The distinctive functions of the two structural calcium atoms in bovine pancreatic deoxyribonuclease

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Abstract

The two amino acid residues, Asp 99 and Asp 201, involved in the coordination of the two calcium atoms in the X-ray structure of bovine pancreatic (bp) DNase, were individually changed by site-directed mutagenesis. The two altered proteins, brDNase(D99A) and brDNase(D201A) were expressed in *Escherichia coli* and purified by anion exchange chromatography. Equilibrium dialysis showed that mutation destroyed one Ca^{2+} -binding site each in brDNase(D99A) and brDNase(D201A). Compared with bpDNase, the V_{max} value for brDNase(D99A) remained unchanged and that for brDNase(D201A) was decreased, whereas the K_m values for the two variants were increased two- to threefold when the DNA hydrolytic hyperchromicity assay was used. Like 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. But under the same conditions, brDNase(D201A) lost the double-scission ability and was not 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 disulfide bond, suggesting that other weaker Ca^{2+} -binding sites not found in the X-ray structure were responsible for these properties. Therefore, the two structural calcium atoms are not for maintaining the overall conformation of the active DNase, as it has been indicated in the X-ray analysis, but rather play the role in the fine-tuning of the DNase activity.

Keywords: DNase; calcium-binding sites; site-directed mutagenesis; DNA double scission; trypsin inactivation; essential disulfide

DNase I is a divalent metal ion-requiring DNase with an alkaline pH optimum and bpDNase is the best-characterized DNase I type enzyme (Moore 1981). The enzyme was found in bovine pancreas in large amounts and was thought

to be one of the digestive enzymes for nutritional purposes. However, owing to the improved assay methods, minute amounts of DNase I activities could be readily detected in various tissues other than pancreas (Nadano et al. 1993). Thus, investigations of other physiological roles of DNase I have emerged in recent years. A good example was the apoptotic DNA fragmentation that was attributed to DNase γ , a member of the DNase I family enzyme (Shiokawa and Tamuma 2001). Although being used widely as a tool in molecular biology, DNase I has been applied in modern medicine (Liao 1997), including the treatments for cystic fibrosis (Durward et al. 2000) and systemic lupus erythematosus (Prince et al. 1998).

For bpDNase, the catalytic efficiency and the mode of action on DNA were very much dependent upon metal ions (Campbell and Jackson 1980). Although Ca^{2+} activated bpDNase with only a minimal activity, it acted synergisti-

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Abbreviations: bp, bovine pancreatic; br, bovine recombinant; brDNase(D99A), the Asp 99→Ala 99 variant of brDNase; brDNase(D201), the Asp 201→Ala 201 variant of brDNase; brDNase(D99A/D201A), the D99A/D201A double mutation of brDNase; NPPP, *p*-nitrophenyl phenylphosphonate; OCPC, *o*-cresolphthalein complexone; AMP, 2-amino-2-methyl propane-1-ol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EGTA, ethylene glycol-bis(β -aminoethyl ether) N,N,N',N'-tetraacetic acid; PVDF, polyvinylidene fluoride; U, units.

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cally when used with other activating ions (Wiberg 1958). At pH 7.5, two strong and several weak Ca^{2+} -binding sites were detected by equilibrium dialysis (Price 1972). Bound to bpDNase, Ca^{2+} had other effects, such as protecting the trypsin inactivation (Price et al. 1969a), preventing the β -mercaptoethanol disruption of the essential disulfide bond (Price et al. 1969b), and producing the conformational changes evidenced by ultraviolet absorption (Tullis and Price 1974), optical rotation (Poulos and Price 1972), and fluorescence (Tullis et al. 1981). The three-dimensional structure of bpDNase, elucidated by X-ray at 2 Å resolution with refinement (Oefner and Suck 1986), showed two structural calcium atoms, located in the two distinctive Sites I and II. Recently, Ca^{2+} -dependent activity of human DNase and its hyperactive variants have been investigated (Pan and Lazarus 1999). To gain insight into the functional roles of the two structural calcium atoms, we produced the variants of bpDNase by replacing Asp 201 and/or Asp 99 with Ala to impair Ca^{2+} -binding at Sites I and II and investigated these variants in response to Ca^{2+} .

Results

Chromatography of the recombinant proteins

Figure 1B showed the purified recombinant proteins as analyzed by anion-exchange chromatography. The chromatographic behavior of the *Escherichia coli* expressed brDNase (Fig. 1B,a) was identical to that of the native bpDNase (Fig. 1B,b). However, the two variants, losing one Ca^{2+} -binding site each, were eluted with higher Ca^{2+} concentrations (Figs. 1B,c,d). Another variant, brDNase(H134Q), which was DNase inactive due to the change of the essential His 134 residue, was eluted at the same position (data not shown) as that of bpDNase, indicating that the elution behavior was not altered by mutation at a site other than the Ca^{2+} -binding sites.

