Original Article

Effects of Mercury on the Structure and Activity of BLM642-1290 Recombinant Helicase*

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Abstract

Objective Bloom's syndrome is an autosomal recessive disorder characterized by genomic instability and a predisposition to many cancers. Mutations of the BLM gene (encoding a BLM helicase) may form a structure of the etiology of this disease. As a global pollutant, mercury poses a major threat to human health. The current study was conducted to elucidate the effects of Hg^{2+} on the structure and activity of BLM642-1290 recombinant helicase, and to further explore the molecular mechanisms of mercury toxicity to the DNA helicase.

Methods The effects of Hg^{2+} on biological activity and structure of BLM642-1290 recombinant helicase were determined by fluorescence polarized, ultraviolet spectroscopic, and free-phosphorus assay technologies, respectively.

Results The helicase activity, the DNA-binding activity, and the ATPase activity of BLM642-1290 recombinant helicase were inhibited by Hg^{2+} treatment. The LMCT (ligand-to-metal charge transition) peaks of the helicase were enhanced with the increase of the Hg^{2+} level. The LMCT peaks of the same concentration of helicase gradually increased over time.

Conclusions The biological activity of BLM642-1290 recombinant helicase is inhibited by Hg^{2+} treatment. The conformation of the helicase is significantly altered by Hg^{2+}. There exist two binding sites between Hg^{2+} and the helicase, which are located in the amino acid residues 1063-1066 and 940-944 of the helicase, respectively.

Key words: BLM642-1290 recombinant helicase; Structure; Enzyme activity; Mercury

Biomed Environ Sci, 2011; 24(1): 47-55 doi:10.3967/0895-3988.2011.01.006 ISSN:0895-3988

www.besjournal.com (full text) CN 11-2816/Q

INTRODUCTION

Mercury (Hg), as a global pollutant, Hg with its toxicity poses a major threat to human health[1]. Mercury chloride ($HgCl_2$) is a widespread environmental pollutant which leads to dysfunction of several tissues in animals and humans[2]. Hg^{2+} is a potential inhibitor of a variety of enzymes, including NAT1 (Human xenobiotic-metab olizing enzyme arylamine

*This research was supported by the Natural Sciences Foundation of China (NSFC, No.30660043), the National Basic Research Program of China (2010CB534912), the Doctoral Program of the Ministry of Education of China (No.200806570003), and the Governor Talents Foundation of Guizhou Province (No.200822).

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Received: Febuary 6,2010; Accepted: Dencember 8,2010

N-acetyltransfer ase 1)[3], antioxidant enzymes[4], cytochrome oxidase[5], pyruvate kinase, cysteine protease[6], and succinic dehydrogenase[7], which has drawn much attention in previous studies[8-9]. Hg^{2+} is able to bind with some proteins or enzymes in metabolic pathways of cells and then cause inactivation of these enzymes. Therefore, it may impact on production of energy and synthesis of protein and nucleic acids etc. [1,10], and also induce a number of disorders such as neurological, nephrological, immunological, cardiac, motor, reproductive, and even genetic disorders[11-12]. Recently, diseases such as Parkinson's disease, autism, lupus, Alzeihemer's dementia, and amyotrophic lateral sclerosis have been justified to be associated with the toxicity of some heavy metals, including mercury[11]. However, the precise molecular mechanism of mercury-induced toxicosis remains unclear.

DNA helicases are a class of enzymes involved in all aspects of DNA metabolism processes, such as DNA replication, repair, transcription, and recombination[13]. The RecQ helicase family is an important member of DNA helicase, and their mutations in human RecQ genes may lead to genomic instability and cancers[14]. Bloom's syndrome (BS) is a rare genetic disorder which is caused by the mutations of Bloom's syndrome helicase gene (*BLM*)[15]. *BLM* gene encodes a protein (BLM) comprising 1,417 amino acids[16]. Previous studies have shown that the RecQ core of BLM helicase (the amino acid residues 642-1290) displays enzymatic properties similar to the full-size BLM helicase[17]. However, there is little information on the pathologic mechanism of BS, especially on the molecular basis of BLM helicase, regardless of a great variety of extensive studies on BLM helicase[18-19].

