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Increasing the refolding efficiency in vitro by site-directed mutagenesis of Cys383 in rat procarboxypeptidase B

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ABSTRACT

This study examines a novel method to reduce the probability of disulfide mismatches during the refolding process by the replacement of cysteines within a protein. Specifically, Cys383 of recombinant rat procarboxypeptidase B was replaced by other amino acids to increase the refolding efficiency in vitro. Mutants C383G, C383A and C383S could refold successfully, but mutants C383R, C383E, C383L and C383Y failed to refold correctly. Compared with wild type, the refolding efficiencies of mutants C383G and C383A were enhanced. The Cys383 mutations changed some of the properties of rat carboxypeptidase B. Mutants C383G, C383A had higher k_{cat}/K_m values which indicated increased catalytic abilities. And both had higher thermal stability. pH had different effects on the activities and stabilities of the mutant and wild type proteins. The studies suggested that mutating Cys383 of rat procarboxypeptidase B could improve the renaturation process by increasing the refolding efficiency. This new method could be taken as a new attempt to improve the refolding efficiency of other recombinant proteins containing disulfide bonds that are expressed as inclusion bodies. While the results also claimed that the potential effects of the substituted amino acid on the protein itself should be seriously considered in addition to its ability to reduce the probability of disulfide mismatches.

1. Introduction

Carboxypeptidase B (CPB), a member of the metallocarboxypeptidase (CPs) family, is an exopeptidase that can catalyze the cleavage of C-terminal peptide bonds formed by basic amino acids in proteins and peptides [1]. Its catalytic domain contains zinc. With the development of modern biotechnology, CPB has been widely used as a tool to engineer proteins. In particular, CPB plays a significant role in the production process of recombinant insulin [2].

Rat carboxypeptidase B (CPB) is activated from rat procarboxypeptidase B (pCPB). The predicted molecular weight of rat pCPB and CPB are 45 kDa and 35 kDa, respectively [3]. Rat pCPB compromises 402 amino acids that form a highly charged globular protein. The pro-peptide, consisting of 95 amino acids, is close to the N-terminus, and the enzyme moiety consists of 307 amino acids [4]. The pro-peptide domain precisely blocks the "pocket" of the enzyme moiety, which contains the catalytic domain, causing it to be inactive. Trypsin specifically targets Arg95 of pCPB, cleaving the pro-peptide away from the enzyme moiety at that amino acid. This cleavage results in the opening of the "pocket", and the enzyme moiety becomes fully activated [5].

Traditionally, pCPB is extracted from the pancreas of animals, which is rather cumbersome and costly [6]. Although we have successfully expressed rat pCPB in *Escherichia coli* [7], the recombinant pCPB is expressed as inclusion bodies (IBs). Much work was required to transform the IBs into a soluble form, and some progress has been made with respect to the refolding process in vitro. The refolding efficiency of pCPB, however, is lower than expected. Misfolding results in the expression of a recombinant protein in an insoluble form. Inappropriate matches between disulfides are one of the major contributors to the protein misfolding process [8]. Because cysteines can form disulfide bonds, changing the cysteines within a protein to different amino acids could potentially avoid inappropriately matched disulfides. We considered this method to enhance the refolding efficiency of procarboxypeptidase B in vitro.

Rat pCPB contains seven cysteines that form three disulfide bonds (Cys158–Cys171; Cys230–Cys253; Cys244–Cys258). Cys383 is, however, free and does not form a disulfide bond with any of the other six cysteines. Thus, Cys383 is likely to form a mismatched disulfide during the process of refolding. In this study, we performed site-directed mutagenesis on Cys383 to reduce, and possibly eliminate, the chance of this free cysteine forming a mismatched disulfide.