K_d of Ca^{2+} binding

It has been shown previously (Price 1972) by equilibrium dialysis that bpDNase had, in addition to several weak sites, two strong Ca^{2+} -binding sites with an average $K_d = 1.4 \times 10^{-5}$ M; but the two calcium atoms in the X-ray structure (Oefner and Suck 1986) bound at Sites I and II were not correlated with these two experimentally measured strong sites. Because the Scatchard plot (Fig. 2) showed only one Ca^{2+} bound per mole for brDNase(D99A), the measured K_d value of 0.3×10^{-5} M was unequivocally assigned to Site I. The two Ca^{2+} bound per mole for brDNase(D201A) with an average $K_d = 1.3 \times 10^{-5}$ M suggested that, in addition to

Fig. 1. Homogeneity of the purified proteins. (A) SDS-PAGE of the purified DNases. Lanes a–d were bpDNase, brDNase, brDNase(D99A), and brDNase(D201A), respectively. Because of glycosylation, the native bpDNase is ~2 kD larger than all of the recombinant proteins. (B) Chromatography of the purified DNases on anion exchange. The sample was loaded on a Mini Q column, pre-equilibrated with Buffer A (20 mM Tris-HCl at pH 7.5). The flow rate was 0.5 mL/min and the sample was eluted with a linear gradient from 0 to 100% Buffer B (15 mM CaCl_2 in Buffer A) for 10 min. The AKTA Purifier System of Amersham Pharmacia Biotech was used for chromatography. (a) bpDNase; (b) brDNase; (c) brDNase(D99A); (d) brDNase(D201A).

Site II, a third weaker site (Site III) also existed, but the Scatchard plot cannot distinguish the two K_d values under the experimental conditions. This was reflected on anion exchange chromatography in which brDNase(D201A), losing Site I, was eluted as a broad peak (Fig. 1B,d). Nevertheless, Ca^{2+} bound at Site II should be stronger than bound at Site III, because calcium was not cocrystallized at Site III. When the three K_d values for Sites I, II, and III were aver-

Fig. 2. The Scatchard plots for Ca^{2+} binding. The lines as well as the K_d and n values were obtained by the linear regression analysis based on SigmaPlot for Windows Version 5.00. (Line *a*) bpDNase, $K_d = 1.0 \times 10^{-5}$ M, $n = 2.6$; (line *b*) brDNase(D99A), $K_d = 0.3 \times 10^{-5}$ M, $n = 1.0$; (line *c*) brDNase(D201A), $K_d = 1.3 \times 10^{-5}$ M, $n = 1.9$.

aged, the calculated value 1.0×10^{-5} [i.e., $(0.3 + 2 \times 1.3) / 3 \times 10^{-5}$] M agreed with the approximately measured K_d value for bpDNase with three Ca^{2+} bound per mole shown in Figure 2 (Curve *c*). The Scatchard plot (Fig. 2, curve *b*) showed only one Ca^{2+} -binding site for brDNase(D99A), probably because, without the medium affinity Site II, its strongest binding Site I predominated, and its Site III was not detectable under the experimental conditions. Another possibility was that the Ca^{2+} -binding at Site II might facilitate the binding of Site III. Therefore, the binding of the three calcium atoms in the native bpDNase, indistinguishable in the Scatchard plot, could be cooperative.

The Scatchard plots (Fig. 2) also provided evidence that replacement of either Asp 99 or Asp 201 with Ala impaired the Ca^{2+} binding, and that a third, weaker Ca^{2+} -binding site in bpDNase, detected by equilibrium dialysis, was not occupied by calcium in the X-ray structure (Oefner and Suck 1986). The fact that Ca^{2+} is coordinated by six oxygen atoms at Site I, whereas by four at Site II in the X-ray structure, may explain why Site I is the strongest binding site.

K_m and V_{max}

The K_m and V_{max} values, as shown in Table 1, were determined by using the DNA hydrolytic hyperchromicity assay. For bpDNase and brDNase the K_m values were about the same, whereas for the two variants they were higher, indi-

cating that the impairment of either one of the two Ca^{2+} -binding sites lowered the enzyme affinity for DNA. As compared with the V_{max} values for bpDNase and brDNase, the value for brDNase(D99A) remained essentially unchanged, but that for brDNase(D201A) was decreased about eightfold. Although the data were obtained with Mn^{2+} plus Ca^{2+} as activating ions, they were comparable with those of human DNase (Pan and Lazarus 1999) in which Mg^{2+} plus Ca^{2+} was used.

