Structure and Function of Bovine Pancreatic Deoxyribonuclease I

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Abstract: Bovine pancreatic deoxyribonuclease I (bpDNase), the first DNase discovered, is the best characterized among various types of DNase. A catalytic mechanism has been suggested based on the X-ray structure of the bpDNase-octamer complex. In this review, we will focus on three aspects: 1) the distinctive functions of the two structural calcium atoms; 2) the biological functions of the two disulfides; and 3) the involvement of the N- and C-terminal fragments in the enzyme folding for activity.

Keywords: Deoxyribonuclease, calcium, disulfide, protein folding, chemical modification, site-directed mutagenesis, enzyme mechanism.

INTRODUCTION

DNase I (EC 3.1.21.1) is a secretory glycoprotein with an endonuclease activity that cleaves double-stranded DNA to yield 5'-phosphorylated polynucleotides. It requires divalent metal ions for catalysis. Bovine pancreatic DNase I (bpDNase) is the best-characterized DNase I type enzyme [1]. It has a molecular mass of 31 kDa and an alkaline pH optimum. DNase I is an enzyme secreted into the alimentary tract by exocrine glands such as pancreas or parotid, and can be found most abundant in these organs. However, it also occurs in other tissues with much less quantities [2], suggesting that it may play roles other than digesting DNA for nutritional purposes. Particularly, DNase activities can be related to DNA degradation in apoptosis [3] and DNA clearance from extracellular media [4]. One of the most peculiar phenomena for DNase I is the very tight 1:1 complex formation with actin (Kd = 5 x 10⁻⁸ M⁻¹) [5], which has led to a proposed physiologic role for DNase I as a regulator of actin polymerization in cells [6]. Besides being used widely as a tool in molecular biology, DNase I has been applied in modern medicine [7], including treatment for cystic fibrosis [8] and systemic lupus erythematosus [9]. DNase I cleaves double-stranded DNA without nucleotide sequence specificity [1]. It was the first DNase sequenced with the conventional protein sequencing technique [10], and the crystal structure was solved at 2.0 Å resolution with refinement [11].

THE PRIMARY, SECONDARY AND TERTIARY STRUCTURES

The enzyme, bpDNase, has many isoforms [12]. They differ according to genetic variations or sialic acid contents [13, 14]. The polypeptide chain is glycosylated at Asn-18, and the presence of sialic acids in the carbohydrate side chain results in charge heterogeneity. The isoforms can be separated and detected by isoelectric focusing [15]. The amino acid sequence of the polypeptide chain has been elucidated by chemical analysis [16]. A gene encoding bpDNase was originally chemically synthesized [17], and later another was cloned from the bp-cDNA [18] and they were expressed in E. coli with DNase activities. The sequence of the 1295-bp polynucleotide from a clone of the bp-cDNA was deduced by DNA sequencing. The sequence shows an open reading frame which can be translated into a 282-amino acid polypeptide, including a 22-amino acid hydrophobic signal peptide and the 260-a.a. mature polypeptide of bpDNase. An expression plasmid was constructed by inserting the bp-cDNA fragment coding for the mature bpDNase plus a hexanucleotide coding for Met-Ala at the 5'-end into the vector pET-15b. The plasmid was transformed into E. coli strain DH5α and the active bovine recombinant (br) DNase was produced after induction of protein synthesis. From the induced culture medium, brDNase was purified by chromatography on a Mono Q column. The purified brDNase showed a molecular mass of 29 kDa with a specific activity close to that of the native bpDNase. The NH₂-terminus of brDNase was Ala, not Met, and Asn at position 19, corresponding to the carbohydrate attachment site of bpDNase, was not glycosylated.