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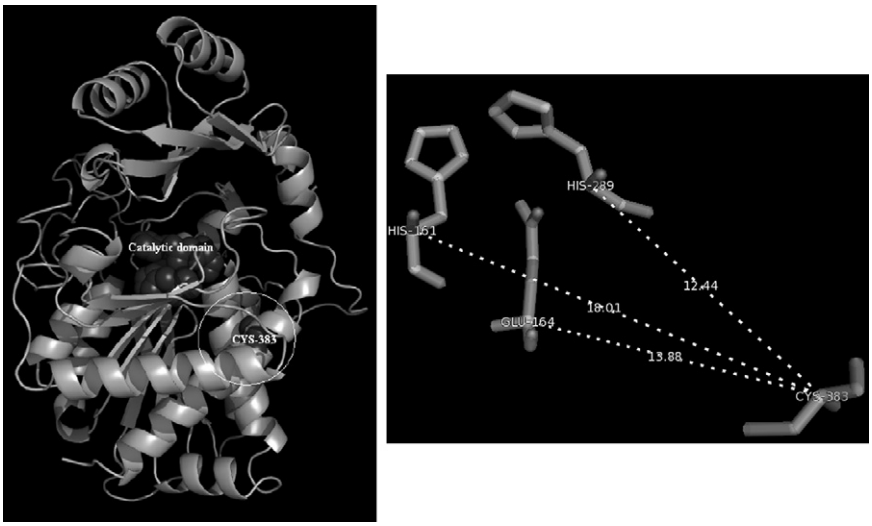


Fig. 1. Three-dimensional model of procarboxypeptidase B (pCPB). The catalytic domain and Cys383 are shown as spheres on the left. The distance (Å) of C383 from the catalytic domain has been specified on the right.

Cys383 of pCPB is located in the C-terminal α-helix (Fig. 1). Because the substitution of different amino acids for Cys383 could change the conformation of the C-terminal α-helix to some extent, the catalytic activity of CPB might also be altered. To investigate this possibility, we also studied the catalytic properties of the CPB mutants.

Although methods for the renaturation of different IBs have been extensively studied, there is little information with respect to the idea of reducing the number of cysteines by site-directed mutagenesis to lower the probability of disulfide mismatches during the refolding process in vitro. Here, the effects of Cys383 mutations in rat pCPB were fully investigated, and the results suggested that the identity of the amino acid selected to replace cysteine was quite important. The potential effects of the substituted amino acid on the protein itself should be seriously considered in addition to its ability to reduce the probability of disulfide mismatches. Our results still suggested this method could be taken as a new attempt to improve the renaturation of other IBs containing disulfide bonds.

2. Materials and methods

2.1. Materials

All PCR primers were synthesized by Sangon Biotechnology Corporation (Shanghai, China). DNA sequence analysis was performed by Invitrogen (Shanghai, China). The PCR enzyme, restriction enzymes and ligation kit were purchased from Takara (Dalian, China). All chemicals were of purest grade commercially available.

2.2. Construction of the expression plasmids for CPB mutants

Because Cys383 is located in the C-terminal α-helix near the C-terminus, the mutations were directly introduced by site-directed mutagenesis using specifically designed 3′-primers (Table 1). After the mutation was incorporated into the gene by PCR, the resulting PCR product was cloned into the pET-28a (+) expression vector (Novagen) using the *NcoI* (upstream) and *HindIII* (downstream) sites. The *E. coli* strain used in this work was BL21 (DE3) which was stored in our laboratory.

2.3. Expression and refolding of CPB mutants

E. coli hosts were routinely cultured at 37 °C in Luria-Bertani medium containing 50 μg/mL kanamycin. When the cells reached a density of 0.9 OD_{600}, protein expression was induced by the addition of 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). After growing for an additional 4 h at 37 °C, the cells were harvested by centrifugation at 6000 rpm for 20 min and lysed by ultrasonication. Then, inclusion bodies were separated by centrifugation at 12,000 rpm for 15 min at 4 °C. Triton X-100 (0.5%, v/v) was used as a detergent to purify the IBs, and the IBs were then washed with 20 mM Tris–HCl pH 8.0 three times to eliminate Triton X-100.

