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Zishuo Cheng
Louisiana State University

Guoqiang Tan
Wenzhou Medical University

Wu Wang
Wenzhou Medical University

Xiaolu Su
Wenzhou Medical University

Aaron P. Landry
Louisiana State University

See next page for additional authors

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Iron and Zinc Binding Activity of *Escherichia coli* Topoisomerase I Homolog YrdD

Zishuo Cheng[#], **Guoqiang Tan**[*], **Wu Wang**[*], **Xiaolu Su**[*], **Aaron P. Landry**[#], **Jianxin Lu**[*,§], and **Huangen Ding**[#,§]

[#]Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803, USA

[*]Laboratory of Molecular Medicine, Wenzhou Medical University, Wenzhou, Zhejiang, China

Abstract

YrdD, a homolog of the C-terminal zinc-binding region of *Escherichia coli* topoisomerase I, is highly conserved among proteobacteria and enterobacteria. However, the function of YrdD remains elusive. Here we report that YrdD purified from *E. coli* cells grown in LB media contains both zinc and iron. Supplement of exogenous zinc in the medium abolishes the iron binding of YrdD in *E. coli* cells, indicating that iron and zinc may compete for the same metal binding sites in the protein. While the zinc-bound YrdD is able to bind single-stranded (ss) DNA and protect ssDNA from the DNase I digestion *in vitro*, the iron-bound YrdD has very little or no binding activity for ssDNA, suggesting that the zinc-bound YrdD may have an important role in DNA repair by interacting with ssDNA in cells.

Keywords

YrdD; topoisomerase I; zinc; iron; metalloprotein

INTRODUCTION

Escherichia coli topoisomerase I belongs to type I subfamily DNA topoisomerases (Wang 2002, Tse-Dinh 2009). The enzyme contains an N-terminal catalytic fragment (67 kDa) and a C-terminal zinc-binding region (30 kDa). While the N-terminal fragment is sufficient for cleaving single-stranded DNA, the C-terminal region is required for relaxing the negatively supercoiled DNA (Tse-Dinh 1991, Ahumada and Tse-Dinh 2002) and for interacting with RNA polymerase (Cheng et al. 2003). It has also been reported that the enzyme activity of topoisomerase I is regulated by the C-terminal and N-terminal domain interactions and by divalent metal ions such as Mg^{2+} and Mn^{2+} (Sissi et al. 2013). Genome-wide search revealed that *E. coli* has two topoisomerase I homologs: topoisomerase III and a function-unknown protein YrdD. Topoisomerase III is homologous to the N-terminal domain of topoisomerase I and has a crucial role in genomic stability (Suski and Marians 2008) and chromosome segregation (Perez-Cheeks et al. 2012). On the other hand, YrdD is homologous to the C-terminal zinc-binding region of topoisomerase I. YrdD is highly

[§]Corresponding author: Huangen Ding, Ph.D., 202 Life Sciences Building, Department of Biological Sciences, Louisiana State University, Baton Rouge, LA, 70803, Tel: (225) 578 4797, Fax: (225) 578 2597, hding@lsu.edu.

conserved among proteobacteria and enterobacteria. Although the function of YrdD is unknown, recent genetic studies indicated that YrdD may have an important role in DNA repair as inactivation of YrdD suppresses the severe growth inhibition phenotype of an *E. coli* mutant with deletions of the key DNA recombination repair protein RecA and the bacterial dNTPase RdgB which removes non-canonical DNA precursors such as dIPT in cells (Budke and Kuzminov 2010).

In previous studies, we reported that topoisomerase I is able to bind both iron and zinc in the C-terminal region in *E. coli* cells, and that unlike the zinc-bound topoisomerase I, the iron-bound enzyme fails to relax the negatively supercoiled DNA (Lu et al. 2011). In the present study, we find that YrdD, the homolog of the topoisomerase I C-terminal region, can also bind both iron and zinc *in vivo*. Furthermore, the zinc-bound YrdD has a strong binding affinity for single stranded (ss) DNA and protects ssDNA from the DNase I digestion *in vitro*. In contrast, the iron-bound YrdD has very little or no binding activity for ssDNA. The results suggest that the zinc-bound YrdD may contribute to the DNA repair activity by interacting with ssDNA and that the ssDNA binding activity of YrdD may be regulated by the iron and zinc binding in the metal binding sites in the protein.

