Putative Secondary Active Site of Bovine Pancreatic Deoxyribonuclease I

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Abstract: Previous structural studies based on the co-crystal of a complex between bovine pancreatic deoxyribonuclease I (bpDNase I) and a double-stranded DNA octamer d(GCGATCGC)2 have suggested the presence of a putative secondary active site near Ser43. In our present study, several crucial amino acid residues postulated in this putative secondary active site, including Thr14, Ser43, and His44 were selected for site-directed mutagenesis. A series of single, double and triple mutants were thus constructed and tested for their DNase I activity by hyperchromicity assay. Substitution of each or both of Thr14 and Ser43 by alanine resulted in mutant enzymes retaining 30-70% of WT bpDNase I activity. However, when His44 was replaced by aspartic acid, either in the single, double, or triple mutant, the enzyme activities were drastically decreased to 0.5-5% that of WT bpDNase I. Interestingly, when cysteine was substituted for Thr14 or Ser43, the specific DNase activities of the mutant enzymes were substantially increased by 1.5-100-fold, comparing to their alanine substitution mutant counterparts. Two other more sensitive DNase activity assay method, plasmid scission and zymogram analyses further confirm these observations. These results suggested that His44 may play a critical role in substrate DNA binding in this putative secondary active site, and introduction of sulfhydryl groups at Thr14 and Ser43 may facilitate Mn2+-coordination and further contribute to the catalytic activity of bpDNase I.

Keywords: Deoxyribonuclease, secondary active site, site-directed mutagenesis.

INTRODUCTION

Bovine pancreatic DNase I (EC 3.1.21.1, bpDNase I) is the best-characterized among DNase I from various species [1]. It requires divalent metal ions for catalysis and cleaves double stranded DNA with no sequence specificity [1]. Recently, the distinctive functions of the two structural calcium atoms [2], the biological significance of the two disulfides [3] and the involvement of the N- and C-termini in the active protein folding [4] of bpDNase I have been investigated and summarized in our previous review article [5]. BpDNase I is formerly known to be secreted into the alimentary tract by pancreas [1]. With a very powerful web-server called “Cell-PLoc” at http://chou.med.harvard.edu/bioinf/Cell-PLoc/ [6], its subcellular location can be further predicted to be the endoplasmic reticulum, lysosome, and nucleus, in addition to being a secretory protein. This suggests that bpDNase I may play roles other than digesting DNA for nutritional purposes. Actually, DNase I has been proved to be involved in apoptosis of several cell types [7, 8].

The three-dimensional structure of bpDNase I was obtained by X-ray analysis at 2.0 Å resolution with refinement [9]. Based on the refined structure with the binding of Cit2+-thymidine 3′,5′-diphosphate (Ca-pTp) at the active site, a model for the interaction of bpDNase I with double-stranded DNA have been proposed [10]. More detailed catalytic mechanism were seen from the two enzyme-oligonucleotide complexes [11, 12] which showed that two histidine residues were near the scissile phosphate, each interacting strongly with a carboxylate group (H134 with E78 and H252 with D212) (Fig. 1). Amino acid sequence alignment of various DNases I also revealed the critical residues (E39, Y76, E78, H134, D168, D212, and H252) at this primary active site and they were all well conserved (Table 1). In our previous study, using chemical rescue, we assign unambiguously the roles for His134 as a general acid, and His252 as a general base, in bpDNase I catalysis [13].

In the crystal structure of the complex between bpDNase I and d(GCGATCGC)2 (Octa I), a dinucleoside-diphosphate (pGpC) was removed from the 3′ end of one of the strands of the nicked octamer duplex [11]. When the bpDNase I-Octa I co-crystals were soaked with Mn2+, but not Ca2+ or Mg2+, a second nick was observed four base-pairs away in the opposite strand. These observations may suggest the presence of a secondary active site in bpDNase I near Ser43, which is only activated by Mn2+ [11]. Possible candidates of amino acid residues that may contribute in a catalytic reaction at this secondary site are Glu13, Thr14, Arg41, Asp42, Ser43, and His44 (Fig. 1, Table 1). This Mn2+-dependent secondary active site could explain the shift from single-stranded scission to a double-stranded cutting mechanism when the divalent cation is changed from Mg2+ to Mn2+ [14].

DNase I has become increasingly important in therapeutic applications in recent years [15]. It may help shed light on protein action mechanisms [16] and provide useful information for drug design by conducting mutagenesis studies through a combination of biochemical experiments with computational modeling approaches [17, 18]. To investigate
Figure 1. The X-ray structure of bpDNase I-DNA complex. The coordinates were obtained from PDB entry 1DNK [11]. Twenty-two residues at the DNA binding interface of bpDNase I (gray) are labeled and colored according to their functional roles: yellow for catalytic residues in the primary active site, blue for residues in the putative secondary active site, magenta for metal ion binding residues, and red for critical DNA binding residues. The two DNA strands are colored in pink and green, respectively.