The three-dimensional structure was obtained by X-ray analysis at 2.0 Å resolution with refinement [11]. The molecule is a compact α,β-protein of an approximate dimension of 45 x 40 x 35 Å. The core of the molecule is formed by two tightly packed, six-stranded β-pleated sheets and is surrounded by eight helices and several loop regions. An extended hydrophobic core is mainly responsible for the structural stability and rigidity of the molecule. Other factors contributing to the stability of the enzyme are numerous intramolecular hydrogen bonds, several salt bridges, two disulfide bonds, and the two structurally bound Ca²⁺ atoms. The bound Ca²⁺ atoms stabilize the loop regions of the molecule. Based on the refined structure with the binding of Ca²⁺-thymidine 3',5'-diphosphate (Ca-pTp) at the active site, a model for the interaction of bpDNase with double-stranded DNA is proposed.
DNA have been proposed that involves the binding of an exposed loop region in the minor groove of B-DNA and electrostatic interactions of phosphates from both strands with basic residues on either side of this loop [19]. A crystal structure of the bpDNase-DNA octamer complex was also solved by molecular replacement and refined to a 2.0 Å [20] resolution. Results from the complexes with the cleavable substrate d(GCGATCGC)_2 and the uncleavable d(GGTATACC)_2 confirm the hypothesis that minor-groove width and depth and the intrinsic flexibility of DNA are major parameters determining the cutting rate. Common features conserved in the two complexes included stacking interactions of a deoxyribose group of DNA onto the side-chain of Tyr76 and the binding of Arg41 in the minor groove. The disposition of residues around the scissile phosphodiester bond suggested that His134 and His252 are involved in catalysis. Based on such a structure, a catalytic mechanism (Fig. 1) was thus suggested [21], and further supported by the crucial amino acid residues identified by chemical modifications [22-25] and by site-directed mutagenesis [26-29]. A second DNA cutting induced by diffusion of Mn^{2+} into the crystals may suggest the presence of a plausible secondary active site in bpDNase.

**CHEMICAL MODIFICATIONS AND SITE-DIRECTED MUTAGENESIS**

Modifications of bpDNase with p-nitrobenzensulfonyl fluoride showed that among the Tyr residues modified, Tyr148 and Tyr175, were crucial for the enzymatic activity [24], and modifications with phenylglyoxal showed that Arg9 and Arg41 were probably involved in substrate binding [25]. In addition to the natural substrate, DNA, bpDNase also hydrolyzes several p-nitrophenyl esters of thymidine phosphates and p-nitrophenyl phenylphosphonate [30]. By far, site-directed mutagenesis has been an excellent strategy for studying the functional aspects of a particular residue in a protein. To select functionally important residues for mutation, quite often one has to rely on information from chemical modifications, molecular modeling or sequence alignment. Sequence alignment of DNases I from various species reveals that 120 of the 260 residues are identical. Among the identical residues are those at the active site (His252, His134, Asp212, Glu39 and Glu78), those involved in one of the two Ca^{2+}-binding sites (Asp99, Asp107, Phe109 and Glu112) and the essential disulfide bridge (Cys173 and Cys209). Thus, the alignment results should provide information to select, with good reasoning, functionally important residues as targets for changes by site-directed mutagenesis. For example, based on X-ray analyses, two variants, bpDNase(H134Q) and bpDNase(Y76A), were created [31], and the very low enzymatic activities of the two variants indicated that His134 was an essential catalytic residue, while Tyr76 contributed to the binding of DNA. Variants of His134, Asp168, Asp212 and His252 demonstrated that the mutated residues played a critical role in the enzymatic catalysis [27]. The roles of Arg41 and Tyr76 in the DNA sequence selectivity [32] and in the coupling of DNA recognition to phosphodiester bond cleavage [26] were also studied by site-directed mutagenesis. Moreover, a loop insertion variant with altered sequence selectivity was used to study the DNA-DNase I interactions [33].

**CELLULAR FUNCTIONS**

Lazarides and Lindberg [6] discovered that actin was a naturally occurring inhibitor of DNase I. Based on this finding, they suggested that DNase I could function as a protein participating in the formation of actin filaments rather than in the degradation of DNA. Because phosphoinositides could dissociate the actin-DNase I complex [34], they also suggested that this interaction may have other biological functions. DNase I has been a useful tool in measuring G-actin levels in cells [35]. However, its role playing in cytoskeletal dynamics is yet to be elucidated. The highly specific and tight binding of DNase I for actin and the widely distributed