MATERIALS AND METHODS

Protein purification

The DNA fragment encoding YrdD was amplified from the wild-type *E. coli* genomic DNA with PCR using two primers: YrdD-1 (5′-CATGCCATGGCGAAATCAGCACTGTTCAC-3′) and YrdD-2 (5′-CCCAAGCTTTTCCGCCGAAACCGGCTTTC-3′). The PCR product was digested with restriction enzymes *Nde*I and *Hind*III, and ligated to an expression vector pET28b$^+$ (Novagen co). The cloned DNA fragment was confirmed by direct sequencing (Genomic Facility, LSU). Recombinant YrdD was expressed in *E. coli* BL21 strain in either LB (Luria-Bertani) medium or M9 minimal medium supplemented with glucose (0.2%), thiamin (5 μg/ml) and 20 amino acids (each at 10 μg/ml). *E. coli* YrdD, IscU (Yang et al. 2006), topoisomerase I (Lu et al. 2011) and the single-stranded DNA binding protein SSB (Cheng et al. 2012) were purified following the procedure described previously. The molecular weight of purified YrdD was confirmed by mass spectrometer. The purity of purified protein was over 95% judging from the SDS/PAGE. The protein concentration of YrdD was measured at 280 nm using an extinction coefficient of 9.6 cm^{-1} mM^{-1}.

Metal content analyses

Total iron content in protein samples was determined using an iron indicator FerroZine following the procedures described in (Cowart et al. 1993). Total zinc content in protein samples was determined using a zinc indicator PAR (4-(2-pyridylazo)-resorcinol) following the procedures in (Bae et al. 2004). The zinc and iron contents in purified proteins were also measured by the Inductively Coupled Plasma-Emission Spectrometry (ICP-ES) (Chemical Analysis Laboratory, University of Georgia). Both metal content analyses produced similar results.

The DNA binding activity assay

The DNA binding activity assay was carried out using a fluorescence labeled 40mer (5′-F*-AATTGCGATCTAGCTCGCCAGUAGCGACCTT ATCTGATGA-3′) (Operon co.). For the single-stranded (ss) DNA binding assay, the fluorescence-labeled 40mer was incubated with protein in buffer containing Tris (20 mM, pH 8.0), NaCl (50 mM), β-mercaptoethanol (1 mM), $MgCl_2$ (1 mM), and bovine serum albumin (BSA) (0.5 mg/mL). For the double-strand (ds) DNA binding assay, the fluorescence labeled 40mer was annealed to a complementary ssDNA in an annealing buffer containing Tris (50 mM, pH 8.0), NaCl (50 mM), and $MgCl_2$ (10 mM). Prepared dsDNA was incubated with protein in buffer at room temperature for 15 min, and the samples were loaded on to a 0.6% agarose gel in TEA (40 mM Tris acetate and 1 mM EDTA, pH 8.0) buffer. The agarose gel was run at 10 V per cm for 30 min at room temperature and photographed in a KODAK Gel Logic 200 Imaging System. The intensity of the DNA bands on agarose gels was quantified using the ImageJ software (NIH).

The DNA protection activity assay

The ssDNA protection activity assays were carried out by incubating ssDNA (70mer) (5′-GAATGAAGGTATGCTGCATTAATCATTTCTTTAATTCAGCATAAGTTGTTGTGTA GG CTGGAGCTGCTTC-3′) (Operon co.) (1 μM) with indicated concentrations of proteins in reaction solutions containing Tris (20 mM, pH 8.0), NaCl (50 mM), β-mercaptoethanol (1 mM), $MgCl_2$ (1 mM), $CaCl_2$ (1.5 mM), and BSA (0.5 mg/mL) at room temperature for 15 minutes. DNase I (Sigma co) was then added to the incubation solutions. Reactions were incubated at 37°C for additional 10 minutes, and terminated by adding 4 μL stop solution (containing SDS (6%), EDTA (60 mM) and Bromophenol Blue (0.3%)). The products were analyzed by loading the samples on a 0.6% agarose gel in TEA buffer (40 mM Tris acetate and 1 mM EDTA, pH 8.0). The agarose gels were stained with 0.5μg/ml Ethidium Bromide for 30 min and photographed in a KODAK Gel Logic 200 Imaging System. The amount of ssDNA on agarose gel was quantified using the ImageJ software (NIH), and compared to that of the undigested ssDNA band to obtain the percentage of the ssDNA protection activity (%).

