Construction and characterization of a bifunctional enzyme with deoxyribonuclease I and thioredoxin-like activities

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Abstract

One large essential (C173–C209) and one small nonessential (C101–C104) disulfide loops occur in bovine pancreatic deoxyribonuclease I (bpDNase I). In our recent study, the reduced nonessential disulfide (-CESC-), which is structurally homologous to the active-site motif (-CGPC-) of thioredoxin, was shown to have thioredoxin-like activity. In order to gain further insight into the potential redox activity of the nonessential disulfide in bpDNase I, four double (GP, PG, WK, and KW) and two quadruple (WGPK, KPGW) mutants were constructed. Most of the mutant enzymes possess similar specific DNase activities as that of WT bpDNase I, while KPGW exhibited only half of the activity, possibly due to gross structural alteration, as revealed by CD analysis. All these mutants were able to accelerate the rate of insulin precipitation. The highest thioredoxin-like activity (66%) measured for WGPK indicated that the conserved sequence (-WCGPK-) of thioredoxin is crucial for its redox activity. Our results suggested that engineering of the nonessential disulfide in bpDNase I was able to generate a novel bifunctional enzyme with enhanced disulfide/dithiol exchange reactivity, while retaining its full DNA-hydrolyzing activity.

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DNase I cleaves double-stranded DNA to yield 5′-phospho polynucleotides in the presence of Mg 2+ or Mn 2+ under neutral or alkaline conditions [1]. Bovine pancreatic DNase I (EC 3.1.21.1, bpDNase I) is the best characterized among DNase I from various species [2]. It was the first DNase whose X-ray structure was resolved at 2.0 Å with refinement [3]. Recently, the biological significance of the two disulfides [4] and the involvement of the N- and C-termini in the active protein folding [5], and its catalytic mechanism was delineated by chemical rescue [6].

One large (C173–C209) and one small (C101–C104) disulfide loops occur in bpDNase I [7]. In a Ca 2+-containing buffer without denaturants, only the small disulfide was reduced by β-mercaptoethanol, resulting in an enzyme still retaining its full activity, while in the Ca 2+-depleted 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 [8]. In our previous report, we further characterized the biochemical functions of these two disulfide loops [4]. The small disulfide loop, albeit not essential for activity, is important for the
structural integrity of bpDNase I. In addition, owing to the structure-based sequence alignment revealing homology between the “nonessential” disulfide (-CESC-) in bpDNase I and the active-site motif of thioredoxin (-CGPC-) (Fig. 1), we measured the thioredoxin-like activity for bpDNase I based on the rate of insulin precipitation [4].

Thioredoxin is a ubiquitous redox-active protein having a highly conserved CXXC active-site motif located in a protruding segment of its three-dimensional structure [9]. Thioredoxin participates in diverse biological functions through dithiol-disulfide interchange reactions. The evolving roles of thioredoxin in cellular proliferation and differentiation [10], defense against oxidative stress [11], control of apoptosis [12], and cancer development [13] strongly suggest that regulation of the redox state of proteins is crucial in many physiological processes.

In the present study, in order to gain further insight into the potential redox activity of the nonessential disulfide in bpDNase I, four double (GP, PG, WK, and KW) and two quadruple (WGPK, KPGW) mutants were constructed, and their DNase and thioredoxin-like activities were thus characterized. Our results suggested that engineering of the nonessential disulfide in bpDNase I was able to generate a novel bifunctional enzyme with additional disulfide/dithiol exchange reactivity, while retaining its full DNA-hydrolyzing activity.

Materials and methods

Materials and analytical methods. The WT bpDNase I (code DP) was obtained from Worthington and further purified as previously described [14]. Dithiothreitol, thioredoxin, bovine insulin, and calf thymus DNA were purchased from Sigma. SDS-PAGE was performed according to Laemmli [15] and gels were silver-stained [16]. Western blot analysis was as described [17] with slight modifications. The refolding of expressed mutant proteins to active DNase I was detected by zymogram analysis, according to Lack et al. [18].

Site-directed mutagenesis. The plasmid pETDNase containing full-length bpDNase I was prepared in our laboratory as previously described [19]. It was used as the wild-type template for construction of the bpDNase I mutants (designated as GP, PG, WK, KW, WGPK, and KPGW) by PCR-based site-directed mutagenesis using the overlap extension method [20] with the synthesized primers as listed in Table 1. Two primers, Ncol (+): 5’-GCTGGCCATGGCCTGAAAGATAG-3’ and Xho (-): 5’-CTGGAGCTGAGAAAGGACTTATGTC-3’, were used to generate restriction enzyme sites (italic) at the 5’ and 3’ ends. After being digested with Ncol and Xho, the PCR products were then cloned into the corresponding sites on pET15b. All mutated genes were sequenced by Mission Biotech Co. to confirm the presence of the mutation sites and to eliminate any PCR errors.