![Figure 1](image-url). A possible catalytic mechanism for bpDNase. H134 and H252 act as catalytic acid and base, respectively. Two Mg^{2+}, interacting with E39 and D168, neutralize the charges on the non-bridging oxygen atoms of the scissile phosphodiester bond and provide electrophilic catalysis. H134 is connected to Y76, a key DNA-binding residue, via E78. This glutamate forms a link between the binding of DNA and its hydrolysis. R(5')O and R(3')O represent the remainder of the DNA molecule.
actin among different cell types may find other biological importance for the enzyme. The crystal structure of the actin-DNase I complex has been determined at 2.8-Å resolution [36]. Although most of the actin binding proteins interact with actin subdomain I, DNase I binds to the part corresponding to the pointed end of actin [37]. It binds so tightly to G-actin ($K_d$ in the nM range) [38], and it essentially removes all free actin monomers in solution. It binds much more weakly to F-actin (100 µM) where a capping protein increases the rate of dissociation from the pointed ends of filaments [37]. Dos Remedios and colleagues found the binary or ternary complexes among DNase I, actin and coflin which suggested that DNase I could act as a stabilizer of actin monomers by effectively removing them from the pool of monomers available for the filament assembly. Thus DNase I could be a natural modulator on the action of coflin in cytoskeleton [39, 40].

DNase I, as well as other DNases, has been implicated in apoptosis [3]. DNase I has been found in the nuclei of apoptotic thymocytes [41] as well as apoptotic prostate epithelial cells after androgen deprivation [42]. DNase I was shown to be constitutively expressed in bovine epithelial lens cells [43] or lens fibers [44], and could be considered as an enzyme associated with nuclear degradation events, particularly with the final phases of the DNA degradation. Furthermore, Puccetti and co-workers recently suggested that DNase I could behave as a transcription factor which selectively regulated cell surface Fas expression in human cells and pointed towards a fundamental role of DNase I in the regulation of the apoptotic machinery [45].

**STRUCTURAL CALCIUM ATOMS**

The catalytic rates and the mode of action for bpDNase on DNA were very much dependent upon metal ions [46]. Although Ca$^{2+}$ activated bpDNase with only a minimal activity, it acted synergistically when used with other activating ions [47]. At pH 7.5, two strong and several weak Ca$^{2+}$-binding sites were found by equilibrium dialysis [48]. When it was bound to bpDNase, Ca$^{2+}$ had other effects. The calcium ion could protect the trypsin inactivation [49] and prevent the β-mercaptoethanol disruption of the essential sulphydryl bond [50]. It could also produce the conformational changes as evidenced by the induced changes of the spectra in ultraviolet absorption [51], optical rotation [52], and fluorescence [53]. The three-dimensional structure of bpDNase [11] showed two structural calcium atoms located in the two distinctive Sites I and II (Fig. 2). Recently, the Ca$^{2+}$-dependent activity of human DNase and its hyperactive variants have been investigated [54].

In order to gain insight into the functional roles of the two structural calcium atoms, we produced the variants of bpDNase by replacing Asp99 or Asp201 with Ala to impair Ca$^{2+}$-binding at Sites I and II by site-directed mutagenesis [65]. The two altered proteins, brDNase(D99A) and brDNase(D201A), were expressed in *E. coli* and purified. Equilibrium dialysis showed that mutation destroyed one Ca$^{2+}$-binding site each in brDNase(D99A) and brDNase (D201A), respectively. Compared to bpDNase, the $V_{max}$ value for brDNase(D99A) remained unchanged and that for brDNase (D201A) was decreased, while the $K_m$ values for the two variants were increased 2-3 fold. Like the native bpDNase, brDNase(D99A) was able to make double-scission on duplex DNA with Mg$^{2+}$ plus Ca$^{2+}$ and was effectively protected by Ca$^{2+}$ from the trypsin inactivation. However, under the same conditions, brDNase(D201A) lost the double-scission ability and was no longer protected by Ca$^{2+}$. Nevertheless, the two variant proteins retained the characteristics of the Ca$^{2+}$-induced conformational changes and the Ca$^{2+}$-protection against the β-mercaptoethanol disruption of the essential sulphydryl bond, suggest-