EPR measurements

The electron paramagnetic resonance (EPR) spectra were recorded at X-band on a Bruker ESR-300 spectrometer equipped with an Oxford Instruments 910 continuous flow cryostat. EPR conditions were: microwave frequency, 9.45 GHz; microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 2 mT; sample temperature, 10 K; receive gain, 1×10^5.

RESULTS

E. coli YrdD is an iron and zinc binding protein

When recombinant YrdD is expressed in *E. coli* cells grown in LB medium, purified YrdD has a reddish color (Figure 1 insert). The UV-visible absorption measurements revealed that purified YrdD has three major absorption peaks at 345 nm, 482 nm and 563 nm, indicative

of iron binding in the protein (Figure 1A). The absorption peaks disappear when the protein is reduced with sodium dithionite and partially restored after the reduced protein is exposed to air (Figure 1A), indicating that the iron center in YrdD is redox active. The total metal content analyses of purified YrdD showed that each YrdD monomer contains 0.46±0.14 iron atoms and 0.62±0.21 zinc atoms (n = 3).

The electron paramagnetic resonance (EPR) was also used to probe the iron binding in YrdD. As shown in Figure 1B, purified YrdD has an EPR signal at $g = 4.3$, reflecting a mononuclear ferric iron center in the protein. Addition of sodium dithionite completely eliminated the EPR signal, confirming that the iron center in YrdD can be reduced with dithionite.

Excess zinc in growth media competes off the iron binding in YrdD in *E. coli* cells

To explore the iron and zinc binding competition in YrdD, the protein is expressed in *E. coli* cells grown in LB media supplemented with increasing amounts of zinc. Figure 2A shows the UV-visible absorption spectra of YrdD proteins purified from the *E. coli* cells grown in LB media supplemented with different amounts of zinc. As the concentration of zinc in LB media is increased, the absorption peaks at 345 nm, 482 nm and 563 nm are gradually decreased, and completely eliminated when LB media are supplemented with 0.25 mM $ZnSO_4$. The total metal content analyses revealed that when the concentration of $ZnSO_4$ in LB media is increased from 0 to 0.25 mM, the iron content in YrdD is progressively decreased (Figure 2B) with concomitant increase of the zinc binding in YrdD to about three zinc atoms per YrdD monomer (Figure 2C) (n = 3).

The zinc-bound YrdD has the ssDNA binding activity

The C-terminal region of the *E. coli* topoisomerase I has been shown to have a strong ssDNA binding activity (Ahumada and Tse-Dinh 2002). To determine whether YrdD also has the similar DNA binding activity, we prepared YrdD protein that contains only iron or zinc by expressing the protein in *E. coli* cells grown in the M9 minimal media supplemented with either iron or zinc, respectively. Figure 3A shows the UV-visible absorption spectra of YrdD purified from the *E. coli* cells grown in the M9 minimal media supplemented with either iron or zinc. The metal content analyses revealed that the iron-bound YrdD contains 1.2±0.3 iron atom and no zinc per YrdD monomer and the zinc-bound YrdD contains 3.1±0.21 zinc atoms and no iron per YrdD monomer (n=3).