Plasmid DNA scission

Duplex DNA was hydrolyzed by bpDNase in the single or double scission mode, depending on metal ions, and these two modes of action could be differentiated from the initial hydrolysis products of the supercoiled plasmid DNA (Campbell and Jackson 1980). In the absence of Ca^{2+} , the native and the two variants cleaved the Mg^{2+} -DNA substrate in a single scission mode with the formation of only the relaxed circular DNA (Fig. 3, top three panels). In the presence of Ca^{2+} , brDNase(D99A), like bpDNase, hydrolyzed the Mg^{2+} -DNA substrate to form, in addition to the relaxed-circular DNA (Form I), some linear duplex DNA (Form II), indicating the double scission (Fig. 3, bottom left two panels). On the other hand, under the same conditions, the Site I-defective brDNase(D201A) completely lost the double scission ability in response to Ca^{2+} (Fig. 3, bottom right). Thus, only Site I is responsible for the DNA double scission. This conclusion is supported by the previous finding that when Site I was bound by an antiserum against bpDNase, the Ca^{2+} binding was inhibited with the loss of the double scission ability on duplex DNA (Liu and Liao 1997). In the data for human DNase (Pan and Lazarus 1999), a contradictory result was presented; a site I mutant had similar linear to relaxed ratios compared with the wild type. However, in the human study, the mutant enzymes in the supernatant of cell culture were used. It was possible that due to the very low DNase activity for the site I mutant, a large amount of the supernatant was used. Under such a condition, the double scission might be created by a trace amount of endogenous nuclease.

Table 1. The V_{max} and K_m values for the four forms of DNase

	V_{max}	K_m	V_{max}/K_m
	U/ μg	$\mu\text{g}/\text{ml}$	$\times 10^3$
bpDNase	0.12	19.7	6.09
brDNase	0.16	26.9	5.94
brDNase(D99A)	0.15	68.2	2.20
brDNase(D201A)	0.02	61.5	0.33

The standard DNase assay method was used with varying amounts of DNA for the measurements of DNase activities. The double reciprocal plots for K_m and V_{max} were obtained by the linear regression analysis based on SigmaPlot for Windows Version 5.00.

Fig. 3. The modes of duplex DNA scission. The reaction mixture (50 μ L) contained 100 μ g/mL bovine serum albumin and 140 μ g/mL plasmid pCRII DNA with 5 mM $CaCl_2$ or 0.1 mM EGTA in 0.05 M Tris-HCl (pH 7.0), 10 mM $MgCl_2$. Hydrolysis was at 25°C and began after addition of the enzyme. At selected time intervals, 5- μ L aliquots of the reaction mixture were quenched with 25 mM EDTA, 6% glycerol, xylene cyanol, and bromophenol blue. Samples were loaded onto 1% agarose gels in the Tris-acetate-EDTA buffer (pH 8.0) containing 0.25 μ g/mL of ethidium bromide and a voltage of 8.5 V/cm was applied. (M) DNA molecular weight makers; I, II and III indicate the relaxed, linear, and supercoiled plasmid DNA, respectively.

Calcium protection against trypsin inactivation

Without Ca^{2+} , bpDNase and the two variants were all readily inactivated (Fig. 4A,a). However, with 10 mM Ca^{2+} , although bpDNase and brDNase(D99A) were protected, brDNase(D201A) lost over 60% of the DNase activity in 1 h (Fig. 4A,b). Figure 4B showed SDS-PAGE of the trypsin-treated samples and the sensitivity of brDNase(D201A) to trypsin. In the presence of 10 mM Ca^{2+} , only brDNase(D201A) produced the initially cleaved products (Fig. 4, lane 8). In another experiment, bpDNase without Ca^{2+} was digested with trypsin and the digested sample, after SDS-PAGE, was blotted to a PVDF membrane. The C-terminal fragment was then subjected to protein sequencing on a Perkin Elmer/ABI PROCISE 494 protein sequencer. The 10 cycles of sequencing were T-S-S-T-F-Q-W-L-I-P, providing evidence that trypsin initially cleaved bpDNase at the peptide bond between Arg 187 and Thr 188 (Liao et al. 1973). As shown in Figure 5A, the guanidinium group of Arg 187 and the β -carboxyl group of Asp 198 had an intramolecular contact distance of 3.05 Å and the beginning amino acid of the Site I loop, Asp 201, was only two residues away from Asp 198 (Fig. 5A). Without Ca^{2+} bound in this loop, the guanidinium group was probably separated from the β -carboxyl group, providing Arg 187 accessible to trypsin. Thus, abolishing the Ca^{2+} binding at Site I has led brDNase(D201A) vulnerable to trypsin even under the Ca^{2+} protection conditions (Fig. 4B).