To date, few reports have been available on the relative sensitivity of the helicase as a target molecule of mercury. Therefore, our study is to evaluate the effects of Hg^{2+} on the structure and activity of $BLM^{642-1290}$ recombinant helicase by fluorescence polarization, ultraviolet (UV) absorption spectrum, and free-phosphorus assay. The results have indicated that Hg^{2+} may serve as a potential inhibitor of the biological activity of BLM helicase.

MATERIALS AND METHODS

Reagents and Buffers

All buffers were prepared with reagent grade chemicals by using distilled water purified through a Milli-Q water purification system (Millipore Corp., USA) as the solvent. $HgCl_2$ and ATP were purchased from Sigma (St.Louis, USA). The ATP assay kit was obtained from Innova Biosciences (England). The reaction buffer was 20 mmol/L Tris-HCl at pH 7.4, containing 20 mmol/L NaCl, 3 mmol/L $MgCl_2$, and 0.1 mmol/L dithiothreitol (DTT). The buffer for detecting the ATPase activity was 0.5 mol/L Tris at pH 7.4, with 10 mmol/L ATP, and 0.1 mol/L $MgCl_2$ included. The values of pH were measured with an ORION pH meter and a 0079 microelectrode (ORION, USA), whose precisions were ±0.01 pH unit at 25 °C.

$BLM^{642-1290}$ Recombinant Helicase

The 6×His-tagged $BLM^{642-1290}$ recombin ant helicase was expressed in *E.coli* strain *BL21* (DE3) via pET15b expression plasmid as described in the previous study[20]. Briefly, the above expressed helicase was purified at 4℃ by fast flow affinity chromatography with a sepharose column of chelating Ni^{2+} (GE Healthcare, USA), and followed by FPLC size exclusion chromat ography by using Superdex 200 (Amersham, Swiss). Based on the bromophenol blue-stained 10% SDS-PAGE analysis, the purity of the helicase product was above 95%. The molar extinction coefficient at 280 nm (ε_{280}) of the helicase was measured by 2100 ultraviolet spectrophotometer (GE Healthcare, USA), which was 9 900 $L \cdot mol^{-1} \cdot cm^{-1}$.

Oligonucleotides

The sequences of DNA substrates are shown in Table 1. The PAGE-purified, fluorescein-unlabeled and labeled synthetic oligonucleotides were purchased from Shanghai Biological Engineering Technology Service Cooperation (China). Double-stranded DNA (dsDNA) was made in a 20 mmol/L Tris-HCl buffer containing 100 mmol/L NaCl (pH 7.2). The mixture was heated to 85 °C, maintained for 5 min, and annealed by slow cooling to room temperature.

Helicase Assay

The DNA unwinding activity was assayed by using a Beacon 2000 polarization instrument (Pan Vera Corp., USA) as described in our previous study[21]. A total of 2 nmol/L fluorescein-labelled dsDNA of different lengths was added to the reaction buffer (150 mL total, pH 7.4) in a temperature-controlled cuvette at 25°C. The anisotropy, with 8 s interval of DNA substrates, was recorded until it was stabilized. The $BLM^{642-1290}$ recombinant helicase was subjected to different concentrations of Hg^{2+} for 2 min, followed

Table 1. Oligonucleotide Sequences of the Substrates used in this Study

Substrate	Length/Mer	Structure and Sequence
A1	63	5′ AATCCGTCGAGCAGagttagggttagggttagggttagttttttttttttttttttttttttt3′
A2	14	3′ FAM-TTAGGCAGCTCGTC5′
B1	45	5′ AATCCGTCGAGCAGAGTTAGGttaggttaggttagttttttttt3′
B2	21	3′ FAM-TTAGGCAGCTCGTCTCAATCC5′
C1	56	5′ AATCCGTCGAGCAGAGTTAGGGTTAGGGTTAGGGTTagttttttttttttttttt 3′
C2	36	3′ FAM-TTAGGCAGCTCGTCTCAATCCCAATCCCAATCCCAA5′

Note. FAM represents the fluorescein chemical group.

by the addition of miscibleliquid to the cuvette and rapid assay for the anisotropy. When the anisotropy became stable, 1 mmol/L ATP was added rapidly to the cuvette. The changes of anisotropy were recorded every 8 s until it was stabilized. The apparent catalytic constants (k_{obs}) were determined by equation (1)[22],

$$A_t=A_1\exp(-k_{obs}t) \quad (1)$$

Where, A_t is the anisotropy at time *t,* and A_1 is the fluorescence anisotropy of the $BLM^{642-1290}$ recombinant helicase before the addition of ATP.