Purified YrdD proteins are then tested for their DNA binding activity. Figure 3B shows that the zinc-bound YrdD has a strong binding activity for ssDNA with an almost stoichiometric binding of YrdD to ssDNA (40mer). In contrast, the iron-bound YrdD has very little or no ssDNA binding activity. In controls, whereas the *E. coli* single-stranded DNA binding protein SSB forms the SSB-ssDNA complex (Shereda et al. 2008, Cheng et al. 2012), the *E. coli* iron-sulfur cluster assembly protein IscU, a zinc-bound protein (Ramelot et al. 2004), fails to bind any ssDNA (Figure 3B), validating the specific ssDNA binding activity of the zinc-bound YrdD. We also compared the ssDNA binding activity of the zinc-bound YrdD and SSB (Figure 3C). Due to stoichiometric binding of YrdD to ssDNA, we were unable to determine the dissociation constant of the zinc-bound YrdD with ssDNA. Nevertheless, the

results clearly demonstrate that the zinc-bound YrdD has a comparable ssDNA binding activity as SSB under the experimental conditions.

We further explored the binding activity of the zinc-bound YrdD for ssDNA and dsDNA under same experimental conditions. As shown in Figure 3D, the zinc-bound YrdD has much less binding activity for dsDNA than for ssDNA, and SSB has no dsDNA binding activity. These results suggest that the zinc-bound YrdD prefers to bind ssDNA with a high binding affinity over dsDNA, while the iron-bound YrdD has very little or no binding activity for ssDNA or dsDNA.

The zinc-bound YrdD protects ssDNA from the DNase I digestion

The finding that the zinc-bound YrdD binds ssDNA led us to inquire whether YrdD can protect ssDNA. In the experiments, ssDNA is incubated with a fixed concentration of YrdD and increasing amounts of DNase I. After incubation, the undigested ssDNA is analyzed by the agarose gel electrophoresis. As shown in Figure 4A, the zinc-bound YrdD can indeed protect ssDNA from the DNase I digestion, while the iron-bound YrdD has no such an activity. We also compared the ssDNA protection activity of the zinc-bound YrdD, IscU and SSB under the same experimental conditions. As shown in Figure 4B, while the zinc-bound YrdD and SSB can effectively protect ssDNA from the DNase I digestion, IscU and the iron-bound YrdD fail to protect ssDNA from the DNase I digestion.

DISCUSSION

In this study, we report that *E. coli* YrdD, previously annotated as one of the topoisomerase I homologs, is an iron/zinc binding protein. When recombinant YrdD is expressed in *E. coli* cells grown in LB media, purified protein contains both iron and zinc. Increasing zinc content in LB media competes off the iron binding in YrdD in *E. coli* cells, suggesting that iron and zinc likely share the same binding sites in YrdD. Our results further reveal that the zinc-bound YrdD has a strong binding activity for ssDNA and protects ssDNA from the DNase I digestion. In contrast, the iron-bound YrdD fails to bind ssDNA or protect ssDNA from the DNase I digestion. The results suggest that the zinc-bound YrdD may have an important role in DNA repair by interacting with ssDNA in *E. coli* cells.

Zinc is an essential trace metal that facilitates correct folding of proteins, stabilizes the domain structure, and plays important catalytic roles in enzymes (Berg and Shi 1996). Depletion of zinc in growth medium results in slow-growth phenotype and activation of zinc uptake systems in *E. coli* cells (Graham et al. 2009). On the other hand, excess zinc is highly toxic to cells (Xu and Imlay 2012). Since iron and zinc have a similar ligand binding coordination (Dauter et al. 1996), it is expected that iron and zinc may compete for the metal binding sites in proteins. In the previous studies, we reported that *E. coli* topoisomerase I is able to bind zinc or iron in the C-terminal zinc-binding region (Lu et al. 2011). While the zinc-bound topoisomerase I is fully active to unwind the negatively supercoiled DNA, the iron-bound topoisomerase I has very little or no enzyme activity (Lu et al. 2011). Here, we find that YrdD, a homolog of the C-terminal region of topoisomerase I, is also capable of binding zinc and iron in *E. coli* cells (Figure 2), supporting the notion that iron and zinc may compete for the metal binding sites in these proteins. Interestingly, only the zinc-bound

YrdD can bind ssDNA (Figure 3), suggesting that zinc binding may result in subtle structural change of YrdD to facilitate the ssDNA binding and protect ssDNA from the DNase I digestion. It appears that that zinc is a preferred metal for the metal-binding sites in YrdD (Figure 3A) and in the C-terminal region of topoisomerase I (Ahumada and Tse-Dinh 2002). Nevertheless, deficiency of zinc or excess iron in cells may produce the iron-bound YrdD and topoisomerase I. In this regard, the ssDNA binding activity of YrdD and the enzyme activity of topoisomerase I may be regulated by intracellular iron and zinc contents.

