wish to thank Dr. Jeanine Harrigan and Mr. Dan McNeill (Laboratory of Molecular Gerontology, NIA-NIH) for critical reading of the manuscript.

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FIGURE LEGENDS

Fig. 1. Schematic representation of the fluorescent DNA substrate design. 19-bp forked duplex with two noncomplementary ssDNA tails of 26 nt (5’ tail) or 25 nt (3’ tail). F is covalently attached to the 3’ blunt end and HF is attached to the 5’ blunt end of the DNA substrate. The asterisk indicates the position of the 5’ 32P label for the radiolabeled DNA substrate. Panel A, When in close proximity, HF quenches the signal emitted from F upon excitation. Panel B, Upon unwinding, the emission from F upon excitation is free to be detected because the HF is no longer in close proximity with F.
Fig. 2. Kinetic analyses of DNA unwinding by WRN helicase using a fluorometric stopped-flow assay. Helicase reaction mixtures contained 0.8 nM forked DNA substrate, 2 mM ATP, 8 mM MgCl₂ and the indicated concentrations of WRN helicase. Panel A, Correlation of kinetic data between fluorometric stopped-flow analyses and radiometric quenched-flow analyses of WRN helicase reactions containing 12 nM WRN. The data from the radiometric assay (large filled circles) were overlayed with the data from the fluorometric assay. The plot shows % unwinding against time (sec). A representative gel of the radiometric time course assay is shown: lane 1, no enzyme control; lanes 2 to 14, reaction time points quenched at 5, 10, 20, 30, 40, 50, 60, 80, 100, 120, 140, 160, and 180 sec, respectively; lane 15, heat-denatured substrate control. Panel B, Fluorometric unwinding data as a function of WRN protein concentration.

Fig. 3. Dependence of WRN helicase activity on Mg²⁺ ion concentration. Helicase reaction mixtures contained 0.8 nM forked DNA substrate, 5 nM WRN, and 2 mM ATP with increasing concentrations of MgCl₂ from 0 to 8 mM (closed circles) or 5 mM ATP with increasing concentrations of MgCl₂ from 0 to 20 mM (open circles). The radiometric assay was used to measure initial rates of WRN helicase activity with quench points at 6, 10, 15, and 20 sec. Initial rates of unwinding (mean value of three independent experiments with standard deviations indicated by error bars) are plotted as a function of [Mg²⁺]:[ATP], mM:mM ratio. Each data set was best fit to a sigmoidal curve as shown with a solid line for 2mM ATP and a dashed line for 5 mM ATP.

Fig. 4. Dependence of WRN helicase activity on ATP-Mg²⁺ concentration. Helicase reaction mixtures contained 0.8 nM forked DNA substrate, increasing concentrations of ATP-Mg²⁺ (1:1) complex (0-2 mM) and 5 nM WRN. Initial rates of unwinding from quantitative analyses of radiometric helicase data (mean value of three independent experiments with standard deviations indicated by error bars) from quench points of 6, 10, 15 and 20 sec are plotted as a function of [ATP-Mg²⁺] concentration.
Fig. 5. **Metal ion substitution for Mg^{2+} in the WRN helicase reaction.** Helicase reaction mixtures contained 0.8 nM forked DNA substrate, 1 mM ATP, the indicated metal (1 mM), and 4.6 nM wild-type WRN (Panel A) or X-WRN (Panel B). The radiometric assay was used to measure WRN helicase activity after a 15 min reaction at 37°C. A phosphorimage of a typical native gel is shown. No enzyme (NE) and heat-denatured substrate (filled triangle) controls are shown.

Fig. 6. **Metal ion inhibition of WRN helicase activity.** Helicase reaction mixtures contained 0.8 nM forked DNA substrate, 1 mM ATP, 1 mM MgCl$_2$, the indicated metal (10 µM), and 4.6 nM WRN. The radiometric assay was used to measure WRN helicase activity after a 15 min reaction at 37°C. A phosphorimage of a typical native gel is shown. Lane 2 is a control reaction in which only Mg$^{2+}$ was present. No enzyme (NE) and heat-denatured substrate (filled triangle) controls are shown.