DNA-Binding Assays

DNA-binding activity was assayed by a Beacon 2 000 polarization instrument[21]. A total of 2 nmol/L fluorescein-labelled dsDNA or single-stranded DNA (ssDNA) of different lengths was added to the reaction buffer (150 mL total volume) in a temperature-controlled cuvette at 25 °C. After the substrate was incubated for 5 min, anisotropy was measured rapidly and continuously until it was stabilized. The $BLM^{642-1290}$ recombinant helicase was subjected to Hg^{2+} stress with different concentrations for 2 min, and afterwards, the miscible liquid was added to the cuvette and the changes of the sample were assayed rapidly. The changes of anisotropy were recorded every 8 s until it became stabilized. The relative DNA-binding activity ($A_{binding}$) was calculated by equation (2)[22],

$$A_{binding}= (A_n/A_0)\times100\% \quad (2)$$

Where, A_n is the fluorescence anisotropy at a given concentration of Hg^{2+} and A_0 is the fluorescence anisotropy in the absence of Hg^{2+}.

ATPase Activity Assays

The ATPase activity was measured with an ATPase assay kit based on a colorimetric estimation of inorganic phosphate produced by ATP hydrolysis[23]. Reactions took place in buffer in the reaction mixture containing 2 nmol/L of 63 mer ssDNA, and got initiated by the addition of the 10 mM ATP and different concentrations of Hg^{2+}. 200μL of reacting mixture was removed from the reactions at various time-points (2.5, 5, 7.5, 10, 15, 20, 25, and 30 min) and mixed with 50 μL Gold mix to terminate ATP hydrolysis. After 2 min, the sample was added with 50 μL stabilizer, and the OD_{650} of the reactions was determined with an ultraviolet spectrophotometer as described[24]. The enzyme activity $A_{activity}$ (units·mL^{-1}) is defined as one unit representing the amount of enzyme that catalyses the reaction of 1μmol of substrate per minute. The activity of undiluted enzyme sample was given by equation (3) according to the instruction of PiColorLock Gold reagent kit,

$$A_{activity} = \frac{A}{500B} C \quad (3)$$

Where, A is the concentration of P_i (μmol/L) determined from the standard curve, B the assay time in minutes and C the reciprocal of the enzyme dilution factor.

Steady-state kinetic parameters (K_m and K_{cat} for ATP) were measured with the helicase in the concentration of 40 nmol/L and in the presence of 0, 40, and 100 mmol/L of Hg^{2+}. The concentration of ATP varied from 0.1 to 1 mmol/L in the measurements. The K_m and k_{cat} values were derived from the Michaelis-Menten equation of the reactions.

Ultraviolet Absorption Spectrum Assays

The UV absorption spectrum of $BLM^{642-1290}$ recombinant helicase (2 μmol/L) between 200 to 340 nm was assayed with the 2 100 ultraviolet spectrophotometer with enzyme-free Tris-HCl buffer (pH 7.9) as the control. Then, Hg^{2+} at varying concentrations (0, 50, 100, 150, 200, 250, 300, and 350 nmol/L) were added to the Tris-HCl buffer. The spectrum of UV absorption of the above mixtures was assayed after incubation for 30 min. In addition, 150 nmol/L of Hg^{2+} was added to the buffer

consisting of 2 μmol/L of the helicase, and the changes of UV absorption spectra were assayed at 0, 3, 6, 9, 12, 15, 18, 21 min.

Statistical Analysis

The obtained results were evaluated under *F*-test statistical analyses by using a commercially available statistical package SPSS 13.0 (SPSS 13.0, 2005, SPSS Inc., Chicago, USA). The general linear model was used to analyze the data of our experiments. A significant difference was declared at $P<0.05$ (*F*test), and an extremely significant difference was declared at $P<0.01$ (*F*test).