The effects of metal ions on DNase activities

In the presence of Ca^{2+} , with Mn^{2+} -DNA as substrate, the DNase activities of brDNase(D99A) and of brDNase(D201A) were reduced (Table 2, column B). This decreasing order of the activities correlated with the decreasing V_{max}/K_m values (Table 1). With Mn^{2+} -DNA as substrate, Ca^{2+} caused bpDNase and brDNase(D99A) about a threefold increase in the DNase activities, whereas brDNase(D201A) was slightly inhibited by Ca^{2+} (Table 2, column B/A), indicating that the Ca^{2+} synergistic effect was due to Ca^{2+} bound to Site I, not to Site II.

The most evident Ca^{2+} synergistic effect was that of bpDNase with Mg^{2+} -DNA as substrate, in which Ca^{2+} caused a 20-fold increase in the DNase activity, whereas the Site II-defective brDNase(D99A) showed an increase of only 2.5-fold (Table 2, column D/C). Under the same conditions, the wild-type and the Site II-defective variant of human DNase also showed similar increases (Pan and Lazarus 1999).

When Sites I and II were simultaneously impaired, only in the presence of Ca^{2+} was the double variant brDNase(D99A/D201A) active against the Mn^{2+} -DNA substrate (Table 2). This double variant was assayed using the DNase activity stain in which one of the steps involved the Ca^{2+} -induced peptide refolding. Because the double variant was devoid of Sites I and II, it must be Site III or other weak binding sites with bound Ca^{2+} that provided necessary information to fold the polypeptide chain of brDNase(D99A/D201A) into the active conformation of DNase.

Calcium protection against inactivation by β -mercaptoethanol

Because in the amino acid sequence of bpDNase, the essential disulfide (Cys 173–Cys 209) is very close to the

Fig. 4. Calcium protection against trypsin inactivation. (A) The inactivation kinetics. The final mixture (150 μ L) containing 33–133 μ g/mL DNase and 3.3–13.3 μ g/mL trypsin in 50 mM Tris-HCl (pH 8.0), with 5 mM EDTA (A,a) or 10 mM CaCl₂ (A,b), was incubated at 25°C. At selected time intervals, 10- μ L aliquots were removed for DNase activity assays. (●) bpDNase; (▼) brDNase(D99A); (○) brDNase(D201A). (B) SDS-PAGE of the trypsin-treated samples. The protein (0.9 μ g) in 15 μ L of 50 mM Tris-HCl (pH 8.0), was incubated for 10 min with the combinations of 0.09 μ g of trypsin, 10 mM Ca²⁺, or 10 mM EDTA. Prior to loading, all samples were treated with β -mercaptoethanol. Gel was stained with Coomassie blue. The arrows indicate the N-terminal fragments of bpDNase (lane 3), brDNase(D99A) (lane 6) and brDNase(D201A) (lanes 8,9). The C-terminal fragment is not shown.

Site I loop (Fig. 5A), it has been suggested that Ca²⁺ bound at Site I was responsible for the protection of the disulfide disruption by β -mercaptoethanol (Oefner and Suck 1986). However, Figure 6 showed that both variants, brDNase(D99A) and brDNase(D201A), resisted β -mercaptoethanol inactivation at 0.1 mM Ca²⁺, a concentration higher than the K_d value for either Site I or Site II. Therefore, the protection of the essential disulfide was not due to the two structural calcium atoms, and it must be the calcium bound to Site III and/or other weaker binding sites that was involved.

Calcium-induced spectral changes

The apparent Stokes radius of bpDNase was increased with increasing pH, but the pH-induced changes could be reversed by the addition of Ca²⁺. At pH 9.5, the transition occurred at 8.9×10^{-5} M Ca²⁺ that was attributed to the weaker Ca²⁺-binding sites (Lizarraga et al. 1978). The Ca²⁺ induced 10% change in protein fluorescence intensity of bpDNase with half of the maximum increase at 6×10^{-5} M Ca²⁺ was also attributed to Ca²⁺ bound to the weaker Ca²⁺-binding sites (Tullis et al. 1981). The difference spectra of bpDNase showed a half maximum change at 2×10^{-5} M Ca²⁺, but 80% of the Ca²⁺ binding was due to the weaker binding sites (Tullis and Price 1974). Figure 7A showed that bpDNase and the two variants had ~10% increase in the fluorescence intensity in response to Ca²⁺. The Ca²⁺ concentrations required for the half increase was $\sim 1 \times 10^{-4}$ M, a value greater than the K_d values for the two structural calcium sites. Figure 7B showed the ultraviolet difference spectra resulting from the addition of Ca²⁺. Again, Ca²⁺ induced bpDNase and the two variants to the same characteristic red shift. Thus, because the two variants, like the native bpDNase, produced the same spectral changes in response to Ca²⁺, it should be Site III or other weaker Ca²⁺-binding sites that was responsible for the overall conformational change of DNase.