Expression and purification of the bpDNase I mutants. For protein expression, the plasmids were transformed into the Escherichia coli strain BL21(DE3)pLysE. The cultured E. coli cells were induced with 1 mM IPTG at 37 °C for 3 h. Only mutants KW and WGPK caused E. coli cells to lyse, resulting in release of the proteins into growth media. For mutants GP, PG, WK, and KPGW, the cultured E. coli cells were centrifuged at 12,000 rpm for 30 min, and the cell pellets were suspended with 1/10 volume of ice-cold 20 mM Tris-HCl, pH 7.0. The suspended solution was

![Fig. 1. The X-ray structures of thioredoxin and bpDNase I depicting their structurally homologous CXXC motifs. The coordinates were obtained from PDB entry 2TRX [29] and 3DNI [3], respectively.](image)

Table 1

<table>
<thead>
<tr>
<th>Oligonucleotide primers used for construction of the bpDNase I mutants</th>
<th>Forward primers</th>
<th>Reverse primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP (-GCPCG-)</td>
<td>5’-GGCTGGCCATGGCCTGAAAGATAG-3’</td>
<td>5’-CCGGCAAGGCCCCGACCGGTTCTGTA-3’</td>
</tr>
<tr>
<td>PG (-GCPCG-)</td>
<td>5’-GGCTGGCCATGGCCTGAAAGATAG-3’</td>
<td>5’-CCGGCAAGGCCCCGACCGGTTCTGTA-3’</td>
</tr>
<tr>
<td>WK (-WCECK)</td>
<td>5’-GACTGGTGCCAGTTCTGACAAGCAGCTT-3’</td>
<td>5’-GTTTTTGCCAGGTACCTGACACCTGCTGACTT-3’</td>
</tr>
<tr>
<td>KW (-KESECK)</td>
<td>5’-GACAAATGGTGCCAGTTCTGACAAGCAGCTT-3’</td>
<td>5’-GTTTACACAGGTAATCCTGACTGCTGACTT-3’</td>
</tr>
<tr>
<td>WGPK (-WGPK-)</td>
<td>5’-GACTGGTGCCAGTTCTGACAAGCAGCTT-3’</td>
<td>5’-GTTTACACAGGTAATCCTGACTGCTGACTT-3’</td>
</tr>
<tr>
<td>KPGW (-KPGW-)</td>
<td>5’-GACAAATGGTGCCAGTTCTGACAAGCAGCTT-3’</td>
<td>5’-GTTTACACAGGTAATCCTGACTGCTGACTT-3’</td>
</tr>
</tbody>
</table>

*The altered nucleotide sequences used for introduction of the mutation sites and the correspondingly mutated residues were underlined.*
frozen-and-thawed 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 these mutant enzymes were detectable and determined to be active in both fractions as shown by Western blots and zymogram analysis. Growth media of KW and WGPK, together with the cytoplasmic fractions of GP, PG, WK, and KPGW were collected for further purification to obtain the purified mutant proteins based on a three-ion exchanger procedure as previously described [4,5].

**DNase and protein assays.** The hyperchromicity method [21] was used as standard DNase assay throughout the study. One unit of activity is defined as the amount of DNase I necessary to cause an increase of one absorbance unit, at 260 nm, per min in 1-mL assay solution containing 0.05 mg of calf thymus DNA, 10 mM CaCl₂, and 10 mM MnCl₂ in 0.1 M Tris–HCl (pH 7.0). For calculation of specific enzyme activities, the protein concentrations were determined using the Bio-Rad protein assay reagent (Bio-Rad Lab) based on the method of Bradford [22] with bovine serum albumin as standard.

**CD measurements.** The CD spectra were recorded using a Jasco J-715 spectropolarimeter over a range of 190–260 nm in a cylindrical cell of 1 mm pathlength in 10 mM Tris–HCl buffer, pH 7.0. The baseline given by the CD spectrum of 10 mM Tris–HCl buffer, pH 7.0, was recorded and subtracted from the CD spectra of each sample. The far-UV CD spectra were analyzed using SELCON 3 algorithms [23] and the secondary structures were thus estimated.