Purified IBs were denatured in 50 mM glycine–NaOH pH 9.5 buffer containing 8 M urea. The denatured IBs were diluted in refolding buffer containing 50 mM glycine–NaOH pH 9.5, 0.1 mM Zn^{2+} and 1 mM GSH as previously reported [7]. The final concentration of protein in the refolding buffer was 1 mg/mL.

2.4. Refolding efficiency assays

After renaturation, the mutant and wild type proteins were activated by 20 U/mL trypsin at 37 °C for 2 h. The method of comparing the amount of trypsin-cleaved product with that before cleavage by band densitometry of SDS-PAGE was used to define the refolding efficiency, the 44.3 kDa Marker was used as the standard band. Electrophoresis Image Analysis System FR 980 (Furi technology corporation, China) was used to determine band densitometry. The activity units per milligram protein (before trypsin activation) in the refolding system were calculated to further determine the refolding efficiency.

CPB activity was measured by the method of Folk and Schirmer [1] with hippuryl-L-arginine (Sigma) as the substrate. One unit of enzyme activity was defined as the amount of the enzyme that catalyzed the hydrolysis of 1 μmol hippuryl-L-arginine per minute at 25 °C, determined by an increase of 0.36 in the absorbance at 254 nm and 1-cm path length. Enzyme concentration was adjusted so as not to exceed 30% hydrolysis within 10 min. The rate of substrate hydrolysis is directly proportional to enzyme concentration within the range employed. The protein concentration was measured according to the method of Bradford [9], using bovine serum albumin as the standard protein. All assays were performed at least three times.

2.5. Purification of CPB mutants

Activated mutant and wild type proteins were purified by DEAE-FF anion-exchange chromatography (GE) as previously described [7]. The activated mutant and wild type proteins were eluted from the column by a linear 0–0.3 M NaCl gradient in 20 mM Tris–HCl pH 8.0. The samples with activity were pooled and stored at −20 °C for further analysis.

2.6. Biochemical properties

2.6.1. Effects of temperature on the activities and thermal-stabilities of CPB mutants and wild type

The effects of temperature on the activities of the CPB mutant and wild type proteins were investigated using hippuryl-L-arginine as the substrate. The substrate solutions were equilibrated for 5 min in a heated water bath to reach the required temperature. The catalytic rates of the enzymes were assayed at different temperatures, ranging from 4 °C to 80 °C, at pH 7.65.

The mutants C383G, C383A and C383S and the wild type were incubated at temperatures of 4–60 °C in a heated water bath. Their activity was determined at different times under standard conditions. Non-heated enzymes were considered as the control (100%).

2.6.2. Effects of pH on the activities and stabilities of CPB mutants and wild type

The activities of the C383G, C383A C383S and wild type proteins were assayed at different pH levels, ranging from 3.0 to 12.0 at 25 °C, using hippuryl-L-arginine as the substrate. The buffers were 25 mM sodium acetate pH 3.0–6.0, 25 mM KPB pH 6.5–7.5, 25 mM Tris–HCl pH 8.0–8.5, 25 mM glycine–NaOH pH 9.5–12.0. The activities of the enzymes investigated at pH 7.65 were considered as the control (100%).

Table 1
3′-primers used for the site-directed mutagenesis.

Mutant	3′-primer
C383L	5′-CCCAAGCTTTCACTAATATAGATGTTCTCGGACATAATTGGCAATGTACTTGACTGCAAGCATTGTCTCCTC**TAA**GGTCTGGCGGA-3′
C383Y	5′-CCCAAGCTTTCACTAATATAGATGTTCTCGGACATAATTGGCAATGTACTTGACTGCAAGCATTGTCTCCTC**ATA**GGTCTGGCGGA-3′
C383R	5′-CCCAAGCTTTCACTAATATAGATGTTCTCGGACATAATTGGCAATGTACTTGACTGCAAGCATTGTCTCCTC**ACG**GGTCTGGCGGA -3′
C383E	5′-CCCAAGCTTTCACTAATATAGATGTTCTCGGACATAATTGGCAATGTACTTGACTGCAAGCATTGTCTCCTC**TTC**GGTCTGGCGGA -3′
C383G	5′-CCCAAGCTTTCACTAATATAGATGTTCTCGGACATAATTGGCAATGTACTTGACTGCAAGCATTGTCTCCTC**ACC**GGTCTGGCGGA-3′
C383A	5′-CCCAAGCTTTCACTAATATAGATGTTCTCGGACATAATTGGCAATGTACTTGACTGCAAGCATTGTCTCCTC**AGC**GGTCTGGCGGA-3′
C383S	5′-CCCAAGCTTTCACTAATATAGATGTTCTCGGACATAATTGGCAATGTACTTGACTGCAAGCATTGTCTCCTC**AGA**GGTCTGGCGGA-3′

