Biological functions of the disulfides in bovine pancreatic deoxyribonuclease

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Abstract

We characterized the biochemical functions of the small nonessential (C101-C104) and the large essential (C173-C209) disulfides in bovine pancreatic (bp) DNase using alanine mutants [brDNase(C101A)] and [brDNase(C173A) and brDNase(C209A)], respectively. We also characterized the effects of an additional third disulfide [brDNase(F192C/A217C)]. Without the Ca^{2+} protection, bpDNase and brDNase(C101A) were readily inactivated by trypsin, whereas 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. Owing to the structure-based sequence alignment revealing homology between the “nonessential” disulfide of bpDNase and the active-site motif of thioredoxin, we measured 39% of the thioredoxin-like activity for bpDNase based on the rate of insulin precipitation ($\Delta A_{650\text{nm}}/\text{min}$). Thus, the disulfides in bpDNase not only play the role of stabilizing the protein molecule but also may engage in biological functions such as the disulfide/dithiol exchange reaction.

Keywords: deoxyribonuclease; cysteine; disulfide; site-directed mutagenesis; thioredoxin; thermal stability; protein refolding

Bovine pancreatic deoxyribonuclease I (bpDNase) cleaves double-stranded DNA with no sequence specificity (Moore 1981). It was the first DNase sequenced with the conven-

tional protein sequencing technique (Liao et al. 1992), and the X-ray structure was resolved at 2.0 Å with refinement (Oefner and Suck 1986). A catalytic mechanism has been suggested from the X-ray structure of the DNase-octamer complex (Weston et al. 1992), partially based on crucial amino acid residues identified by chemical modifications (Liao and McKenzie 1979; Sartin et al. 1980; Liao et al. 1982; Liao et al. 1991) and by site-directed mutagenesis (Doherty et al. 1995; Jones et al. 1996; Warren et al. 1997; Evans et al. 1999). The distinctive functions of the two structural calcium atoms were recently delineated (Chen et al. 2002).

One large (C173-C209) and one small (C101-C104) disulfide loop occur in bpDNase (Liao et al. 1992). In a Ca^{2+} -

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Abbreviations: bp, bovine pancreatic; br, bovine recombinant; brDNase (F192C/A217C), the brDNase double mutant with changes of Phe192 to Cys192 and Ala217 to Cys217; brDNase(C101A), the brDNase mutant with change of Cys101 to Ala101; brDNase(C173A), the brDNase mutant with change of Cys173 to Ala173; brDNase(C209A), the brDNase mutant with change of Cys209 to Ala209; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; cm-Cys, S-carboxymethyl Cys.

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Figure 1. SDS-PAGE analysis of the purified proteins. Each lane contained 10 μ g of total protein. Lanes 1–6: bpDNase, brDNase(wt), brDNase(F192C/A217C), brDNase(C101A), brDNase(C173A), and brDNase(C209A), respectively. The gel was run under nonreducing conditions and was silver-stained.

containing buffer without denaturing agents, only the small loop was reduced by β -mercaptoethanol, resulting in an enzyme still retaining its 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 “nonessential” and “essential” disulfides, respectively (Price et al. 1969b). During molecular evolution of proteins, a change in the number of disulfides in DNase occurs. Thus, fish DNase (Hsiao et al. 1997) contains only the “essential” disulfide, and chicken DNase (Hu et al. 2003), with the same two disulfides as in bpDNase, has one extra disulfide (C192-C217). Because enzymatic and physical properties of DNase from these three species were not quite the same, it prompted us to investigate, by site-directed mutagenesis in bpDNase, the possible biological functions of the two disulfides and an engineered disulfide corresponding to the third disulfide present in chicken DNase. In addition, because the structure-based sequence alignment revealed that the “nonessential” disulfide (CESC) in bpDNase was homologous to the active-site motif of thioredoxin (CGPC) (Holmgren 1979a), we also present data on the “nonessential” disulfide for the new biological function in the protein disulfide/dithiol exchange reaction. In this present study of bpDNase, the importance of all of the disulfides for stabilization of the protein molecule was delineated, and the significance of thioredoxin-like activities for the small disulfide was demonstrated.