The C-terminal region of *E. coli* topoisomerase I is essential for relaxing the negatively supercoiled DNA (Tse-Dinh 1991, Ahumada and Tse-Dinh 2002), likely through strong interaction with ssDNA (Ahumada and Tse-Dinh 2002) and direct interaction with the N-terminal domain of the protein (Sissi et al. 2013). Here we find that the zinc-bound YrdD, but not the iron-bound YrdD, retains strong binding affinity for ssDNA and relatively weak binding activity for dsDNA (Figure 3D). While the physiological function of YrdD remains elusive, our results suggest that YrdD is able to protect ssDNA via interaction with ssDNA. This idea is consistent with the recent study showing that inactivation of the gene encoding YrdD can suppress the severe growth inhibition phenotype of an *E. coli* mutant with deletion of the DNA recombination repair protein RecA and the bacterial dNTPase RdgB which degrades non-canonical DNA precursors (Budke and Kuzminov 2010). Deficiency of RdgB will result in accumulation of the clastogenic DNA precursors such as dIPT in cells and endonuclease V may nick DNA near the base analogues to initiate excision repair (Lukas and Kuzminov 2006). Further inactivation of RecA protein may block recombinational repair which leads to severe growth inhibition. Deletion of endonuclease V or YrdD appears to inhibit the endonuclease V-initiated repair pathway and suppresses the severe growth inhibition phenotype of the *E. coli* cells with deletions of RecA and RdgB (Budke and Kuzminov 2010). Thus, YrdD and endonuclease V may work in concert in the DNA repair pathways. Since YrdD is a relatively small protein (180 amino acids), it is most likely that YrdD will have partners such as endonuclease V for its physiological functions in cells. Additional partners of YrdD remain to be further identified in cells.

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Abbreviations

YrdD	*E. coli* topoisomerase I homolog
Zn-YrdD	the zinc-bound YrdD
Fe-YrdD	the Fe-bound YrdD
EPR	electron paramagnetic resonance

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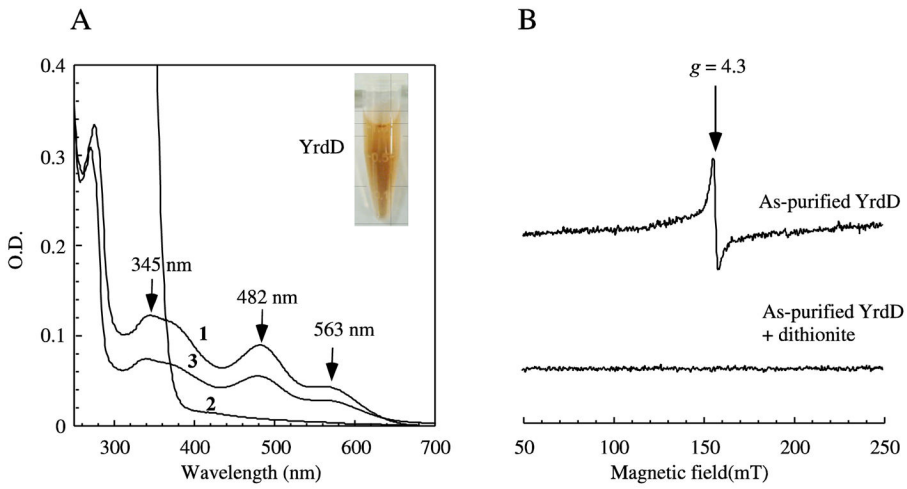

Figure 1. The *E. coli* topoisomerase I homolog YrdD binds a mononuclear iron center

Recombinant YrdD was purified from *E. coli* cells grown in LB media under aerobic growth conditions. ***A)***, UV-visible absorption spectra of purified YrdD. YrdD (32 μM) (spectrum 1) was reduced with sodium dithionite (1.5 mM) (spectrum 2), followed by exposure to air for 30 min (spectrum 3). Insert is a photograph of purified YrdD (200 μM). ***B)***, EPR spectra of purified YrdD. YrdD (200 μM) was dissolved in buffer containing Tris (20 mM, pH 8.0) and NaCl (500 mM) before and after reduced with sodium dithionite (1.5 mM).