Fig. 7. **Inhibition of WRN helicase activity by Fe$^{2+}$ or Cu$^{2+}$ is dependent on metal ion concentration.** Helicase reaction mixtures contained 0.8 nM forked DNA substrate, 1 mM ATP, 1 mM MgCl$_2$, the indicated concentration of Fe$^{2+}$ (closed circles) or Cu$^{2+}$ (open circles), and 4.6 nM WRN. Metal titrations contained 0, 0.3, 0.6, 1.25, 2.5, 5, and 10 µM of Fe$^{2+}$ or Cu$^{2+}$. The radiometric assay was used to measure WRN helicase activity after a 15 min reaction at 37°C. Quantitative analyses of radiometric helicase data (mean value of three independent experiments with standard deviations indicated by error bars) are plotted as a function of [Fe$^{2+}$] or [Cu$^{2+}$] concentration.

Fig. 8. **Inhibition of helicase activity by Fe$^{2+}$ or Cu$^{2+}$ is specific to RecQ helicases.** Helicase reaction mixtures contained 0.8 nM forked DNA substrate, 1 mM ATP, 1 mM MgCl$_2$, 10 µM of the indicated metal (Fe$^{2+}$ or Cu$^{2+}$), and 5 nM BLM (Panel A) or 20 nM RECQ1 (Panel B). The radiometric assay was used to measure helicase activity after a 15 min reaction at 37°C. A phosphorimage of a representative gel is shown. Lane 2 is a control reaction in which only Mg$^{2+}$ was present. No enzyme (NE) and heat-denatured substrate (filled triangle) controls are shown.
Fig. 9. **Analysis of WRN helicase kinetics under pre-steady state conditions.**
Helicase reaction mixtures contained 75 nM forked DNA substrate and 1 mM ATP-Mg$^{2+}$. Kinetic time courses of WRN helicase activity were determined from the fluorometric assay at 4, 8, and 16 nM WRN as shown.

Fig. 10. **Differential effects of Zn$^{2+}$ and Mg$^{2+}$ on WRN exonuclease activity.**
Reaction mixtures contained 0.8 nM forked DNA substrate, 4.6 nM WRN, and the indicated concentrations of ATP, MgCl$_2$ or ZnCl$_2$. Each 15 min reaction was incubated at 37°C and products were resolved on 14% polyacrylamide denaturing gels. No enzyme (NE) controls and markers are shown for each gel. **Panel A**, Phosphorimage of a typical denaturing gel showing products from WRN exonuclease reactions where Zn$^{2+}$ was titrated in the presence of 1 mM ATP. Lane 2 is a control reaction in which no metal was present. Lanes 3 to 7 contain 10, 50, 100, 500, and 1000 µM Zn$^{2+}$, respectively. Lane 8 contains 1000 µM Mg$^{2+}$. **Panel B**, Phosphorimage of a typical denaturing gel showing products from WRN exonuclease reactions where Zn$^{2+}$ and Mg$^{2+}$ were titrated in the absence of ATP. Lane 2 is a control reaction in which no metal was present. Lanes 3 to 12 contain the indicated (+/-) concentration of Zn$^{2+}$ or Mg$^{2+}$. **Panel C**, Phosphorimage of a typical denaturing gel showing products from reaction mixtures containing 0.8 nM forked DNA substrate, 4.6 nM X-WRN, 1 mM ATP where indicated by +/-, and the indicated (+/-) concentrations (100 or 1000 µM) of MgCl$_2$ or ZnCl$_2$. Lane 2 and 5 are controls (- or + ATP, respectively) in which no metal was present. **Panel D**, same as **Panel C** except that the reactions contained 18 nM N-WRN. **Panel E**, Phosphorimage of a typical denaturing gel showing products from WRN exonuclease reactions where Zn$^{2+}$ was titrated in the presence of 1 mM ATP-Mg$^{2+}$. Lane 2 is a control reaction in which only Mg$^{2+}$ was present. Lanes 3 to 7 contain 10, 50, 100, 500, and 1000 µM Zn$^{2+}$, respectively. The inset shows the same products on a native polyacrylamide helicase gel, and also includes a no enzyme (NE) control which contained 1000 µM Zn$^{2+}$ (lane 8) and a heat-denatured substrate (filled triangle) control (lane 9). **Panel F**, same as **Panel E** except that the reactions contained 1 mM Mg$^{2+}$ but no ATP.
Table I