Results

SDS-PAGE analysis of the purified proteins

The expressed wild-type brDNase, brDNase(C101A) and brDNase(F192C/A217C), with full DNase activity, caused *E. coli* cells to lyse, resulting in release of the recombinant proteins into growth media, and were thus purified to ho-

mogeneity (Fig. 1, lanes 2–4) according to a procedure of ion-exchange chromatography described in the Materials and Methods section. However, brDNase(C173A) and brDNase(C209A) were expressed in inclusion bodies and the pellets were washed extensively several times and used directly without further purification, as the purity was greater than 95%, as evidenced by the SDS-PAGE analysis (Fig. 1, lanes 5,6). Because the enzymatic properties for bpDNase and brDNase were practically the same (Chen et al. 2002), bpDNase was used instead of brDNase in many experiments. Because of glycosylation, the native bpDNase was about 2 kD larger than all of the recombinant proteins.

Determination of sulfhydryl groups

The purified proteins (200 μ g/mL) were vacuum-dried and resuspended in 40 μ L of 0.1 M Tris-HCl (pH 8.0) containing 8 M urea, allowing complete denaturation of proteins to assess all free sulfhydryls prior to iodoacetamide or β -mercaptoethanol treatments. Without β -mercaptoethanol treatment (Table 1, column 1), no free sulfhydryl groups were detected in brDNase(F192C/A217C), indicating that the newly created two Cys residues were paired to form a disulfide. Under the same conditions, as expected, no free sulfhydryl groups for bpDNase and one free sulfhydryl group each for brDNase(C101A), brDNase(C173A), and brDNase(C209A) was detected. Reduction with β -mercaptoethanol revealed four sulfhydryl groups for bpDNase, six for brDNase(F192C/A217C), and three each for brDNase(C101A), brDNase(C173A), and brDNase(C209A) (Table 1, col. 2). It is, therefore, evident that brDNase(C101A), brDNase(C173A), and brDNase(C209A) had an unpaired

Table 1. The number of cm-Cys residues in various forms of DNase

Forms of DNase	Free sulfhydryl groups ^a	Free sulfhydryl groups plus half-cystine residues ^b
	cm-Cys per mole	
bpDNase	0.0 (0)	3.94 $\pm$ 0.10 (4)
brDNase(F192C/A217C)	0.0 (0)	5.50 $\pm$ 0.10 (6)
brDNase(C101A)	0.82 $\pm$ 0.05 (1)	3.19 $\pm$ 0.13 (3)
brDNase(C173A)	1.05 $\pm$ 0.05 (1)	2.95 $\pm$ 0.15 (3)
brDNase(C209A)	0.85 $\pm$ 0.05 (1)	2.85 $\pm$ 0.05 (3)

The number of cm-Cys residues was obtained from the amino acid composition of each protein. See the text for the details of amino acid analyses. The experimental value for each determination represents an average of triplicates. Theoretical values are in parentheses.

^a The purified proteins (200 μ g/mL) were vacuum-dried and resuspended in 40 μ L of 0.1 M Tris-HCl (pH 8.0) containing 8 M urea. The samples were then incubated at 25°C for 30 min. For determination of free sulfhydryl groups, iodoacetamide was added directly to a final concentration of 125 mM, and the reaction mixture was allowed to stand in the dark for another 30 min.

^b For determination of free sulfhydryl groups plus half-cystine residues, samples were treated with 0.1 M β -mercaptoethanol for 40 min, followed by the addition of iodoacetamide to a final concentration of 125 mM.

Cys residue and that one disulfide each was disrupted in these three mutants. Expression of the brDNase(C104A) mutant was unsuccessful, preventing its inclusion in the present study.