Discussion

The present studies on the calcium function for bpDNase were based on the assumption that impairment of the Ca²⁺-binding sites caused the enzyme to become insensitive to Ca²⁺ with respect to the Ca²⁺ synergistic property. However, the mutation also produced a certain amount of changes at the catalytic site. As shown in Table 2, except for brDNase (D99A) with Ca²⁺-DNA or Mg²⁺-DNA as substrate, the two variants showed decreased DNase activities. This activity reduction was probably not due to the overall protein conformational change because the two variant proteins still retained the characteristics of the Ca²⁺-induced spectral changes and the Ca²⁺ protection against the β -mercaptoethanol disruption of the essential disulfide bond.

Fig. 5. The X-ray structure and sequence homology of Sites I and II. (A) Site I with bound calcium. The calcium atom is coordinated by the oxygens of Asp 201, Thr 203, Thr 205, and Thr 207. The loop shown by light ribbon consists of the amino acid residues between Cys 173 and Cys 209, which form the essential disulfide. (B) Site II with bound calcium. The calcium atom is coordinated by the oxygens of Asp 99, Asp 107, Phe 109, and Glu 112. The arrow points toward the flexible loop, GCESCGN. (C) Sequence homology within the motifs of Sites I and II. The alignment for human DNase I, DNase X, DNase γ, and DNAS1L2 was from Shiokawa and Tanuma (2001) and that for bovine and fish DNase I was from Hsiao et al. (1997).

The variant brDNase(D99A), like the native bpDNase, exhibited the same Ca^{2+} synergistic property, but brDNase(D201A) was devoid of the Ca^{2+} activation (Table 2). On the basis of these characteristics of the metal-dependent DNase activities, a model of the Ca^{2+} synergistic effect on bpDNase is proposed (Fig. 8). In this model, Mn^{2+} -DNA, Mg^{2+} -DNA, and Ca^{2+} -DNA can all serve as substrate. Binding of Ca^{2+} at Site III, or other weaker sites, facilitates the protein conformational change enabling the active site. Because Mn^{2+} or Mg^{2+} alone was sufficient to activate the enzyme (Table 2), the overall protein conformational change from the inactive to active DNase was not restricted to the binding of Ca^{2+} at Site

III. The previous studies on the metal ion-induced protein conformational changes also included the Mg^{2+} binding (Tullis and Price 1974; Tullis et al. 1981) and the metal ion bound to the catalytic site was considered to be associated with these changes (Poulos and Price 1972). Additionally, the X-ray structures of the Ca^{2+} -thymidine-3',5'-diphosphate-DNase and of the O2'-methylated (GGUUAUACC)2 duplex-DNase crystallized in the Mn^{2+} -containing solution, revealed a metal bound at the catalytic pocket (Weston et al. 1992). Therefore, the suggested Site III could very likely be the catalytic site.

Equilibrium dialysis showed (Price 1972) that Ca^{2+} bound at one of the two strong Ca^{2+} -binding sites was not

Table 2. The specific DNase activities

	A	B		C	D	E	
	Mn ²⁺	Mn ²⁺ + Ca ²⁺	B/A	Mg ²⁺	Mg ²⁺ + Ca ²⁺	Ca ²⁺	D/C
		U/μg			U/μg × 10 ³		
bpDNase	1.03	2.92	2.8	37	735	4.0	19.9
brDNase(D99A)	0.66	1.80	2.7	82	203	25.6	2.5
brDNase(D201A)	0.52	0.39	0.8	1.5	5.2	5.0	1.0 ^a
brDNase(D99A/D201A) ^b	0.00	0.03	—	0	0	0	—

The standard DNase assay method was used except for brDNase(D99A/D201A). The concentrations for all ions were 10 mM.

^a Because Ca^{2+} alone had about the same activity as that of Mg^{2+} plus Ca^{2+} , the activation due to Ca^{2+} did not occur. This figure was the E/D value.

^b The DNase activities were determined from the DNase activity stain gel. The final incubation buffer for revealing the activity contained 10 mM of various metal ions in 40 mM Tris-HCl (pH 7.0). The protein concentration was determined using Western blotting.