RESULTS

Effects of Hg^{2+} on Helicase Activity of $BLM^{642-1290}$ Recombinant Helicase

The effects of the Hg^{2+} on the helicase activity were examined by using fluorescence polarization technology (Figure 1). Hg^{2+} at the concentration of 0.2 mmol/L displayed different inhibitive effects on the helicase activity with three substrate dsDNA of different lengths (Figure 1A). The effects of Hg^{2+} on DNA unwinding activity was negatively correlated with the length of dsDNA ($P<0.05$). The helicase activity was inhibited by Hg^{2+} in a concentration- dependent manner, with the lowest level attained at 0.4 mmol/L (Figure 1B). The calculated rate constant of k_{obs} was $4.5(\pm1.3)\times10^{-3}$. The inhibition constant (K_i) for helicase activity was 0.18(±0.02) mmol/L (Table 2).

Effects of Hg^{2+} on DNA-binding Activity of $BLM^{642-1290}$ Recombinant Helicase

The effects of Hg^{2+} on DNA-binding activity are shown in Figure 2. Hg^{2+} inhibited the DNA-binding activity of $BLM^{642-1290}$ recombinant helicase in a concentration-dependent manner, with little difference ($P>0.05$) among three segments of either ssDNA (Figure 2A) or dsDNA of different lengths (Figure 2B). Hg^{2+} showed effects of great difference ($P<0.01$) between the two kinds of DNA substrates. The inhibition constant (K_i) of Hg^{2+} for the DNA-binding activity with ssDNA and dsDNA as the substrates were 3.0(±0.3) mmol/L or 0.5(±0.1) mmol/L (Table 2), respectively, indicating that the effects of Hg^{2+} on the DNA-binding activity were related to the structure of substrates. In other words, the inhibition effects with dsDNA as the substrate were much stronger than those with ssDNA ($P<0.05$) as the substrate. There was neither difference in the effects of Hg^{2+} between ssDNA of three lengths as substrates ($P>0.05$), nor difference in the effects of Hg^{2+} between dsDNA of three lengths as substrates. The K_i values of Hg^{2+} for the DNA-binding activity were 3.0(±0.3) mmol/L (with ssDNA as substrate) and 0.5(±0.1) mmol/L (with dsDNA as substrate) (Table 2).

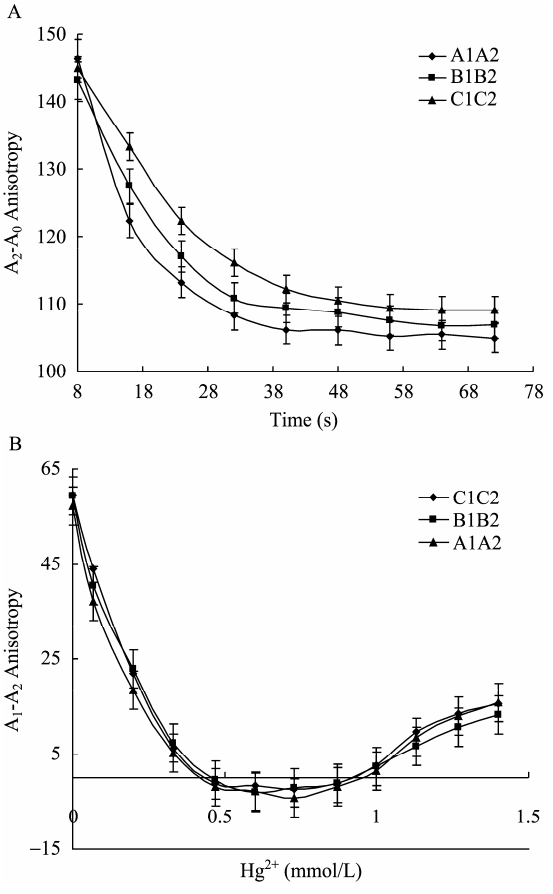


Figure 1. Effects of Hg^{2+} on the helicase activity of $BLM^{642-1290}$ recombinant helicase. The helicase activity determined by the fluorescence polariz ation assay was described in the "Methods" section. A: The time course for the relative DNA unwinding rate of the helicase in the presence of 0.2 mmol/L Hg^{2+}. B: Effects of Hg^{2+} on the helicase activity. Experiments were performed in the helicase activity assay buffer (20 mmol/L Tris-HCl, pH 7.5) at 25 °C. Data were means ± SD with five replicates and the same below.