Plasmid DNA scission

In the absence of Ca^{2+} , the native (bpDNase) and the two mutants [brDNase(F192C/A217C) and brDNase(C101A)] cleaved the Mg^{2+} -DNA substrate in a single nicking mode with the formation of only the relaxed open-circular DNA. In the presence of Ca^{2+} , the two mutants, like the native, hydrolyzed the Mg^{2+} -DNA substrate forming some linear duplex DNA in addition to the relaxed open-circular DNA, indicating double scission (data not shown). The ability to retain this double-scission mode in the presence of Ca^{2+} suggested that micro-environment of the Ca^{2+} -binding Site I (Chen et al. 2002) in the single disulfide form [brDNase (C101A)] or the triple disulfide form [brDNase (F192C/A217C)] was not disrupted and thus the global protein structure was not altered.

Thermal stability

As shown in Figure 2, the native bpDNase was inactivated at a transition temperature of 65°C. The brDNase(C101A) mutant, losing the “nonessential” disulfide, was inactivated at a transition temperature of 60°C. Fish DNase, also lacking the “nonessential” disulfide, exhibited a similar transition temperature and was relatively thermal-labile (Hsiao et

al. 1997). The transition temperature of 73°C for the mutant with an extra disulfide [brDNase(F192C/A217C)] was similar to the transition temperature for chicken DNase, which, having the corresponding third disulfide, was thermally more stable than bpDNase (Hu et al. 2003). However, amphibian DNase with the two disulfides conserved was reported to lose 50% of the activity when heated at 50°C (Takeshita et al. 2001). It was more heat-labile than all of the other vertebrate DNases discovered to date, perhaps owing to an additional C-terminal cysteine-rich stretch and the insertion of Ser205 at Ca^{2+} -binding Site I. The three mutants [brDNase(C101A), brDNase(C173A), and brDNase(C209A)] with only one disulfide were all inactivated at a transition temperature of 60°C. Thus, the results underline the importance of the enzyme stability in relation to the number of disulfides.

Reactivation of guanidine-HCl-denatured DNases by Ca^{2+}

Treatment with 6 M guanidine-HCl caused complete denaturation and inactivation of DNase, as shown by the direct addition of the treated samples into the assay buffer with about 100-fold dilution of guanidine-HCl (data not shown). However, when the treated samples were diluted 10-fold into a Ca^{2+} -containing buffer (0.1 M Tris-HCl [pH 7.0], 10 mM Ca^{2+}) prior to the assay, the native bpDNase with two disulfides, the mutant [brDNase(C101A)] with one disulfide, and the mutant [brDNase(F192C/A217C)] with three disulfides, were reactivated within 2 h as assayed (Fig. 3).

Figure 2. Thermal stability. (A) Purified DNases in 50 mM Tris-HCl (pH 7.5) were heated at the specified temperature for 10 min and cooled immediately on ice. The activity was measured using the hyperchromicity assay. (B) First derivative of the relative DNase activities shown in (A) to indicate the melting temperature for each DNase at the curve peak. (Filled circles) bpDNase, (hollow circles) brDNase(F192C/A217C), (filled triangles) brDNase(C101A), (hollow triangles) brDNase(C173A), (squares) brDNase(C209A).

Figure 3. Reactivation of the guanidine-HCl-treated DNases. Purified DNases (2.5–9 μg) in 20 μL of 6 M guanidine-HCl was incubated for 30 min at 25°C and then diluted 10-fold into 100 mM Tris-HCl (pH 7.0) with 10 mM EDTA or 10 mM CaCl_2 . At selected time intervals, 10- μL aliquots were removed for measurements of DNase activities using the hyperchromicity assay. (Filled circles) bpDNase with Ca^{2+} , (hollow circles) bpDNase with EDTA, (filled triangles) brDNase(F192C/A217C) with Ca^{2+} , (hollow triangles) brDNase(F192C/A217C) with EDTA, (filled squares) brDNase(C101A) with Ca^{2+} , (hollow squares) brDNase(C101A) with EDTA, (filled down triangles) brDNase(C173A) with Ca^{2+} , (hollow down triangles) brDNase(C173A) with EDTA, (hollow diamonds) brDNase(C209A) with Ca^{2+} , (filled diamonds) brDNase(C209A) with EDTA.