Table 1. Oligonucleotide Primers Used for Construction of the bpDNase I Mutants

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the existence of this putative secondary active site and the dual active site mechanism of bpDNase I, here we perform site-directed mutagenesis of three critical residues, Thr14, Ser43 and His44. The cDNA of bpDNase I [19] was used to construct a series of single, double, and triple mutants using PCR-based overlap extension method. The mutants were expressed in E. coli strain BL21(DE3)pLysE, purified, and tested for enzyme activity by hyperchromicity assay and supercoiled plasmid scission analysis. Evidence is shown to indicate that Thr14, Ser43 and His44 may combine to form a putative secondary Mn^{2+}-dependent active site, which in conjunction with the primary active site (Glu39, Tyr76, Glu78, His134, Asp168, Asp212, and His252), could be responsible for the double-stranded DNA cleavage mode of action of bpDNase I.

MATERIALS AND METHODS

Materials and Analytical Methods

The WT bpDNase I (code DP) was purchased from Worthington Biochemical Corporations and further purified as previously described [2]. Calf thymus DNA, DTNB, sodium sulfite, and HEPES were purchased from Sigma. All other reagents were of analytical grade. SDS-PAGE was according to the method of Laemmli [20] and gels were stained with silver [21]. Western blotting was as described [22] with
slight modifications using rabbit anti-bpDNase I polyclonal antibody (1000-fold dilution). Zymogram for measuring DNase activity after SDS-PAGE was according to Lacks et al. [23]. The CD spectra were measured as previously described [4].

Site-Directed Mutagenesis

A series of single (T14A, T14C, S43A, S43C, H44D), double (T14A::S43A, T14A::S43C, T14A::H44D, T14C::S43A, T14C::H44D, S43A::H44D), and triple (T14A::S43A::H44D, T14C::S43A::H44D) mutants of Thr14, Ser43 and His44 in bpDNase I was constructed by PCR-based site-directed mutagenesis using the overlap extension method [24] with the synthesized primers as listed in Table 2. Two primers used for introduction of the restriction enzyme sites (italic) were: NcoI (+): 5’-GCTGGCCATGGCCCTGAAGATGTC-3’ and XhoI (–): 5’-CTGGACTCGAGAAGGGACTTC-3’. The genes encoding the mutants were cloned into the NcoI and XhoI sites of pET15b. All mutated genes were sequenced by Mission Biotech Co. to confirm the presence of the mutation sites and to ensure no alterations at other sites.

Expression and Purification of the bpDNase I Mutants

For protein expression, the plasmids were transformed into the E. coli strain BL21(DE3)pLysE. The cultured E. coli cells were induced with 1 mM IPTG for 3 h and centrifuged at 12,000 rpm for 30 min. The expressed proteins with DNase I activity (WT, T14A, T14C, S43A, S43C, T14A::S43A, T14A::S43C, T14C::S43A, T14C::H44D, and T14C::S43A::H44D) caused E. coli cells to lyse, resulting in release of the proteins into growth media. After a brief centrifugation of the growth media, the supernatant fractions were used as the sources for a standard purification procedure according to our previous report [3].

The cell pellets of the inactive mutants (H44D, T14A::H44D, S43A::H44D, and T14A::S43A::H44D) were suspended with 1/10 volume of ice-cold 20 mM Tris-HCl, pH 7.0. The suspended solution was frozen-and-thawed for three times followed by sonication. The solution was then centrifuged at 12,000 rpm for 10 min. The supernatant fraction contained the soluble cytoplasmic proteins and the pellet fraction, insoluble inclusion bodies. All the mutant enzymes were detectable in both fractions as shown by Western blots (data not shown). Only the cytoplasmic fractions were collected for further purification to obtain the purified proteins based on a three-ion exchanger procedure as previously described [3].

DNase Activity and Protein Assays

Measurements of the DNase activities of wild-type and mutant bpDNase I in this study were based on the hyperchromicity method due to duplex DNA hydrolysis [25] with slight modifications. One unit of activity was defined as the amount of DNase I necessary to cause an increase of one A260 unit/min in 1 mL of assay solution containing 0.05 mg of calf thymus DNA, 10 mM CaCl₂, and 10 mM MnCl₂ at 25 °C. Protein concentrations were determined using the Bio-Rad protein assay reagent (Bio-Rad Lab) based on the method of Bradford [26], with BSA as the standard.