Note. A_0 is the fluorescence anisotropy in the presence of a DNA segment; A_1 is the fluorescence anisotropy of the $BLM^{642-1290}$ recombinant helicase; A_2 is the fluorescence anisotropy of the $BLM^{642-1290}$ recombinant helicase after the addition of ATP and Hg^{2+}.

Effects of Hg^{2+} on ATPase Activity of $BLM^{642-1290}$ Recombinant Helicase

To elucidate the mechanism of enzyme inhibition, we then examined the effects of Hg^{2+} on the ATPase activity with increasing Hg^{2+} concentrations (Figure 3). Hg^{2+} treatment showed a significant difference in the ATPase activity (P<0.05) over time (Figure 3A). The ATPase activity was markedly inhibited (P<0.01) by 100 μmol/L of Hg^{2+}, as compared with the control (0 μmol/L). The ATPase activity of the $BLM^{642-1290}$ recombinant helicase was completely inhibited by Hg^{2+} at the concentration of 100 μmol/L (Figure 3B). The K_m was invariable (P>0.05) and V_{max} (P<0.01) was decreased by Hg^{2+} in a concentration-dependent manner, leading to decreased K_{cat} along with the decrease in V_{max} (P<0.05, Table 3), indicating that Hg^{2+} was a noncompetitive inhibitor of $BLM^{642-1290}$ recombinant helicase and the converse efficiency was affected by Hg^{2+}. The inhibition constant (K_i) of the ATPase activity was 35(±2.5) μmol/L (Table2).

Effects of Hg^{2+} on the Structure of $BLM^{642-1290}$ Recombinant Helicase

The ligand-to-metal charge transition (LMCT) was increased in the different UV spectra of $BLM^{642-1290}$ recombinant helicase with the addition of Hg^{2+} (Figure 4). Hg^{2+} could increase the absorbance of LMCT peaks with the addition of Hg^{2+} (P<0.01) and the wavelengths of maximum absorption were found to be a little varied (P<0.05) (from 235 to 240 nm) (Figure 4A). The LMCT peaks at the same concentration of the helicase were gradually increased over time (P<0.01) (Figure 4B). The regression curve of $[Hg^{2+}]/[BLM^{642-1290}]$ and absorbance showed an obvious inflexion, suggesting the existence of two binding sites. The slope of the first line (solid line) was bigger than that of the second line (broken line), indicating that the affinity of the first binding site was becoming stronger than that of the second one (Figure 4C).

Table 2. Comparison of Effects of Hg^{2+} on the DNA-binding Activity, Helicase Activity, and ATPase Activity of $BLM^{642-1290}$ Recombinant protein

Apparent K_i $BLM^{642-1290}$	DNA-binding activity(mmol/L)	Helicase activity(mmol/L)	ATPase activity(mmol/L×10^{-3})
ssDNA	3.0±0.3*	—	35±2.5
dsDNA	0.5±0.1	0.18±0.02	—

Note. * Means within the same column differ (P<0.01), compared with the activity with dsDNA as the substrates.