When diluted into a Ca^{2+} -free buffer, the native with two disulfides and the mutant with one disulfide remained denatured and inactive, whereas the mutant with three disulfides had 60% of its activity restored.

Calcium protection against trypsin inactivation

It has been known that bpDNase was protected by Ca^{2+} against the protease inactivation (Price et al. 1969a). In the Ca^{2+} -free buffer (50 mM Tris-HCl, pH 8.0), the native (bpDNase), the two mutants devoid of the “essential” disulfide [brDNase(C209A) and brDNase(C173A)], and the mutant with the disrupted “nonessential” disulfide [brDNase(C101A)] were readily inactivated by trypsin, whereas the mutant with an extra disulfide [brDNase(F192C/A217C)] remained active (Fig. 4A, a). With 10 mM Ca^{2+} , all enzymes were effectively protected from trypsin inactivation except for the mutant lacking the “nonessential” disulfide [brDNase(C101A)], which exhibited the first-order rate of inactivation (Fig. 4A, b). SDS-PAGE analyses of the trypsin-treated samples showed that, in the presence of Ca^{2+} , only the brDNase(C101A) mutant produced the initially cleaved products (Fig. 4B, lane 8), indicating that the DNase lacking the “nonessential” disulfide was not protected by Ca^{2+} and was sensitive to trypsin cleavage. The bands of the initially cleaved products in the gel of Figure 4B were shown previously (Chen et al. 2002) to have the sequence of

T-S-S-T-F-Q-W-L-I-P, providing evidence that trypsin cleaved bpDNase at the peptide bond between Arg 187 and Thr 188.

Reactivation of brDNase mutants devoid of the “essential” disulfide

The sense of nonessential and essential disulfide bonds in bpDNase was based on the results of chemical modification with β -mercaptoethanol (Price et al. 1969b). However, the meaning of absolutely essential in chemical terms is difficult to define. In the present study, two mutants devoid of the “essential” disulfide [brDNase(C173A) and brDNase(C209A)] were constructed to investigate the essentiality of the disulfide. Expression of these two mutants in *E. coli* strain BL21(DE3)plysE resulted in inclusion bodies. However, they were able to regain the DNase activity as shown in the zymogram analyses (data not shown), despite the relatively low activities. For quantitative measurements of the DNase activity, pellets of the two mutants were dissolved in 6 M guanidine-HCl and then diluted 10-fold into 100 mM Tris-HCl (pH 7.0) containing 10 mM EDTA or 10 mM CaCl_2 . Figure 3 shows the time-dependent regain of DNase activities as determined using the hyperchromicity assay. When diluted into the Ca^{2+} -containing buffer, the maximum activities were obtained within 1 h, representing 10% and 18% of the native bpDNase activity for brDNase(C173A) and brDNase(C209A), respectively. On the other hand, when diluted into a buffer without Ca^{2+} (10 mM EDTA), very little activity was found. Thus, the mutants with the disrupted “essential” disulfide in inclusion bodies, after dissolved in 6 M guanidine-HCl, can regain partial DNase activities by a refolding process with assistance of Ca^{2+} .