**Insulin reduction assays.** For measurement of disulfide reduction, catalyzed by thioredoxin or WT and mutant bpDNases I with dithiothreitol as reducing agent, the procedure was as described [24] with slight modifications. The reactions were carried out in 100 mM potassium phosphate buffer (pH 7.0), 0.13 mM bovine insulin, 2 mM EDTA, 1.32 mM DTT, and 1.95 μM thioredoxin or WT and mutant bpDNase I with a final reaction mixture of 600 μL. The measurements were performed at 650 nm using 0.5-min recording. The nonenzymatic reduction of insulin by dithiothreitol alone was recorded as control. The time for the start of precipitation, defined as an increase by 0.02 at A650 nm over a stable baseline recording, was determined. The second parameter calculated was the rate of precipitation at 650 nm.

**Results and discussion**

**SDS-PAGE analysis of the purified proteins**

Upon IPTG induction, the expressed proteins of mutants KW and WGPK, with full DNase activity, caused *E. coli* cells to lyse, resulting in release of the recombinant proteins into the growth medium (Fig. 2A and B) and were thus purified to homogeneity (Fig. 2C and D). However, mutants GP, PG and KPGW, without causing cell lysis, were expressed in both the soluble cytoplasmic and insoluble pellet fractions of the *E. coli* cells, as shown by Western blotting (Fig. 2A), and were all proved to be actively folded as revealed by zymogram analysis (Fig. 2B). The cytoplasmic fractions were collected and used as sources for further purification (Fig. 2C and D). Since the enzymatic properties for the commercially available WT bpDNase I and the *E. coli*-expressed native bpDNase I were practically the same [14], purified WT bpDNase I, together with all the purified mutant proteins, was used throughout the current study. WT bpDNase I was about 2–3 kDa larger than all of the recombinant proteins due to glycosylation (Fig. 2A–D). Expression of mutant WK was unsuccessful (Fig. 2A and B), preventing its inclusion in the present study.

**Specific DNase activity and structural analysis of the mutant proteins**

DNase activities of the purified mutant proteins were measured using hyperchromicity assay. The protein concentrations were determined based on Bradford dye-bind-

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![Fig. 2. Gel electrophoresis of WT and mutant bpDNases I. Western blot analysis (A) and zymogram analysis (B) for expressed mutant proteins secreted into culture medium (lanes m), in the soluble cytosolic fractions (lanes S), and in the insoluble pellet fractions (lanes P). The arrow heads in (B) indicated endogenous DNase activity of *E. coli* at around 23 kDa. Silver-stained gel (C) and zymogram analysis (D) for purified bpDNases I. Each lane in (C) and (D) contained 10 μg of the purified proteins. Lane M: pre-stained molecular weight marker.]
ing method, and the specific DNase activities were thus calculated and are summarized in Table 2. All mutants had almost identical specific DNase activities as that of the WT bpDNase I (1000 U/mg) except mutant KPGW, which exhibited only half of the activity (531 U/mg). To determine whether these mutants were correctly folded, we compared the secondary structures of the WT and mutant proteins by examining their CD spectra. Similar CD spectra were observed in WT bpDNase I and all the mutant proteins except mutant KPGW (Fig. 3), which showed an apparent increase in β-sheet, turn, and random structures, accompanied by a significant decrease in α-helix structure (Table 3). These results indicated that the dramatic decrease in the specific activity for mutant KPGW may be attributed to the gross structural alteration of the protein molecule.

The thioredoxin-like activity

The DTT-dependent reduction of insulin by thioredoxin, or WT and mutant bpDNases I is shown in Fig. 4. Two major parameters, rates of insulin precipitation and times to the beginning of precipitation, are summarized in Table 4. Similar times to the beginning of precipitation were recorded for all the mutants (20 min), as compared to those of thioredoxin (6.5 min) and no protein control (DTT only, 40 min). WT bpDNase I and mutant KW exhibited 11% of the thioredoxin-like activity, while mutants GP and PG, carrying mutations within the CXXC motif, increase the thioredoxin-like activity to 45%. Furthermore, exchange of the nonessential disulfide loop (-GCESCG-) in bpDNase I with the complete sequence of the conserved CXXC motif (-WCGPCK-) in thioredoxin generated a bpDNase I mutant (WGPK) with the highest thioredoxin-like activity of 66%. These results indicated that the conserved sequence (-WCGPCK-) is crucial for the redox activity, both for thioredoxin, and for the engineered bpDNase I mutants.