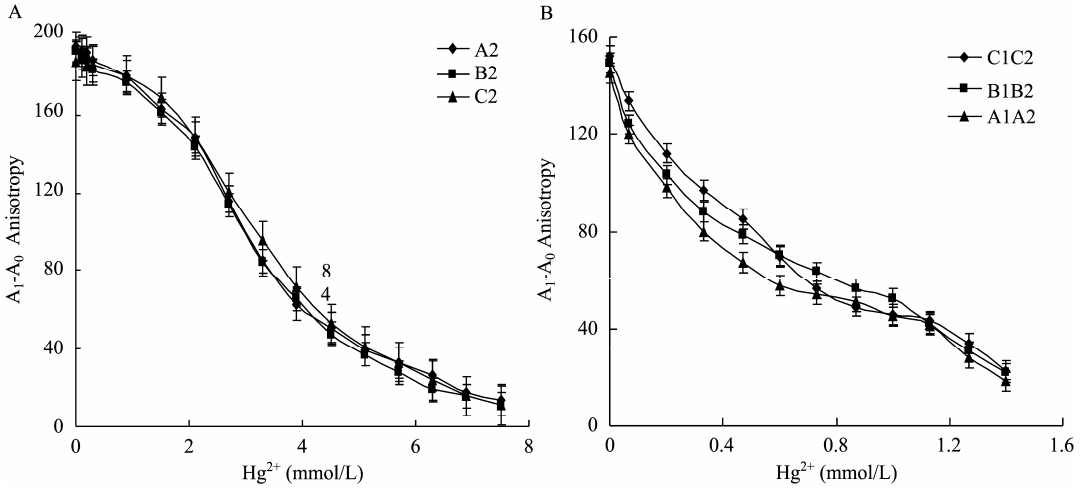


Figure 2. Effects of Hg^{2+} on the DNA-binding activity of $BLM^{642-1290}$ recombinant helicase. Fluorescence aniso tropy values were determined as a function of the helicase concentration with ssDNA (A) and dsDNA of different lengths (B) as substrates. Experiments were performed in DNA-Bind ing activity assay buffer (20 mmol/L Tris-HCl, pH 7.5) at 25 °C. Fluorescein- labelled DNA (2 nmol/L) reacted with the miscible liquids of $BLM^{642-1290}$ recombinant helicase at differrent concentrations of Hg^{2+}.

Note. A_0 is the fluorescence anisotropy in the presence of the correspo nding DNA segment; A_1 is the fluorescence anisotropy of the miscible liquid of the $BLM^{642-1290}$ recombinant helicase and Hg^{2+}.

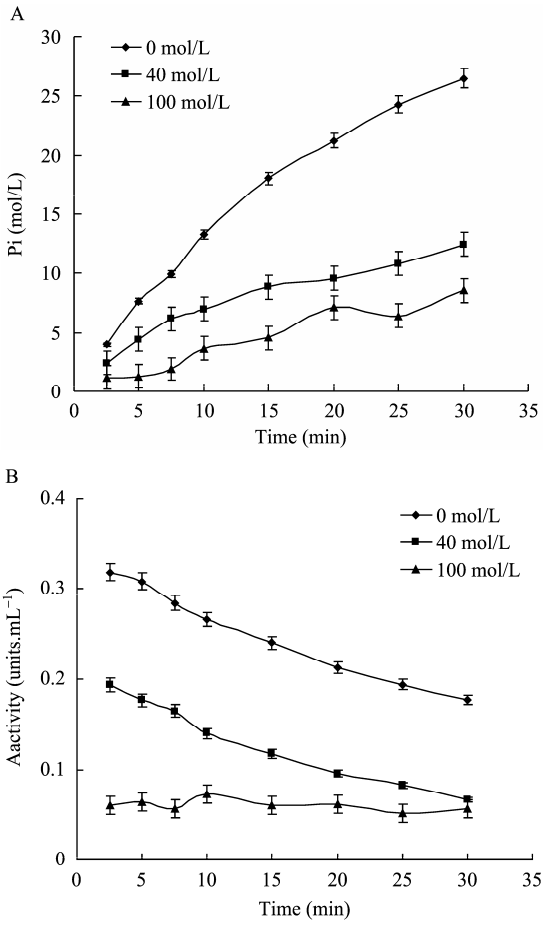


Figure 3. Effects of Hg^{2+} on the ATPase activity of the $BLM^{642-1290}$ recombinant helicase. A: Time course of ATP hydrolysis by 400 nmol/L $BLM^{642–1290}$ recombinant helicase in the presence of 0 μmol/L (closed circles), 40 μmol/L (closed squares), and 100 μmol/L (open triangles) of Hg^{2+}. B: Curves of activity [based on the equation (3)] of the miscible liquids of $BLM^{642–1290}$ recombinant helicase and Hg^{2+} concentration. In each reaction, 63 mer DNA was present at the concentration of 2 nmol/L, and ATP was at 500 μmol/L. Experiments were performed in ATPase activity assay buffer (50 mmol/L Tris-HCl, 2.5 mmol/L $MgCl_2$, and pH 7.5) at 25 °C.