The thioredoxin-like activity

Rates of insulin precipitation and times to the beginning of precipitation, catalyzed by dithiothreitol/thioredoxin or by dithiothreitol/bpDNase, are shown in Figure 5A. The native DNase and the mutant with an extra disulfide [brDNase(F192C/A217C)] exhibited 39% of the thioredoxin-like activity, and addition of Ca^{2+} further increased the activity to 50% (Table 2A). In contrast, the mutant with the change of CESC to AESC in the CXXC motif [brDNase(C101A)] failed to catalyze the reduction reaction. Thus, it is evident that the CXXC motif in bpDNase possesses the thioredoxin-like activity. The kinetics for NADPH-dependent reduction of insulin by the thioredoxin/thioredoxin reductase and bpDNase/thioredoxin reductase systems are shown in Figure 5B. Although the catalytic rates for thioredoxin/thioredoxin reductase were normal, bpDNase/thioredoxin reductase failed to catalyze the oxidation of NADPH (Table 2B),

Figure 4. Calcium protection against trypsin inactivation. (A) The inactivation kinetics. The mixture (150 μ L) containing 15–55 μ g/mL DNase and 1.5–5.5 μ g/mL trypsin in 50 mM Tris-HCl (pH 8.0) was incubated at 25°C. (a) with 10 mM EDTA; (b) with 10 mM CaCl_2 . At selected time intervals, 10- μ L aliquots were removed for measurements using the hyperchromicity assay. (Filled circles) bpDNase, (open circles) brDNase(F192C/A217C), (filled triangles) brDNase(C101A), (open triangles), brDNase(C173A), (squares), brDNase(C209A). (B) SDS-PAGE analyses of the trypsin-treated samples. The protein (1 μ g) in 15 μ L of 50 mM Tris-HCl (pH 8.0) was incubated for 10 min with the combination of 0.1 μ g of trypsin, 10 mM CaCl_2 , 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 cleaved-bpDNase in lane 3, and brDNase(C101A) in lanes 8,9.

indicating that bpDNase was not recognized by thioredoxin reductase.

Discussion

The effects of engineered disulfides on protein stability are poorly understood because they can influence the structure,

dynamics, and energetics. The engineered disulfides if paired correctly would normally increase the stability of the protein. Thus, the removal or addition of disulfides in *Cucurbita maxima* trypsin inhibitor-V showed an enthalpy-entropy compensation (Zavodszky et al. 2001). Many other examples also illustrate these effects. In barnase, the disul-

Figure 5. The thioredoxin-like activity of bpDNase. (A) The dithiothreitol-dependent reduction of insulin by thioredoxin or bpDNase. The reaction mixture contained, in a final volume of 0.6 mL: 0.1M potassium phosphate (pH 7.0), 2 mM EDTA, 0.13 mM bovine insulin, 0.33 mM dithiothreitol, and 1.95 μ M thioredoxin or DNases. (Open squares) Thioredoxin, (filled squares) bpDNase plus 5 mM Ca²⁺, (open circles) bpDNase, (filled circles) brDNase(F192C/A217C), (open triangles) brDNase(C101A), (filled triangles) control, without thioredoxin or DNase. (B) NADPH oxidation catalyzed by the thioredoxin/thioredoxin reductase system. The assay mixture was the same as in (A) except for NADPH (0.4 mM) replacing dithiothreitol. The reaction was started by addition of 6.95×10^{-8} M thioredoxin reductase. The NADPH oxidation was measured by recording the increase of absorbance at 340 nm until turbidity appeared. The cuvettes then were shifted as indicated by an arrow, and the increase of absorbance at 650 nm was followed to obtain the rate of precipitation. (Open circles) Thioredoxin, (filled triangles) bpDNase, (open triangles) control, without thioredoxin or DNase.

fides constrained the denatured state, and a specific extended β -sheet structure was detected in the mutant protein (Clarke et al. 2000). Enhancement of the thermal stability by introduction of a disulfide was found in ovalbumin with $\Delta T_m = 8.7^\circ\text{C}$ (Arii et al. 1999), in cellulase C with $\Delta T_m = 3^\circ\text{C}$ (Nemeth et al. 2002), in ferredoxin with

$\Delta T_m = 8^\circ\text{C}$ (Meyer et al. 2002), and in haloalkane dehalogenase with $\Delta T_m = 5^\circ\text{C}$ (Pikkemaat et al. 2002). In the present study, engineering the additional third disulfide (Cys192-Cys217) into bpDNase was found to increase the melting temperature from 65° to 73°C ($\Delta T_m = 8^\circ\text{C}$). We also found that the stability of bpDNase was increased with the increased number of disulfides, as shown by thermostability and guanidine-HCl renaturation analyses. The increased thermostability may have some relevance to body temperature, which in chicken is 40°C , human 37°C , and fish cold-blooded.