Table 2. Sequence Alignment of Active Site Residues of DNase I from Various Species

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The sequences were obtained from GenBank (accession numbers: bovine, AAA72974; ovine, P119377; porcine, P11936; human, AAA63170; canine, BAD6000; rabbit, BAA21724; rat, BAB62091; mouse, CAA03895; chicken, CAA10503; fish, O42446; snake, BAB65863; frog, BAA89002). Residues that are conserved across all 12 species are in bold. 18 crucial residues are classified into four groups and labeled with “C” for the catalytic residues in the primary active site, “S” for residues in the putative secondary active site, “M” for the metal ion binding residues, and “D” for critical DNA binding residues.
Plasmid DNA Scission Analysis

Depending on metal ions, duplex DNA was hydrolyzed by bpDNase I in a single or double scission mode. The two modes of action could be differentiated from the initial hydrolysis products of the supercoiled plasmid DNA [14]. The reaction mixture of 50 mM Tris-HCl, pH 7.0 (40 μL) contained 4 μg of BSA and 5.6 μg of plasmid pCRII with 10 mM MgCl₂ and 10 mM CaCl₂ or 1 mM EGTA, or 10 mM MnCl₂ and 1 mM EGTA. Hydrolysis was at 25 °C and began after addition of the enzyme. At selected time intervals, 5-μL aliquots of the reaction mixture were quenched with sample buffer (25 mM EDTA, 6% glycerol, 0.25% xylene cyanol, and 0.25% bromophenol blue) and then analyzed on 1% agarose gel.

RESULTS AND DISCUSSION

Expression and Purification of bpDNase I Mutants at Thr14, Ser43 and His44

WT and all mutant bpDNases I were purified to homogeneity as evidenced by SDS-PAGE analysis with coomassie blue staining (Fig. 2A). Since the enzymatic properties for E. coli-expressed WT bpDNase I and bpDNase I from Worthington were practically the same [2], the latter was used for comparison with the mutant enzymes in the current study. Because of glycosylation, the native bpDNase I was about 2 kDa larger than all of the recombinant proteins (Fig. 2A). To determine whether these mutants were correctly folded, we compared the secondary structures of the WT and mutant proteins by examining their CD spectra. The CD spectra of all the mutant proteins were similar to that of the WT bpDNase I (Fig. 3), indicating that the loss of DNase activity of some mutant enzymes (H44D, T14A::H44D, S43A::H44D, and T14A::S43A::H44D) was apparently due to the removal of the side chains rather than any gross structural changes of the protein molecule.

Hyperchromicity Assay and Zymogram Analysis

We measured the DNase I activity for the purified WT and mutant proteins based on the hyperchromicity method and the results were summarized as a bar chart showing the average of triplicate assays (Fig. 4). Replacing each or both of Thr14 and Ser43 with alanine results in mutant enzymes (T14A, S43A, and T14A::S43A) retaining 30-70% of WT bpDNase I activity. However, when His44 was substituted by aspartic acid, either in the single (H44D), double (T14A::H44D and S43A::H44D), or triple mutant (T14A::S43A::H44D), the enzyme activities were dramatically decreased to 0.5-10% that of WT bpDNase I. Interestingly, when cysteine was substituted for Thr14 or Ser43, the specific DNase activities of the mutant enzymes were substantially increased by 1.5-100-fold, comparing to their alanine substitution mutant counterparts (Fig. 4). These results suggested that His44 plays a more important role than either Thr14 or Ser43 in this putative secondary active site, and introduction of sulfhydryl groups at Thr14 and Ser43 may further contribute to the catalytic activity of bpDNase I. Zymogram analyses (Fig. 2B) further confirms the above observations.

Plasmid DNA Scission Analysis

This Mn²⁺-dependent secondary active site, as postulated by the previous report [11], could explain the shift from single-stranded scission to a double-stranded cutting mechanism when the divalent cation is changed from Mg²⁺ to Mn²⁺ [14]. If this Mn²⁺-dependent active site indeed exists, mutations at this region of bpDNase I should reduce the linear to
relaxed product ratio in the supercoiled plasmid scission assay in the presence of Mn\(^{2+}\) but not Mg\(^{2+}\).

Figure 3. The CD spectra for WT and mutant bpDNases I. All the spectra were collected with 3.5-4.0 μM proteins in 10 mM Tris-HCl, pH 7.0, containing 10 mM Ca\(^{2+}\). The spectra for: ○, WT; ▼, T14A; ▽, T14C; △, S43A; ■, H44D; □, T14A::H44D; ♦, T14C::H44D; ◊, T14A::S43A::H44D; ▲, T14C::S43A::H44D.