The mutants C383G, C383A and C383S and the wild type were incubated in different pH buffers, as indicated above, at 25 °C. The activities were measured after a 12-h incubation. The enzyme activities before incubation were considered as the control (100%).

2.6.3. Kinetic parameters

The Michaelis constant kinetic parameters K_m and V_{max} were determined according to the Michaelis–Menten equation by the double-reciprocal method. The activities of the mutants C383G, C383A and C383S and the wild type were precisely measured using hippuryl-L-arginine as the substrate at various concentrations (0.05–0.3 mM). All determinations were performed at least three times at 25 °C. The value of the turnover number, k_{cat}, was calculated by the following equation: $k_{cat} = V_{max}/[E_0]$, where $[E_0]$ is the active enzyme concentration [10].

2.7. Analysis of the three-dimensional structure of CPB mutants and wild type

SWISS-MODEL (http://swissmodel.expasy.org/) was used for homologous modeling of the CPB mutants [11,12]. Software PyMOL 0.99 (http://www.pymol.org/) was used for all PDB visualization files and figure captures.

3. Results

3.1. Comparison of refolding efficiencies between CPB mutants and wild type

The refolding efficiencies of the CPB mutant and wild type proteins were determined by comparing the amount of trypsin-cleaved product with that before cleavage by band densitometry of SDS-PAGE. The band densitometries before cleavage were almost the same. After activation, however, the band densitometries performed differently. Mutants C383R, C383E, C383L and C383Y could not refold correctly and all of them were degraded after the trypsin treatment. In contrast, mutants C383G, C383A and C383S could refold, although to different extents (Fig. 2A). A comparison of the refolding efficiencies revealed that the mutant C383A had the highest level at 47.55%, followed by the mutant C383G, which was slightly better than the wild type, with efficiencies of 35.22% and 29.98%, respectively. Mutant C383S had the lowest efficiency at 22.19%. These differences in the refolding efficiencies could be clearly further proved by the activity units per milligram protein (before trypsin activation) in the refolding system, they were 19.32 ± 1.20 U/mg for C383A, 13.41 ± 0.65 U/mg for C383G, 12.14 ± 0.10 U/mg for wild type, and 9.58 ± 0.33 U/mg for C383S, respectively.

3.2. Purification of active CPB mutants and wild type

DEAE-FF anion-exchange chromatography was used to concentrate and purify active mutants C383G, C383A, C383S and wild type successfully. The resulting eluents for the CPB mutant and wild type protein were all highly pure, as shown by SDS-PAGE analysis (Fig. 2B).

3.3. Comparison of the properties between CPB mutants and wild type

3.3.1. Kinetic parameters

The kinetic parameters K_m, k_{cat} and V_{max} of the CPB mutant and wild type proteins were investigated using hippuryl-L-arginine as the substrate (Table 2). The catalytic efficiency was assessed by the value of k_{cat}/K_m. Mutants C383G, C383A and C383S all exhibited higher K_m and V_{max} values than the wild type. The k_{cat}/K_m value of mutant C383A was slightly higher than that of the wild type. In contrast, both mutants C383G and C383S had much higher k_{cat}/K_m values, which were 1.9- and 1.49-fold increased, respectively, when compared with wild type. Significantly, the mutant C383G had the highest catalytic efficiency, even though it exhibited the highest K_m. In addition, the mutants C383A and C383S both had higher catalytic efficiencies than that of the wild type. Although the wild type protein had the lowest K_m value, it also had the lowest V_{max}, directly leading to its poor catalytic efficiency. These results suggested that mutation of the Cys383 in pCPB could affect the catalytic domain of the enzyme, causing the altered kinetic profiles of the mutant proteins.