Introduction of new disulfides in some cases altered protein conformation and biological functions. In cytochrome b5, temperature- and urea-induced denaturation caused significant differences in Trp22 fluorescence between the wild-type and an S18C/R47C disulfide mutant, and thus inhibited cleft mobility (Storch et al. 1999). In G-actin, locking a hydrophobic loop by a new disulfide bridge prevented filament formation (Shvetsov et al. 2002). Between the lactose repressor homodimers, an engineered disulfide linking the hinge regions increased operator affinity, decreased sequence selectivity, and altered allostery (Falcon and Matthews 2001). In the X-ray structure of the Ca²⁺-bound bpDNase (Fig. 6), the intramolecular contact distance between the guanidinium group of Arg187 and the β -carboxyl group of Asp198 is 3.05 Å (Oefner and Suck 1986) and the beginning amino acid of the Ca²⁺-binding Site I, Asp 201, was only two residues away from Asp 198. When Ca²⁺ was not bound in the vicinity, the guanidinium group was separated from the β -carboxyl group, providing Arg187 accessible to trypsin (Chen et al. 2002). In the pres-

Table 2. Kinetics of insulin reduction

(A) The rate and time of the thioredoxin- or DNase-catalyzed precipitation of insulin with dithiothreitol as reducing agent

Protein	Time to precipitation (min)	Rate of precipitation (absorbance at 650 nm/min)
Thioredoxin	14	0.083
bpDNase	22	0.032
bpDNase + 5 mM Ca ²⁺	16	0.042
brDNase(F192C/A217C)	24	0.032
brDNase(C101A)	51	0.012
Control	52	0.012

(B) Rates of NADPH oxidation and insulin precipitation catalyzed by thioredoxin reductase with thioredoxin or DNase

Protein	Rate of NADPH oxidation (absorbance at 340 nm per min)	Rate of precipitation (absorbance at 650 nm/min)
Thioredoxin	0.055	0.125
bpDNase	0.002	0.005
Control	0.002	0.003

Figure 6. Ribbon diagram for bpDNase based on the crystal structure (Oefner and Suck 1986). The structure illustrates the two intrinsic disulfides (Cys101-Cys104 and Cys173-Cys209) and the two Ca^{2+} -binding sites. The newly engineered third disulfide, Cys192-Cys217, not shown in the structure, corresponds to Phe192 and Ala217, whose side chains are as shown. The distance between the guanidinium group of Arg187 and the β -carboxyl group of Asp198 is 3.05 Å. N' and C' indicate the NH_2 - and COOH -termini, respectively.

ent study, 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. This argument is supported by the fact that, after denaturation in 6 M guanidine-HCl, brDNase(F192C/A217C), due to an extra disulfide, can readily refold back to the native conformation and regain enzymatic activity without assistance of Ca^{2+} (Fig. 3).

The native bpDNase without assistance of Ca^{2+} was readily inactivated by trypsin, whereas under identical conditions fish DNase remained active (Hsiao et al. 1997). Because the change of Cys101 to the corresponding Pro100 in fish DNase caused the flexible loop to become a rigid turn due to restriction of conformational freedom by proline with a side chain fixed to the main chain, whether Ca^{2+} is present or not, fish DNase always remains in a tight and compact conformation and thus resists trypsin inactivation. It appears that disruption of the “nonessential” disulfide, although not affecting DNase activity, may loosen the loop structure, thus making the brDNase(C101A) mutant vulnerable to trypsin, even in the presence of Ca^{2+} .