Kinetics of WRN-catalyzed DNA unwinding as a function of WRN protein concentration

Fluorescence stopped-flow kinetic experiments were performed using the forked duplex DNA substrate (0.8 nM) at the indicated concentrations of WRN as described in Materials and Methods. Initial rates of unwinding were determined from the initial linear regime (first 10 sec) of each time course plotted in Fig. 2B.

| [WRN] (nM) | Initial Rate of Unwinding (nM bp / s) |
|-----------|-------------------------------------|
| 0.6       | 0.01976                             |
| 3         | 0.1672                              |
| 6         | 0.2128                              |
| 12        | 0.3648                              |
| 16        | 0.5624                              |
| 32        | 0.5624                              |
Fluorescence stopped-flow kinetic experiments were performed as described in Material and Methods under pre-steady state conditions, where the enzyme was saturated with excess forked duplex DNA substrate (75 nM), at the indicated concentrations of WRN. The amplitude of the pre-steady state and rates of unwinding were determined from the initial pre-steady state and also for the steady state of each time course plotted in Fig. 10.

| [WRN] (nM) | Amplitude (nM DNA unwound) | Amplitude/ nM WRN | Pre-steady State Initial Rate (nM bp /s) | Steady State Rate (nM bp /s) |
|------------|---------------------------|-------------------|------------------------------------------|-----------------------------|
| 4          | 3.75                      | 0.9               | 228                                      | 13                          |
| 8          | 7.5                       | 0.9               | 270                                      | 18                          |
| 16         | 15                        | 0.9               | 356                                      | 27                          |
Fig. 1 A-B
Fig. 3

Initial Rate of Unwinding (nM bp / s)

[\text{Mg}^{2+}]:[\text{ATP}], \text{mM}:\text{mM}

- 2 mM ATP
- 5 mM ATP

Fig. 3
Initial Rate of Unwinding (nM bp / s) vs. [ATP], mM

Fig. 4
Fig. 5A
Fig. 6
Fig. 7

Unwinding (%) vs. [Metal], µM

- • Fe
- ○ Cu
Fig. 8 A-B
Fig. 9

- 16 nM WRN
- 8 nM WRN
- 4 nM WRN

Unwinding (%) vs. Time (s)
**Fig. 10B**

| [Metal] (µM):NE | 10  | 50  | 100 | 500 | 1000 |
|-----------------|-----|-----|-----|-----|------|
| MgCl₂:          | -   | -   | +   | -   | +    |
| ZnCl₂:          | -   | +   | -   | +   | -    |

- 44-mer
- 39-mer
- 34-mer
- 29-mer
- 24-mer
|     | MgCl₂: |  |  | + |  |  | + |  |
|-----|--------|---|---|---|---|---|---|---|
| ZnCl₂: | - | - | - | + | - | - | + |
| ATP: | + | - | - | - | + | + | + |
| [Metal] (µM): | NE | - | 100 | - | 1000 |  |  |

Fig. 10C

![Gel Image](http://www.jbc.org/)

- 44-mer
- 39-mer
- 34-mer
- 29-mer
- 24-mer

Fig. 10C
|        | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 |
|--------|----|----|----|----|----|----|----|----|----|----|----|----|
| MgCl₂: | -  | -  | +  | -  | -  | +  | -  |    |    |    |    |    |
| ZnCl₂: | -  | -  | -  | +  | -  | -  | +  |    |    |    |    |    |
| ATP:   | +  | -  | -  | -  | +  | +  | +  |    |    |    |    |    |
| Metal (µM): | NE | 100 | - | 1000 | - |    |    |    |    |    |    |    |

Fig. 10D
Biochemical and kinetic characterization of the DNA helicase and exonuclease activities of Werner syndrome protein
Saba Choudhary, Joshua A. Sommers and Robert M. Brosh Jr.

J. Biol. Chem. published online June 8, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M401901200

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