3.3.2. The effects of temperature on the activities of CPB mutants and wild type

For all of the mutant and wild type proteins, an increase in the temperature resulted in an increase in the activity level. The wild type had the quickest enhancement compared with that of the mutants over the temperature ranging from 40 to 80 °C. Temperature had similar effects on the activities of the mutants when the temperature was below 50 °C. When the temperature ranged between 50 °C and 80 °C, however, the activity of mutant C383G was higher than that of the other mutants. The catalytic rate of mutant C383S did not increase further as the temperature increased to 60 °C. Mutant C383A had the lowest increase in the activity rate among all the mutant and wild type proteins.

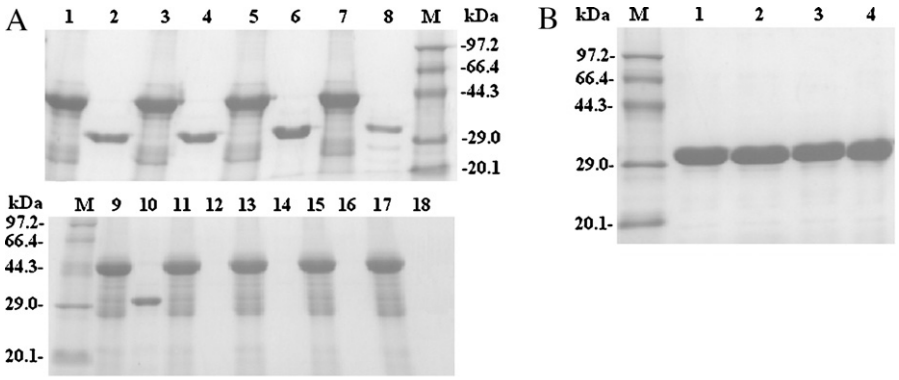
3.3.3. Thermal stability of mutants and wild type

The mutants C383G, C383A and C383S and the wild type were all quite stable below 40 °C. After incubation for 8 h at 50 °C, mutant C383S retained approximately 35% of its activity. In contrast, mutants C383G and C383A retained less than 15% of their activities, and no activity could be detected for the wild type. After incubation at 60 °C for 15 min, mutant C383G and the wild type lost over 90% of their activity, mutant C383A lost 65% of its activity, and

Table 2
Kinetic parameters for the wild type and the CPB mutants C383G, C383A and C383S.

	K_m (mM)	V_{max} (μmol/min)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\ S^{-1}$)
Wild type	0.31 ± 0.04	205.73 ± 9.65	1.03×10^4	3.32×10^4
C383G	0.56 ± 0.03	353.61 ± 20.28	3.54×10^4	6.32×10^4
C383A	0.47 ± 0.06	276.10 ± 19.69	1.66×10^4	3.53×10^4
C383S	0.50 ± 0.03	492.90 ± 33.42	2.47×10^4	4.94×10^4

All determinations were performed at least three times at 25 °C using hippuryl-L-arginine as the substrate.



	Line	Relative concentration (%)	Pixels value	Remaining percentage (%)
	Marker			
	44.3kDa	100.00	637	
	1	281.16	1791	
Wild type	2	84.30	537	29.98
	3	277.71	1769	
C383G	4	97.80	623	35.22
	5	275.67	1756	
C383A	6	131.08	835	47.55
	7	284.46	1812	
C383S	8	63.11	402	22.19