Generation of a bifunctional enzyme

The disulfide/dithiol exchange reaction plays a critical role in the regulation of many cellular processes, including cell proliferation, differentiation, and cancer development [10–13]. Furthermore, during cell differentiation and development, it is known that apoptosis can occur [25]. According to our results, since bpDNase I is able to participate in the disulfide/dithiol exchange reaction, the nonessential disulfide in bpDNase I may thus possess certain cellular activities. It is possible that a DNase/DNase thioreductase system, other than the thioredoxin/thioreductase system, may exist in some tissues or cell types. Therefore, the “nonessential” disulfide, albeit not crucial for

<table>
<thead>
<tr>
<th>bpDNase I</th>
<th>ΔG (kJ/mol)</th>
<th>ΔS (J/K mol)</th>
<th>ΔH (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (-GCESCG-)</td>
<td>0.12</td>
<td>0.11</td>
<td>0.10</td>
</tr>
<tr>
<td>KW (-KCESCW-)</td>
<td>0.11</td>
<td>0.10</td>
<td>0.09</td>
</tr>
<tr>
<td>GP (-GCCPCG-)</td>
<td>0.10</td>
<td>0.09</td>
<td>0.08</td>
</tr>
<tr>
<td>PG (-GCPGC-)</td>
<td>0.09</td>
<td>0.08</td>
<td>0.07</td>
</tr>
<tr>
<td>KPGW (-KCPCGW-)</td>
<td>0.08</td>
<td>0.07</td>
<td>0.06</td>
</tr>
<tr>
<td>WGPK (-WCPCPK-)</td>
<td>0.07</td>
<td>0.06</td>
<td>0.05</td>
</tr>
</tbody>
</table>
DNase activity, may have yet another unknown physiological function.

DNase has become increasingly important in therapeutic applications in recent years [26]. Earlier Studies have shown that hyperactive and actin-resistant human recombinant DNases I proved successful for treatment of cystic fibrosis and systemic lupus erythematosus [27]. A chimeric protein comprising a scFv fragment and bpDNase I was designed and investigated for its cytotoxic potential and possible use as immunotoxin in tumor-targeting strategies in cancer therapy [28]. In the current study, the mutant with an engineered thioredoxin-like CXXC motif (WCGPCK) was proved to have 66% that of the thioredoxin-like activity, while retaining its full DNA-hydrolyzing activity. Such a bifunctional enzyme is likely to be more favorable for clinical uses.

Fig. 4. The thioredoxin-like activity of DTT-dependent reduction of insulin by thioredoxin, WT, and mutant bpDNases I. The reactions were carried out in 100 mM potassium phosphate buffer (pH 7.0), containing 0.13 mM bovine insulin, 2 mM EDTA, 1.2 mM DTT, and 1.95 μM thioredoxin or WT and mutant bpDNase I with a final reaction mixture of 0.6 mL. Symbols: ○, Thioredoxin; ●, WT bpDNase I; ▽, KW; ▼, GP; ■, PG; □, KPGW; ◦, WGPK; ○, control (no protein).

<table>
<thead>
<tr>
<th>Table 4</th>
<th>The thioredoxin-like activity of WT and mutant bpDNases I</th>
</tr>
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<tbody>
<tr>
<td>Protein</td>
<td>Time to precipitation (min)</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------</td>
</tr>
<tr>
<td>Trx(-WCGPCK-)</td>
<td>6.5</td>
</tr>
<tr>
<td>WT (-GCESCG-)</td>
<td>21.5</td>
</tr>
<tr>
<td>KW (-KCESCW-)</td>
<td>21.5</td>
</tr>
<tr>
<td>GP (-GGPCGC-)</td>
<td>19.5</td>
</tr>
<tr>
<td>PG (-GCPGCG-)</td>
<td>20.0</td>
</tr>
<tr>
<td>KPGW (-KCPGWC-)</td>
<td>19.5</td>
</tr>
<tr>
<td>WGPK (-WCGPCK-)</td>
<td>19.5</td>
</tr>
<tr>
<td>Control (no protein)</td>
<td>40.5</td>
</tr>
</tbody>
</table>

a Trx, thioredoxin.

b Time to precipitation is recorded as the incubation time for the absorbance at 650 nm to reach 0.02.

c Relative to Trx activity(%) = [V_{Dnase} - V_{control}]/V_{Trx} - V_{control} × 100, where V is the rate of precipitation.

Acknowledgments

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