Fig. 6. Effect of Ca^{2+} on the β -mercaptoethanol inactivation of DNase. The reaction mixture containing 50 mM β -mercaptoethanol in 50 mM Tris-HCl (pH 8.0), with varied amounts of Ca^{2+} was incubated at 25°C for 15 min and then an equimolar amount of iodoacetamide was added to stop the reaction prior to assay for DNase activities. Samples used, (a) bpDNase; (b) rbDNase(D99A); (c) rbDNase(D201A).

readily competed by Mn^{2+} or Mg^{2+} . This Ca^{2+} -specific site was assigned to Site I, because of all of the Ca^{2+} bindings, Site I was the strongest (Fig. 2). The Ca^{2+} synergistic effect (Table 2) also agreed with this assignment because the Site II-defective brDNase(D99A), with the intact Site I, still maintained approximately threefold DNase activity increase in response to Ca^{2+} , whereas the Site I-defective brDNase(D201A) lost the Ca^{2+} synergistic property. The same argument also suggested that Ca^{2+} , Mn^{2+} , and Mg^{2+} bound to Site II with about equal strengths. This observation was consistent with the previous finding (Price 1972) that Mn^{2+} or Mg^{2+} competed Ca^{2+} at a less strong binding site. Nevertheless, the two structural calcium atoms in bpDNase must play an important role in the fine-tuning of the DNase activity. Thus, Site I is evidently responsible for the synergistic activation of the DNase activity. Although Site II is not responsive to Ca^{2+} , its impairment created a structure for better hydrolysis of Mg^{2+} -DNA or Ca^{2+} -DNA as substrate.

The Ca^{2+} -binding proteins were classified into two categories (Strynadka and James 1989). One group consisted of extracellular proteins or enzymes that were similar to bpDNase in that Ca^{2+} activated and stabilized the enzyme. An example was thermolysin that became temperature sensitive when the Ca^{2+} binding was destroyed by mutation at Asp 57 or Asp 59 (Veltman et al. 1997). The other group consisted of intracellular proteins having the EF-hand. For example, the enzymatic activity of phospholipase C was decreased by mutation at one of the Asp residues in the EF-hand of the Ca^{2+} -binding motif (Drayer et al. 1995). Figure 5C showed the sequence homology of three extracellular (bovine, human, and fish DNases I) and three

intracellular (human DNase X, DNase γ , and DNAS1L2) DNases. At Site II, the Ca^{2+} -binding loop in the extracellular DNases had an extra pentapeptide (GCESC), which in the X-ray structure, showed as a flexible loop (Fig. 5B). Therefore, this motif was unlikely to be required for the integrity of the Ca^{2+} binding. On the other hand, Asp 201 and Thr 203 in the Site I loop were unchanged, whereas Thr 205 and Thr 207 were conserved with variation. This was understandable because, for the Ca^{2+} coordination, the side-chain oxygens of Asp 201 and Thr 203 were involved, whereas at the positions 205 and 207, the amide oxygens were contributed.

The K_d values for most of the intracellular proteins were within the intracellular Ca^{2+} concentrations at 10^{-7} to 10^{-6} M (Hiraoki and Vogel 1987). It is possible that the intracellular DNase X and DNase γ , having an extra residue between Thr 205 and Thr 207 (Fig. 5C), may lower the K_d value to 10^{-6} M to increase the affinity for Ca^{2+} . Or, perhaps, in the progression of apoptosis, the Ca^{2+} concentration in nuclei increases to 10^{-5} M to activate DNase X activity (Los et al. 2000) for DNA fragmentation.

The experimental data of the bovine enzymes were comparable with those of human DNase (Pan and Lazarus 1999) except for the Site I-defective variant. In the presence of Ca^{2+} , the bovine variant lost the DNA double-scission ability and the synergistic effect, whereas both characteristics were still maintained by the human variant. Additional new data, including the K_d values for Ca^{2+} , the Ca^{2+} -induced spectral changes, and the tryptic cleavage site, which were difficult to investigate with the impure human enzymes, were obtained by use of the purified bovine enzymes. The data included also in the bovine study were the Ca^{2+} protection against the trypsin and the β -mercaptoethanol inactivations of DNase that were useful in discussing the Ca^{2+} -binding to DNase. It has been shown (Shak et al. 1990) that when the bovine and the human enzymes were aligned, the amino acid sequence identity was ~77 %, indicating that the two proteins might have the same overall three-dimensional structure but micro-environmental differences could exist. In contrast to the double mutation used in the human study, the single mutation was created for the Site I-defective bovine variant. As shown by the difference absorption and fluorescence spectra, practically no conformational changes occurred in the bovine variants. The single mutation resulting in the impairment of the Ca^{2+} -binding to DNase was verified by the K_d measurements. Therefore, any discrepancy in the results between the two studies could be very likely due to the sample preparation or the species variation.