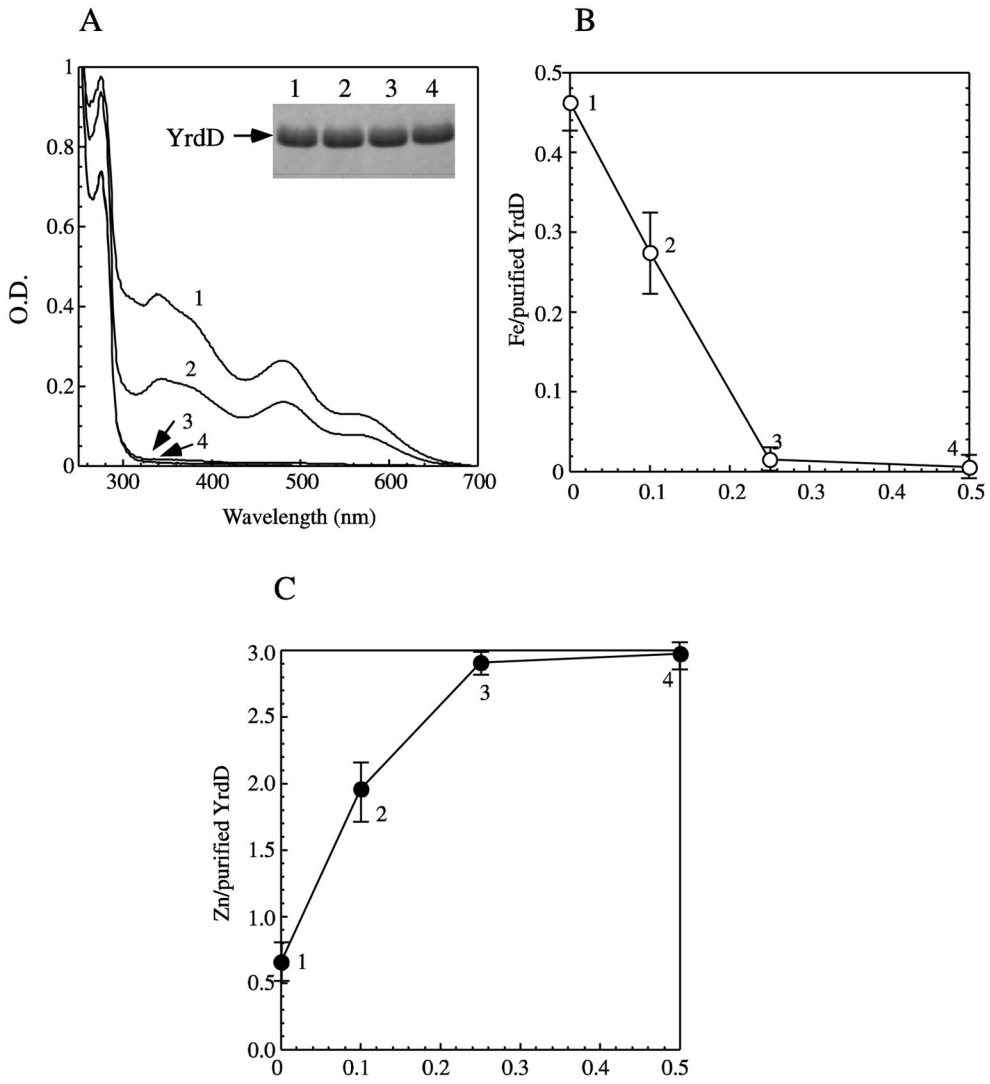

Figure 2. Competition of iron and zinc binding in YrdD in *E. coli* cells

A), UV-visible absorption spectra of YrdD purified from *E. coli* cells grown in LB medium supplemented with 0.0 mM (spectrum 1), 0.1 mM (spectrum 2), 0.25 mM (spectrum 3) and 0.5 mM $ZnSO_4$ (spectrum 4). The concentration of each purified protein was about 80 μM. Insert is a photograph of the SDS polyacrylamide electrophoresis gel of purified YrdD proteins. ***B)***, iron contents of YrdD purified from *E. coli* cells grown in the LB growth medium supplemented with 0.0 mM, 0.1 mM, 0.25 mM and 0.5 mM $ZnSO_4$. ***C)***, zinc contents of YrdD purified from *E. coli* cells grown in LB growth medium supplemented with 0.0 mM, 0.1 mM, 0.25 mM and 0.5 mM $ZnSO_4$. The data in B and C are the averages with standard deviation from three independent experiments.

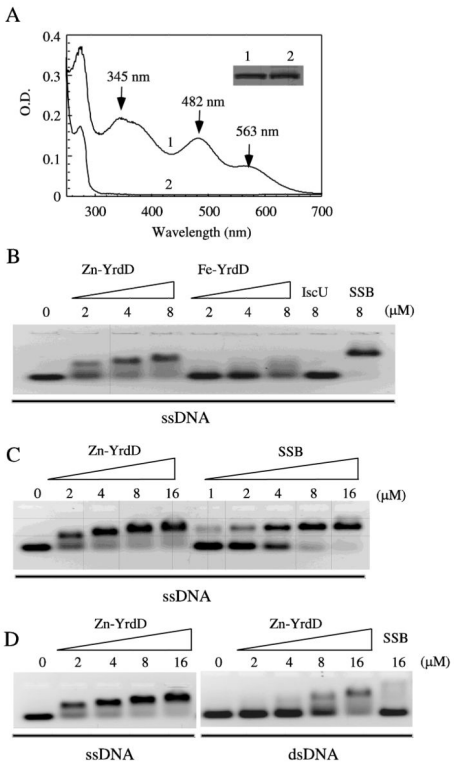

Fig. 3. The Zn-bound YrdD has the ssDNA binding activity

A), UV-visible absorption spectra of Zn-YrdD and Fe-YrdD. Recombinant YrdD was expressed in *E. coli* cells grown in M9 minimal media supplemented with 40 μM $Fe(NH_4)_2(SO_4)_2$ (spectrum 1) or 40 μM $ZnSO_4$ (spectrum 2). The protein concentration was about 16 μM. Insert is a photograph of the SDS polyacrylamide electrophoresis gel of Fe-YrdD (1) and Zn-YrdD (2). ***B),*** ssDNA binding activity of YrdD. Zn-YrdD, Fe-YrdD, IscU, and SSB were incubated with the fluorescein-labeled ssDNA (4 μM) at room temperature for 15 min. The samples were loaded onto a 0.6% agarose gel to resolve the protein-ssDNA complex from "free" ssDNA. ***C),*** comparison of the ssDNA binding activity of Zn-YrdD and SSB. The fluorescein-labeled ssDNA (4 μM) was incubated with increasing concentrations of Zn-YrdD and SSB at room temperature for 15 min. The samples were loaded onto a 0.6% agarose gel to resolve the protein-ssDNA complex from "free" ssDNA. ***D)***, comparison of the ssDNA and dsDNA binding activity of Zn-YrdD. Fluorescein-labeled ssDNA (left panel) or dsDNA (right panel) (4 μM) was incubated with increasing concentrations of Zn-YrdD for 15 min at room temperature. The samples were loaded into a 0.6% agarose gel to resolve the protein-DNA binding complex from "free" DNA. SSB (last lane) had no dsDNA binding activity. The data are representative of three independent experiments.