The disulfide/dithiol exchange reaction plays an important role in the regulation of cell growth and proliferation (Nakamura et al. 1997), in human cancer development (Baker et al. 1997), and in the development of postirradiation effects (Kojima et al. 1998). Furthermore, during cell differentiation and development, it is known that apoptosis

can occur (Shiokawa and Tanuma 2001). Because DNase is able to take part in the disulfide/dithiol exchange reaction, the “nonessential” disulfide in DNase may thus possess certain cellular activities. It is possible that a DNase/DNase thioreductase system, other than the thioredoxin/thioredoxin thioreductase system, may exist in some tissues or cell types. Therefore, the “nonessential” disulfide, albeit not essential for DNase activity, may have yet another unknown physiological function.

DNase has become increasingly important in therapeutic applications in recent years (Liao 1997). The most successful use of DNase has been as a drug for the relief of cystic fibrosis symptoms (Shak et al. 1990). Recent studies with human recombinant DNase have shown that a hyperactive and actin-resistant DNase was biochemically superior in vitro for the treatment of cystic fibrosis (Pan et al. 1998). DNase γ , an actin-resistant DNase I-like endonuclease, may also have clinical benefits for cystic fibrosis patients (Shiokawa and Tanuma 2001). A chimeric molecule comprising an scFv (immunoreactive against the human placental alkaline phosphatase) and bpDNase was also designed and investigated for its cytotoxic potential and possible use as immunotoxin in tumor-targeting strategies in cancer therapy (Linardou et al. 2000). In the present study, the mutant with a newly engineered disulfide [brDNase(F192C/A217C)] was proved to be thermostable and resistant against trypsin inactivation. Because immunotoxins should have a long half-life regarding proteolytic degradation after endocytosis, a more stabilized bpDNase is likely to be more favorable for clinical uses.

Materials and methods

Materials

The native bpDNase (code DP) and TPCK-trypsin were purchased from Worthington Biochemical. This DP-grade bpDNase was further purified by anion-exchange (Mono Q) chromatography as described (Chen et al. 2002). Dithiothreitol, β -mercaptoethanol, EGTA, NADPH, thioredoxin, thioredoxin reductase and bovine insulin were purchased from Sigma. The anion-exchange resin (Source 15Q) and Mono Q (HR 5/5), and Mono S (HR 5/50) columns were from Amersham Pharmacia Biotech. The pCRII vector was from Invitrogen.

Site-directed mutagenesis

The gene encoding bpDNase has been cloned in pET15b as pETDNase (Chen et al. 2002). It was used as the wild-type template for site-directed mutagenesis by an overlap extension method using PCR (Ho et al. 1989) with the synthesized primers. The primers used were: at the 5' NcoI site, 5' forward primer, 5'-GCTGGCCATGGCCCTGAAGATAG-3', and at the 3' XhoI site, 3' reverse primer, 5'-CTGGACTCGAGAAGGGACTTATGTC-3'; for brDNase(C101A), 5' forward primer, 5'-ACGGCGCCGAGTCTGCGGGAACGACA-3' and 3' reverse primer, 5'-GACTCGGCGCCGTCGTCGTAAGGTA-3'; for brDNase(C173A), 5' forward primer, 5'-GCTGACGCTAGCTACGTGACCTCCTC-3'