Materials and methods

Materials

The preparation of rabbit antibodies against bpDNase have been described (Liu and Liao 1997). TPCK-Trypsin and bpDNase

Fig. 7. Calcium-induced spectral changes. (A) The fluorescence spectra in response to Ca^{2+} . (a) bpDNase, 5.2 $\mu\text{g/mL}$; (b) brDNase(D99A), 7.5 $\mu\text{g/mL}$; (c) brDNase(D201A), 7 $\mu\text{g/mL}$. The tracings from bottom to top in each panel represent the addition of CaCl_2 to the final concentrations of 1×10^{-7} , 10^{-6} , 10^{-5} , 10^{-4} , 10^{-3} , to 5×10^{-2} M in a and 1×10^{-7} , 2×10^{-5} and 5×10^{-2} M in b and c. (B) The Ca^{2+} -induced UV difference spectra. (a) bpDNase, 0.22 mg/mL; (b) brDNase(D99A), 0.25 mg/mL; (c) brDNase(D201A), 0.29 mg/mL.

(code DP) was purchased from Worthington Biochemical Corporation. The purchased bpDNase was further purified on anion-exchange chromatography (Chen et al. 1998). NPPP, β -mercaptoethanol, EGTA, OCP, AMP, and horseradish peroxidase-conjugated secondary antibodies were purchased from Sigma. The anion-exchange resin (Source 15Q), the Mono Q (HR5/5), and the Mini Q (PE4.6/50) columns, and ECL Western blotting detection reagents were from Amersham Pharmacia Biotech. The pCRII vector was from Invitrogen. PVDF membranes were from Millipore.

DNase and protein assays

The standard DNase assay method was based on hyperchromicity due to DNA hydrolysis (Liao 1974). Unless otherwise stated, the assay solution was 0.1 M Tris-HCl (pH 7.0), containing 10 mM CaCl_2 , 10 mM MnCl_2 , and 0.05 mg/mL calf thymus DNA. One unit causes an increase of one absorbance unit at 260 nm in 1-mL assay medium at 25°C. For calculation of the specific DNase activities, the protein concentrations were determined using the Bio-Rad protein assay kits (Bio-Rad Lab) based on the method of Bradford (1976) with bovine serum albumin as standard.

The amount of brDNase(D99A/D201A) in the growth medium was determined indirectly by Western blotting. Proteins were transferred to a PVDF membrane using the Semi-Dry Transfer Unit TE 70 of Amersham Pharmacia Biotech. Rabbit antibodies against bpDNase were used as the primary antibodies. The horseradish peroxidase-conjugated secondary antibodies coupled with the ECL Western blotting detection reagents were used for chemiluminescent detection on a Kodak Biomax Light film. The very low DNase activity of brDNase(D99A/D201A), not measurable by the standard DNase assay method, was determined from the *in situ* DNase activity stain after SDS-PAGE (Ho and Liao 1999). The amounts of proteins and DNase activities were calculated from the intensities relative to those of bpDNase by scanning the bands using Image-Pro Plus Version 3.0 by Media Cybernetics.

Site-directed mutagenesis

The gene encoding bpDNase was cloned in pET15b as pETDNase and was used for site-directed mutagenesis by PCR (Ho et al.

1989) with the synthesized primers. The primers were: at the 5' *NcoI* site, 5' forward primer, 5'-GCTGGCCATGGCCCTGAA GATAG-3', and at the 3' *XhoI* site, 3' reverse primer, 5'-CTG GACTCGAGAAGGGACTTATGTC-3'; for brDNase(H134Q), 5' forward primer, 5'-GGAATTCGCCATTGTTGCTGCCCTG CAGTC-3' and 3' reverse primer, 5'-GCAGGGCAACAATGGC GAATTC-3'; for brDNase(D99A), 5' forward primer, 5'-TAC CAGTACGACGCAGGCTGCGA-3' and 3' reverse primer, 5'-TCGCAGCCTGCGTCGTACTGGTA-3'; for brDNase(D201A), 5' forward primer, 5'-TGATTCCTGACAGTGCCTGCCACCA-3' and 3' reverse primer, 5'-TGGTGGCGGCACTGTCTAGGAATCA 3'. In all cases, the codon used to bring about the mutation is underlined. The genes encoding the variants were cloned into the *NcoI* and *XhoI* sites of pET15b. Analysis of cDNA for bpDNase with PC GENE showed a *SacI* site between the codons for Asp 99 and Asp 201. When pETDNase(D99A) and pETDNase(D201A) were cut at the *NcoI* and the *SacI* sites, a 0.6-kB fragment was released. The 0.6-kB fragment from pETDNase(D99A) was then inserted into the DNA of pETDNase(D201A) minus the 0.6-kB fragment to yield the plasmid for the double variant, brDNase(D99A/D201A). All of the mutated genes were sequenced to confirm the presence of the mutation sites and to ensure no alterations at other sites.