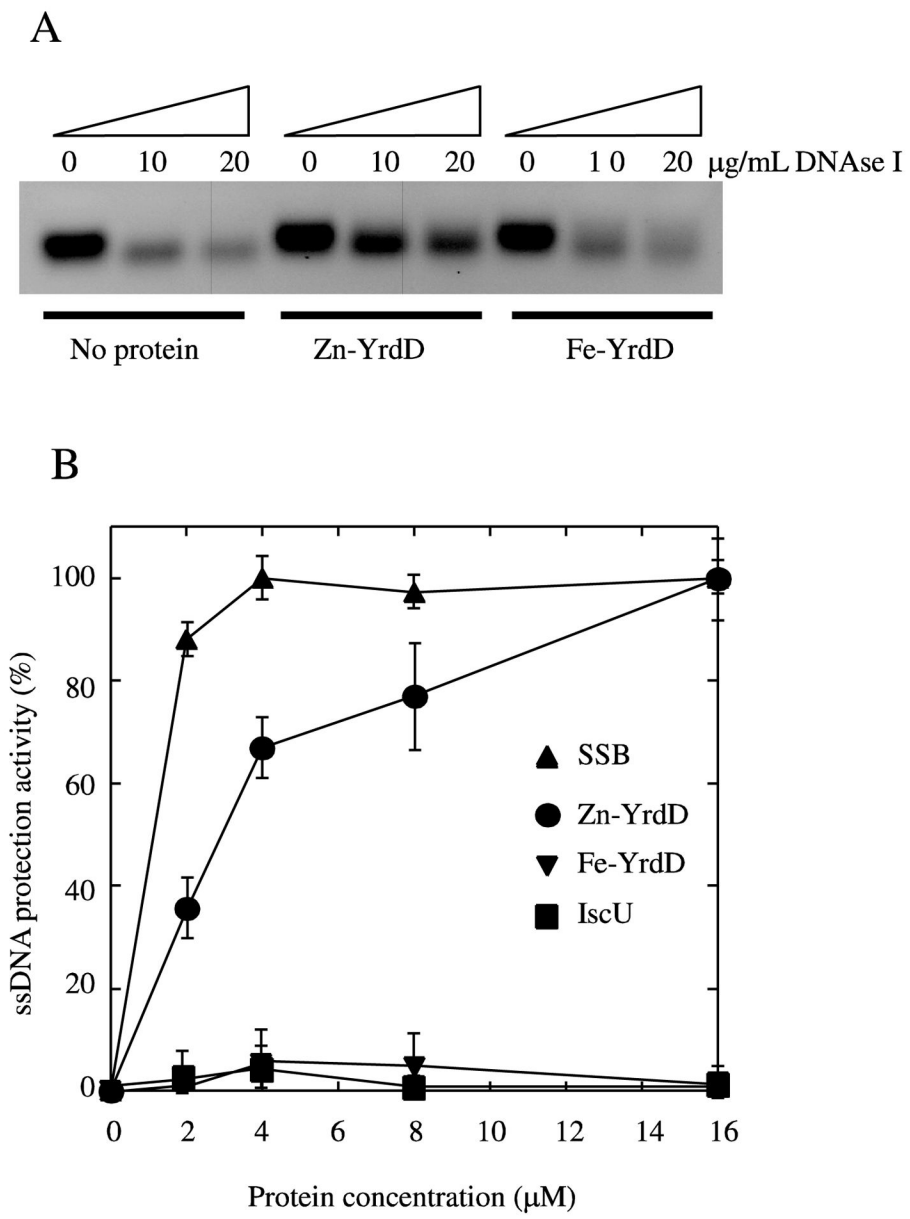

Fig. 4. The Zn-bound YrdD protects ssDNA from DNase I digestion

A), protective activity of Zn-YrdD and Fe-YrdD on ssDNA. The ssDNA (70mer) (1 μM) was incubated with 8 μM Zn- or Fe-YrdD at room temperature for 15 min, followed by digestion with 0, 10 or 20 μg/ml DNase I for 15 min. The samples were then loaded onto 1% agarose gel for quantification of intact ssDNA. ***B),*** protective activity of Zn-YrdD, Fe-YrdD, IscU, and SSB on ssDNA. The ssDNA (70mer) (1 μM) was incubated with indicated concentrations of proteins and treated with DNase I (10 μg/ml) at room temperature for 15 min. The samples were loaded onto 1% agarose gel for quantification of intact ssDNA. The amount of the ssDNA band was analyzed with ImageJ software (NIH) and compared to that of the undigested ssDNA band to obtain the percentage of the ssDNA protection activity (%). The data are the averages with standard deviations from three independent experiments.