DISCUSSION

Mercury has long been recognized as a health hazard to humans, and it still poses a health risk at present for its presence in some industrial uses. However, it may influence the biological functions of the endogenous biomacromolecules such as DNA and enzymes *in vivo*[25-26]. Little information is available about the inhibitory effects of Hg^{2+} on the DNA-binding activity, helicase activity, and ATPase activity of the helicase. The present study used a modified established method of fluorescence polarization and based on the observation of 3' fluorescein-labelled DNA, which had more rapid rotational diffusion than BLM-DNA complexes[21, 27]. The DNA unwinding reaction can be followed in real time by measuring the change of fluorescence polarization values.

As shown by our results, the DNA-binding activity, helicase activity, and ATPase activity are inhibited by Hg^{2+}, but the mechanism of the actions remain to be determined. Hg^{2+} could inhibit the activity of superoxide dismutase, catalase, and glutathione peroxidase activities in human erythrocytes *in vitro* in the range of 1.052-10.524 μmol/L[4]. Recent studies have shown that Hg^{2+} inhibits the biotransformation functions of NAT1[3], papain[6], NADPH -cytochrome P450 reductase[28], etc., at or near to the μmol/L level. Our results have also indicated that Hg^{2+} could affect the ATPase activity of $BLM^{642-1290}$ helicase at the μmol/L level, providing a basis for our further research of the molecular mechanisms of mercury toxicity from the aspects of DNA helicase.

The Hg^{2+} could also inactivate a number of enzymes by blocking their functional sites and binding with sulfhydryl groups, which are part of catalytic or binding domains[29]. Hg^{2+} could covalently bind with sulfhydryl groups of some proteins, which would alter the conformation of the protein, and create some protein adducts by modifying the side chains and finally result in changes in the shape and activity of the protein[30]. The sole CXXC sequence (with C being cysteine, X a quelconque amino acid and with two to four possible repeats) is the specific binding site in a wide variety of metal-binding proteins, including ferredoxins and rubredoxins[31]. The primary structure of $BLM^{642-1290}$ recombinant helicase is composed of two sole CXXC sequences, i.e., CDNC and CQVIC, which are located in the amino acid residues 1063-1066 and 940-944, respectively. A scheme of the mechanism of Hg^{2+} actions on the $BLM^{642-1290}$ recombinant helicase is illustrated in Figure 5.

The $BLM^{642-1290}$ recombinant helicase consists of the DEAH, RecQ-Ct (RecQ Conserved-Terminal), and HRDC (helicase and RNAseD C-terminal) domains[17]. DEAH helicase domain is a seven amino acid This can be explained by the fact that Hg^{2+} is bound with the catalytic "helicase core" domain; at the lower level of the Hg^{2+}, the conformation of the helicase is altered without inhibition of the biological activity. When the concentration of Hg^{2+} reached a higher

level, the activities are inhibited. Overall, these results have indicated that mercury may affect the biological activities and the structure of the BLM helicase, and the use of this enzyme may be helpful for further understanding of the molecular mechanisms of mercury toxicity from the aspects of DNA helicase and the molecular basis of BS pathogenicity.

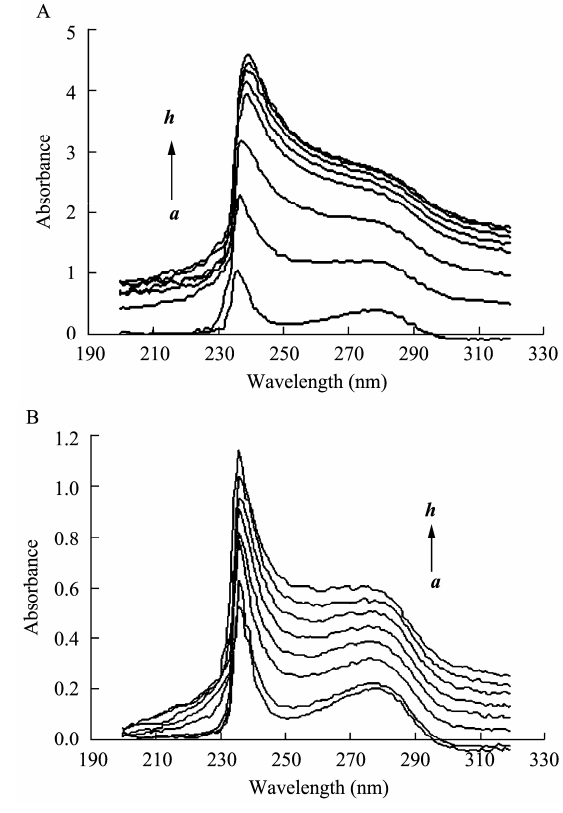
Table 3. Effects of Hg^{2+} on the ATPase Activity Constants of $BLM^{642-1290}$ Recombinant Helicase