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**Figure 2.** (A) The X-ray structure of bpDNase showing the calcium-binding Sites I and II. The calcium atom in Site I is coordinated by the oxygens of Asp201, Thr203, Thr205, and Thr207, while the calcium atom in Site II by the oxygens of Asp99, Asp107, Phe109, and Glu112. (B) Sequence homology within the motifs of Sites I and II of DNases from various species. Sequences were taken from bovine, ovine, porcine [55], human [56], canine [57], rabbit [58], rat [59], mouse [60], chicken [61], snake [62], frog [63], and fish [64] DNases.
ing that other weaker Ca\(^{2+}\)-binding sites not found in the X-ray structure, but found in the equilibrium dialysis experiment [48], were responsible for these properties. Therefore, the two structural calcium atoms can not only maintain the overall conformation of the active DNase, but also play the role in the fine-tuning of DNase activities.

**BIOLOGICAL FUNCTIONS OF THE DISULFIDES**

One large (Cys173-Cys209) and one small (Cys101-Cys104) disulfide loops occur in bpDNase (Fig. 3A, [66]). In a Ca\(^{2+}\)-containing buffer without denaturing agents, only the small loop was reduced by β-mercaptoethanol, resulting in an enzyme structure still retaining the full activity. In the Ca\(^{2+}\)-free buffer, both loops were reduced by β-mercaptoethanol with concomitant loss of the enzymatic activity. Therefore, the small and large loops were referred to as the “non-essential” and “essential” disulfides, respectively [50]. During molecular evolution of proteins (Fig. 3B), changes in the number of disulfides in DNase occur. Thus, fish DNase [64] contains only the “essential” disulfide and chicken DNase [61], with the same two disulfides as in bpDNase, has one extra disulfide (Cys192-Cys217). Since enzymatic and physical properties of DNase from these three species were not quite the same, it prompted us to investigate the possible biological functions of the two disulfides and the disulfide corresponding to the third disulfide present in chicken DNase [66]. Thus, the small non-essential and the large essential disulfides in bpDNase were changed by using alanine mutations. Three variants, [brDNase(C101A)] and [brDNase(C173A) and brDNase(C209A)] as well as the variant with an additional third disulfide [brDNase(F192C/A217C)] were produced. Without the Ca\(^{2+}\) protection, bpDNase and brDNase(C101A) were readily inactivated by trypsin while brDNase(F192C/A217C) remained active. With Ca\(^{2+}\), all forms of DNase, except for brDNase(C101A), were protected against trypsin. All forms of DNase, after being dissolved in 6 M guanidine-HCl, were fully reactivated by diluting into a Ca\(^{2+}\)-containing buffer. However, when diluted into a Ca\(^{2+}\)-free buffer, bpDNase and brDNase(C101A) remained inactive, but 60% of the bpDNase activity was restored with brDNase (F192C/A217C). When heated, bpDNase was inactivated at a transition temperature of 65 °C, brDNase(C101A) at 60 °C, and brDNase(F192C/A217C) at 73 °C, indicating that the small disulfide, albeit not essential for activity, is important for the structural integrity, and that the introduction of a third disulfide can further stabilize the enzyme. When pellets of brDNase(C173A) and brDNase(C209A) in inclusion bodies were dissolved in 6 M guanidine-HCl and then diluted into a Ca\(^{2+}\)-containing buffer, 10-18% of the bpDNase activity was restored, suggesting that the “essential” disulfide is not absolutely crucial for enzymatic catalysis. Because there was a sequence homology between the “non-essential” disulfide (–CESC–) of bpDNase and the active site motif (–CGPC–) of thioredoxin [67], a 39% thioredoxin-like activity could be detected in bpDNase. Addition of Ca\(^{2+}\) in the assay buffer further increased the activity to 50%. Thus, the disulfides in bpDNase not only play the role in stabilizing the protein molecule but also may engage in other biological functions such as the disulfide/dithiol exchange reaction.