and, 5'-GTAGCTAGCGTCAGCATTGAAATCGC-3'; for brDNase (C209A), 5' forward primer, 5'-ACGAACGCTGCCTATGACAGGATCGT-3' and 3' reverse primer, 5'-ATAGGCAGCGTTCCGTGGACGTAGCCGT-3'. For the double mutant brDNase(F192C/A217C), 5' forward primer, 5'-TCCACCTGCCAGTGGCTGATTCCTGA-3' and 3' reverse primer, 5'-CACTGGCAGGTGGAGCTCGTACG-3' were used first to prepare the polynucleotide with F192C mutation, and this polynucleotide was then used as template for the second round of PCR mutagenesis with 5' forward primer, 5'-TGGTCTGCGGGTCTCTGCTCCAGA-3' and 3' reverse primer, 5'-GACCCGCAGACCACGATCCTGTCATA-3' to produce the A217C mutation. In all cases, the codon used to bring about the mutation is underlined. The genes encoding the mutants were cloned into the NcoI and XhoI sites of pET15b. The entire 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 plasmids were transformed into the *E. coli* strain BL21(DE3)pLysE. The expressed proteins with DNase activities [wild-type brDNase, brDNase(F192C/A217C), and brDNase(C101A)] 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 purification of brDNase and its mutants. Collected supernatant fractions, after concentration with an Ultrafiltration Cell (Amicon), were applied to a Source 15Q column (1.0 × 7.0 cm) for the initial clean-up. The recombinant proteins were eluted within the 0–15 mM CaCl₂ gradient in 20 mM Tris-HCl (pH 7.5). Fractions with DNase activities were concentrated, desalted, and applied to a Mono Q (HR5/5) column with the same gradient for chromatography. When necessary, fractions with DNase activities were concentrated, desalted, acidified with 1 M acetic acid, and placed through a Mono S (HR5/50) column. Proteins were eluted within the 0–0.5 M NaCl gradient in 50 mM sodium acetate (pH 4.7). The protein purity was checked by SDS-PAGE (Laemmli 1970) with silver staining (Merril et al. 1981). The cell pellets containing the expressed proteins in inclusion bodies [brDNase (C173A) and brDNase(C209A)] were washed several times and used directly without further purification, as the purity was greater than 95%.

DNase and protein assays

The standard DNase assay was based on hyperchromicity due to DNA hydrolysis (Liao 1974). Unless otherwise stated, the standard assay buffer was 0.1 M Tris-HCl (pH 7.0), containing 10 mM CaCl₂, 10 mM MnCl₂, 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 specific activities, the values of 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.

Determination of sulfhydryl groups

The purified proteins (200 µg/mL) were vacuum-dried and resuspended in 40 µL of 0.1 M Tris-HCl (pH 8.0) containing 8 M urea. The samples were then incubated at 25°C for 30 min. For determination of free sulfhydryl groups, iodoacetamide was added di-

rectly to a final concentration of 125 mM, and the reaction mixture was allowed to stand in the dark for another 30 min. For determination of free sulfhydryl groups plus half-cystine residues, samples were treated with 0.1 M β-mercaptoethanol for 40 min, followed by the addition of iodoacetamide to a final concentration of 125 mM. The reaction mixtures were then dialyzed against H₂O overnight and vacuum-dried. These samples were hydrolyzed with 6 N HCl vapor at 150°C for 2 h. Precolumn derivatization of the hydrolysate with phenylisothiocyanate was performed, and the phenylthiocarbamoyl amino acid derivatives were analyzed using HPLC with a Pico-Tag column (Waters; Heinrikson and Meredith 1984). The amino acid composition of each protein was determined, and sulfhydryl groups were obtained as the amount of cm-Cys.

Plasmid DNA scission analysis

Depending on metal ions, duplex DNA was hydrolyzed by bpDNase 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 (Campbell and Jackson 1980). The reaction mixture (40 µL) contained 100 µg/mL bovine serum albumin and 140 µg/mL plasmid pCRII DNA with 10 mM CaCl₂ or 1 mM EGTA in 50 mM Tris-HCl (pH 7.0), 10 mM MgCl₂. 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 25 mM EDTA, 6% glycerol, xylene cyanol, and bromophenol blue and then analyzed on a 1% agarose gel.

Insulin reduction assays

For measurements of disulfide reduction, catalyzed by thioredoxin or bpDNase with dithiothreitol as reducing agent, the procedure was that of Holmgren (1979a). The NADPH-dependent reduction of insulin by the thioredoxin/ thioredoxin reductase or the bpDNase/thioredoxin reductase was measured as described (Holmgren 1979b).

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