$BLM^{642-1290}$	ATPase			
	V_{max}	K_m	K_{cat}	K_{cat} / K_m
	μmol · L^{-1} · min	μmol/L	s^{-1}	s^{-1} · μmol · L^{-1}
Control	146.2±25.6	187.6±2.4	6.1±1.7	2.6±0.2
40 μmol/L Hg^{2+}	58.4±8.6**	186.9±2.7	2.5±0.5*	1.0±0.3**
100 μmol/L Hg^{2+}	2.4±0.6**	188.1±3.4	0.1±0.05*	0.04±0.01**

Note. *: $P < 0.05$, **: $P < 0.01$, compared with the control group.

ACKNOWLEDGEMENTS

The authors thank Dr. XU GuangXi (Curie institute of 11th university in Paris, France) for providing the recombinant $BLM^{642-1290}$ *Escherichia coli BL21*.



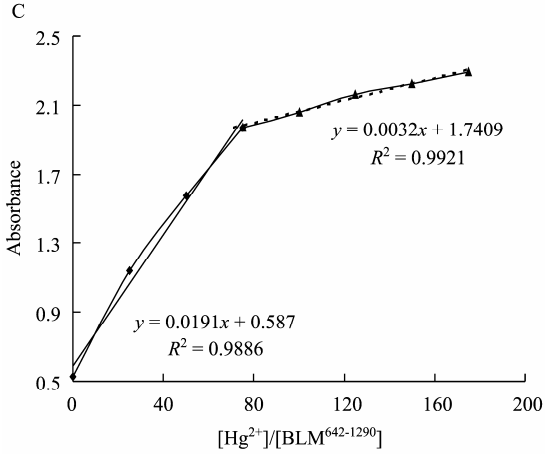


Figure 4. Effects of Hg^{2+} on the ultraviolet absorption spectrum of the $BLM^{642-1290}$ recombinant helicase. A: Differential UV spectra produced by the addition of different concentr ations of Hg^{2+} to 2.0 μmol/L of the helicase in the buffer (20 mmol/L Tris-HCl, pH 7.9) at 25 °C. The concentrations of Hg^{2+} (*a* to *h*) were 0, 50, 100, 150, 200, 250, 300, and 350 nmol/L. B: Differential UV spectra produced by different time of reac tion between 2 μmol/L of $BLM^{642-1290}$ recombi nant helicase and 150 nmol/L Hg^{2+}. The time of *a* to *h* was 0, 3, 6, 9, 12, 15, 18, 21 min. C: The titration curve of $BLM^{642-1290}$ recombinant helicase for the addition of Hg^{2+}.

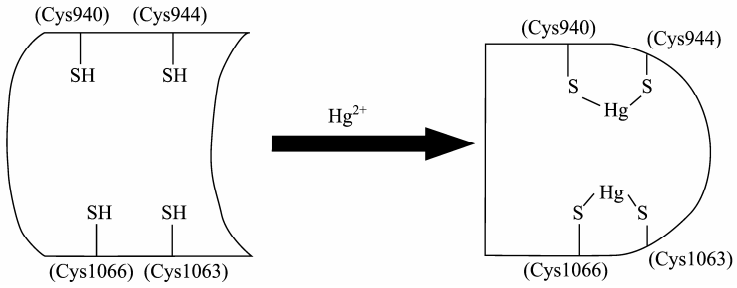


Figure 5. Schematic representation of the interaction of Hg^{2+} and $BLM^{642-1290}$ recombinant helicase. Hg^{2+} could bind with the helicase at a molar ratio of 2:1 for an inhibitor to the helicase as shown by mass spectra analysis. Therefore, it was proposed that between the two cysteine residues (Cys940-Cys944 and Cys1063-Cys1066), two intramolecular bridges be formed with Hg^{2+}, potentially leading to changes in the whole structure and function of $BLM^{642-1290}$ recombinant helicase.

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