In the X-ray structure of the Ca\(^{2+}\)-bound bpDNase (Fig. 3A), the intramolecular contact distance between the guanidinium group of Arg187 and the β-carboxyl group of Asp198 is 3.05 Å [11] and the beginning amino acid of the Ca\(^{2+}\)-binding Site I, Asp201, was only two residues away from Asp198. When Ca\(^{2+}\) was not bound in the vicinity, the guanidinium group was separated from the β-carboxyl group, providing Arg187 accessible to trypsin [65]. In our study [66], brDNase(F192C/A217C) was resistant to trypsin inactivation without assistance of Ca\(^{2+}\), probably because the third disulfide provided the increased ionic interaction between Arg187 and Asp198, making Arg187 inaccessible to trypsin.

![Figure 3](image-url)

**Figure 3.** (A) The X-ray structure of bpDNase illustrating the two intrinsic disulfides (Cys101-Cys104 and Cys173-Cys209) and the corresponding residues (Phe192 and Ala217) for the newly engineered third disulfide (Cys192-Cys217). (B) Sequence homology within the disulfide-containing motifs of DNases from various species. Sequences were taken from the same sources as described in the legend for Fig. 2.
PARTICIPATION OF THE N- AND C-TERMINAL SEQUENCES IN ACTIVE PROTEIN FOLDING

The three-dimensional structure of bpDNase revealed that its N- and C-termini were in close proximity with the backbone hydrogen bonding forming an anti-parallel β-sheet structure (Fig. 4A). Previous studies showed [68] that removal of only two amino acid residues from the C-terminus could lead to a complete inactivation of bpDNase, suggesting the possible role of the C-terminal sequence in the final active protein folding. The loss of the DNase activity was very likely due to the loss of the stabilizing effect contributed by the anti-parallel β-sheet structure because alignment of the N- and C-terminal amino acid sequences of DNases from various species (Fig. 4B) revealed that residues within the two terminal sequences were well conserved. Alignment also showed that most of the polypeptide chains were 260 amino acid residues in length, but DNases from pig [55], dog [57], rat [59], mouse [60], chicken [61] and snake [62] contained a dipeptide protruding at the C-terminus, while amphibian DNases were characterized by an additional C-terminal fragment of 70-amino acids with a unique cysteine-rich stretch [63]. Thus, heterogeneity at the C-terminus beyond the residue 260 was not an unusual phenomenon among species and probably would not affect the DNase activity. The involvement of the N- and C-terminal fragments in the active protein folding of bpDNase was thus investigated via a series of deletion and substitution variants [69].

Several substitution variants of N-terminal Leu1 and C-terminal Leu259, and one variant with only the last Thr260 deleted, remained fully active. However, the other deletion variants, in which 2 to 10 amino acid residues were removed from the C- or N-terminus, all lost the DNase activity. The results indicate that the backbone hydrogen bonding in the anti-parallel β-sheet, rather than the side-chain interactions, is crucial for the correct protein folding. When the deletion variants were complemented with synthetic peptides of the deleted N- or C-terminal sequences, the DNase activity could be generated. The highest DNase activity was observed when the C-terminal 10 residue-deleted brDNase(Δ251-260) was admixed with the C-terminal 10 residue-peptide (Peptide C10) in a molar ratio of 1:400. The non-covalent binding between brDNase(Δ251-260) and Peptide C10 exhibited a dissociation constant of 48 µM. Circular dichroism spectra showed that the deletion variants were partially folded with...
mainly helical structures, and that admixture with corresponding peptides facilitated their folding into the native-like β-sheet-rich structure. Thermal denaturation profiles also revealed that the transition temperature for brDNase(Δ251-260) was increased from 55 to 63 °C after incubation with Peptide C10.

The folding-activation process for the deletion variant occurred in two stages as illustrated in Fig. 5. The first stage was the folding associated with Ca²⁺-binding for acquisition of a partially-folded intermediate with mainly helical structures. The second stage was the formation of a structure rich in β-sheet upon Peptide C10 binding followed by a tight packing of the preformed secondary structure segments to lead to the final native structure through the formation of the N- and C-terminal anti-parallel β-sheets. Ca²⁺ participates in the folding-activation process of the deletion variant by stabilizing the partially-folded species, facilitating the formation of the N- and C-terminal anti-parallel β-sheets, and enabling the efficient conversion of the polypeptide into its packed native structure.

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