Expression and purification of the recombinant proteins

For protein expression, the plasmid was transformed into the *E. coli* strain BL21(DE3)pLysE. When protein synthesis was initiated with IPTG, the expressed DNase caused the *E. coli* cells to lyse, resulting in release of DNase activity into the growth medium. After a brief centrifugation, the supernatant fractions of the growth medium were used as the sources for protein purification. Collected samples, after concentrated with an Ultrafiltration Cell (Amicon Inc.), were applied first to a Source 15Q column (1.0×7.0 cm). The recombinant proteins were eluted within the 0–15 mM CaCl_2 gradient in 20 mM Tris-HCl (pH 7.5). Fractions with DNase activities were concentrated, desalted, and applied to a Mono Q column. Proteins were eluted with the same 0–15 mM CaCl_2 gradient in 20 mM Tris-HCl (pH 7.5). When necessary, the enzyme was placed through another Mono Q column under the

Fig. 8. The reaction schemes illustrating the conformational changes, metal bindings, and metal ion-DNA hydrolysis rates for the native and the two variant DNases. I, II, and III represent Sites I, II, and III for the metal ion binding, respectively. C is the catalytic site. CSE refers to as the Ca^{2+} synergistic effect. Squares and circles represent the inactive and the active conformations of DNases, respectively. The dark areas indicate the impaired sites.

same conditions except that a higher ionic strength buffer (100 mM Tris-HCl) with shallower gradient (0–5 mM CaCl_2) was used. The protein purity was checked by SDS-PAGE (Laemmli 1970) using silver stain (Merril et al. 1981). As shown in Figure 1A, all recombinant proteins were homogeneous. Because the enzymatic properties (Chen et al. 1998) for brDNase and bpDNase were practically the same, in many experiments bpDNase instead of brDNase was used. The double variant brDNase(D99A/D201A) was not affinity eluted by Ca^{2+} on the Source 15Q column; the cell growth medium containing the expressed protein was used directly for all its investigations.

Calcium-binding assay

Approximately 55 μL of the purified proteins (0.5–1.0 mg/mL) were first dialyzed for 1 h each against two changes of 200 mL of 5 mM Tris-HC, (pH 7.5), using a Pierce Microdialyzer System 100 and then dialyzed with the same buffer containing various concentrations of CaCl_2 for 16 h at 25°C. Aliquots (20 μL) were

removed from each well for Ca^{2+} measurements (see below) and additional 20 μL aliquots were diluted 15-fold for determinations of the protein concentrations by measuring the absorbance at 220 nm with a micro cuvette. The molar quantities of the recombinant proteins were calculated using bpDNase as the standard whose concentration was determined on the basis of the absorbance at 280 nm for the 0.1% solution in 1-cm path cuvette = 1.23 (Lindberg 1967).

Ca^{2+} measurements

For determination of Ca^{2+} , the method (Corns and Ludman 1987) used was based on an indicator, OCPC, which underwent a spectral change upon Ca^{2+} binding. To accommodate the very small volume, samples were measured on the AKTA Purifier System without an analytical column. The moving phase, at a flow rate of 0.1 mL/min, was 2.5 mM Tris-HCl (pH 7.5), containing OCPC (50 mg/L), 0.4 M AMP. A 20- μL sample to be measured was mixed with 4 μL of OCPC (500 mg/L), 4 μL of 4 M AMP, and adjusted with H_2O to a final volume of 40 μL , from which 10 μL was injected. Absorbance at 575 nm was continuously monitored and the areas above the base line were integrated for determination of the amount of Ca^{2+} by comparing with the areas for the Ca^{2+} standards.

Fluorescence spectra

The fluorescence emission spectra were obtained on a Hitachi fluorometer Model F-3010 with excitation at 283 nm. Calcium ion concentrations were adjusted by mixing 8.5–85- μL aliquots of the CaCl_2 standards with the protein solutions in 5 mM Tris-HCl (pH 7.2) to a final volume of 1.7 mL. The solution was gently mixed and allowed to equilibrate for 20–30 min prior to the spectral recording.

Difference UV spectra

The difference spectra were recorded on a Hitachi spectrophotometer Model U-3200. One milliliter each of a protein solution in 5 mM Tris-HCl (pH 7.5) was placed in the reference and the sample cuvettes, and the base line was recorded. An aliquot of a CaCl_2 solution was then added to the sample cuvette to make a final $[\text{CaCl}_2] = 10$ mM, whereas an equal volume of water was added to the reference cuvette. The samples were allowed to equilibrate for 5 min prior to each recording.

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