To test the above hypothesis, we performed plasmid scission analyses for WT and all the mutant bpDNases I in the present study. In the presence of Mg\(^{2+}\) only, WT and all mutant enzymes cleaved the Mg\(^{2+}\)-DNA substrate in a single nicking mode with the formation of only the relaxed open-circular DNA (Fig. 5). In the presence of Mn\(^{2+}\), WT and all single mutants (T14A, T14C, S43A, S43C) except H44D were able to hydrolyze the Mn\(^{2+}\)-DNA substrate forming 10-50% linear duplex DNA in addition to the relaxed open-circular DNA, indicating double scission, while H44D mutant could only cleave the Mn\(^{2+}\)-DNA substrate in a single nicking mode (Fig. 5A-F). These results indicated that mutations of Thr14 or Ser43 only have little effect on the catalytic activity of bpDNase I, while His44 may play a more significant role at this putative secondary active site.

In order to gain further insight into the roles of these crucial residues in the secondary active site, several double and triple mutants were constructed and characterized. In the presence of Mn\(^{2+}\), double mutant T14A::S43A, though being less active than WT bpDNase I, could still cleave the plasmid DNA in the double scission mode. Interestingly, DNase activity for double mutants T14C::S43A and T14A::S43C were similar to that of the WT enzyme, suggesting that replacing Thr14 and Ser43 with cysteine may further increase the catalytic activity of bpDNase I. Similar to mutant H44D, double mutants T14A::H44D and S43A::H44D could only cleave the Mn\(^{2+}\)-DNA substrate in a single nicking mode (Fig. 5G-L). Results from the triple mutants T14A::S43A::H44D and T14C::S43A::H44D (Fig. 5M, N) further confirm the contributions of His44 and the introduction of sulfhydryl groups at Thr14 and Ser43 to the catalytic activity of bpDNase I.

Recently, Lazarus and co-workers performed a mutational analysis of human DNase I at the DNA binding interface [27]. In their study, single mutants E13A, T14A, S43A and H44A showed no significant difference on the supercoiled plasmid digestion pattern from the WT protein in the presence of either Mg\(^{2+}\) or Mn\(^{2+}\) ions, prompting them to conclude that a Mn\(^{2+}\)-dependent secondary active site may not exist in human DNase I. In our present study, similar results could be found for single mutants T14A and S43A. H44A was constructed and expressed with similar activity to the WT enzyme (data not shown), while H44D showed very little activity, indicating that His44 may play a critical role in substrate DNA binding. Since bovine and human DNase I
are homologous but not identical, the possibility of the presence of this putative secondary active site does exist for the bovine enzyme.

**CONCLUSION**

In our present study, we found that His44 may play a critical role in substrate DNA binding in the putative secon-
dary active site, at least in bovine pancreatic DNase I. Moreover, an interesting phenomenon was observed that substitution of Thr14 and Ser43 with Cys substantially increase the DNase activities, as compared to either the WT enzyme or their Ala-substitution mutant counterparts. It is apparent that T14C and S43C form a metal ion binding site that is even more attractive as it already is in the WT enzyme for a “thiophilic” metal ion, such as Mn$^{2+}$. In contrast to Mg$^{2+}$, which can be readily coordinated by Asn, Thr, Ser, Asp and also His residues, Mn$^{2+}$ is also readily coordinated by the thiol group of a Cys residue. Significant increase in DNase I activity measured for the double (T14C::H44D) and triple mutants (T14C::S43A::H44D) further confirm our hypothesis, suggesting a possible role of Thr14 and Ser43 for metal ion coordination in this putative secondary active site of bpDNase I. Delineating this dual active site catalytic mechanism in the present study not only solve the long-lasting mystery of bpDNase I, but also may provide very useful insights for design of hyperactive recombinant DNase I as potential drugs for clinical uses.

ACKNOWLEDGEMENTS
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ABBREVIATIONS

bpDNase I = Bovine pancreatic deoxyribonuclease I
WT = Wild-type
T14X = bpDNase I mutants with Thr$^{14}$ replaced by Ala or Cys
S43X = bpDNase I mutants with Ser$^{43}$ replaced by Ala or Cys
H44D = bpDNase I mutants with His$^{44}$ replaced by Asp
T14X::S43X = bpDNase I mutants with Thr$^{14}$ replaced by Ala or Cys and Ser$^{43}$ replaced by Ala or Cys
T14X::H44D = bpDNase I mutants with Thr$^{14}$ replaced by Ala or Cys and His$^{44}$ replaced by Asp
T14X::S43A::H44D = bpDNase I mutants with Thr$^{14}$ replaced by Ala or Cys, Ser$^{43}$ replaced by Ala, and His$^{44}$ replaced by Asp

REFERENCES