Fig. 2. (A) Comparison of the refolding efficiencies of the CPB mutants and the wild type by SDS-PAGE (13.5%) analysis. Lanes 1, 3, 5, 7, 9, 11, 13, 15 and 17 refer to wild type, C383G, C383A, C383S, wild type, C383R, C383E, C383L and C383Y prior to trypsin activation, respectively. Lanes 2, 4, 6, 8, 10, 12, 14, 16 and 18 refer to wild type, C383G, C383A, C383S, wild type, C383R, C383E, C383L and C383Y after trypsin activation, respectively. M refers to the low molecular weight marker protein ladder, with the band sizes indicated. The same volume was used for all samples. The results of band densitometry of SDS-PAGE were shown at the bottom. (B) SDS-PAGE (13.5%) analysis of the results of purification by DEAE-FF anion-exchange chromatography. M, low molecular weight marker protein ladder (band sizes indicated on the left). Lane 1, wild type. Lane 2, mutant C383G. Lane 3, mutant C383A. Lane 4, mutant C383S.

mutant C383S still retained 55% of its activity. The same trend was seen for incubation periods of 30 min and 60 min at 60 °C. Overall, the CPB mutants were more thermally stable than the wide type (Fig. 3).

3.3.4. Optimal pH levels

Using hippuryl-L-arginine as the substrate, the optimal pH was determined to be 7.5 for the CPB mutants and the wild type. At pH 6.5–7.5, the mutants C383A and C383S had higher activities

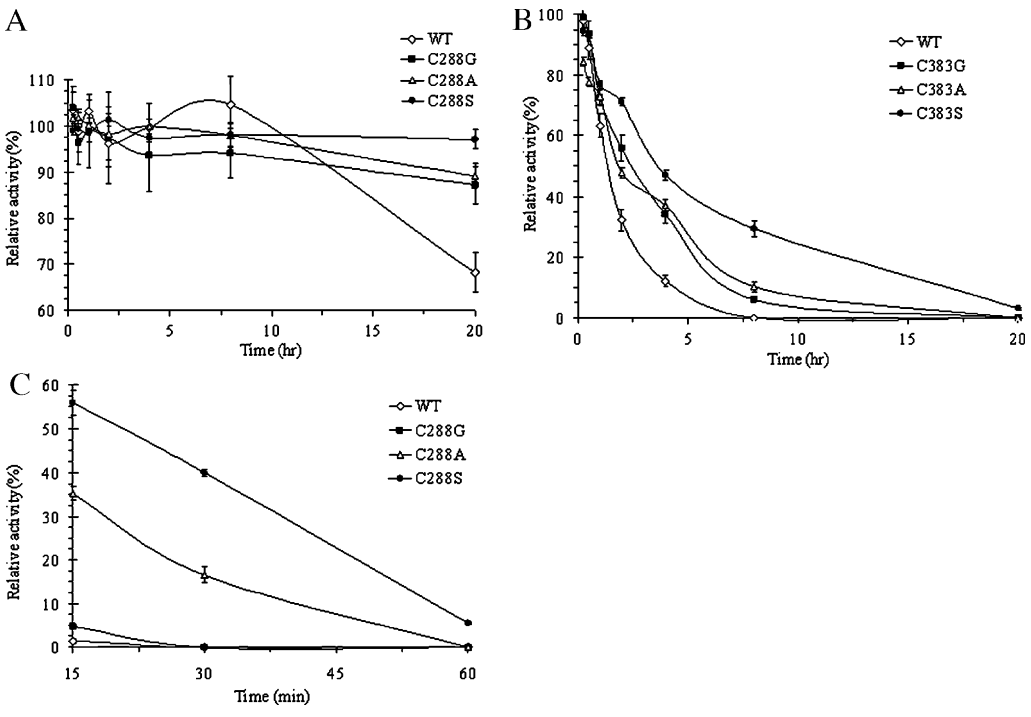


Fig. 3. The thermal stabilities of the mutants C383G, C383A and C383S and the wild type. (A–C) Temperatures of 40 °C, 50 °C and 60 °C, respectively. The activity was determined at different times at pH 7.65 and 25 °C. The non-heated enzyme was considered as the control (100%).

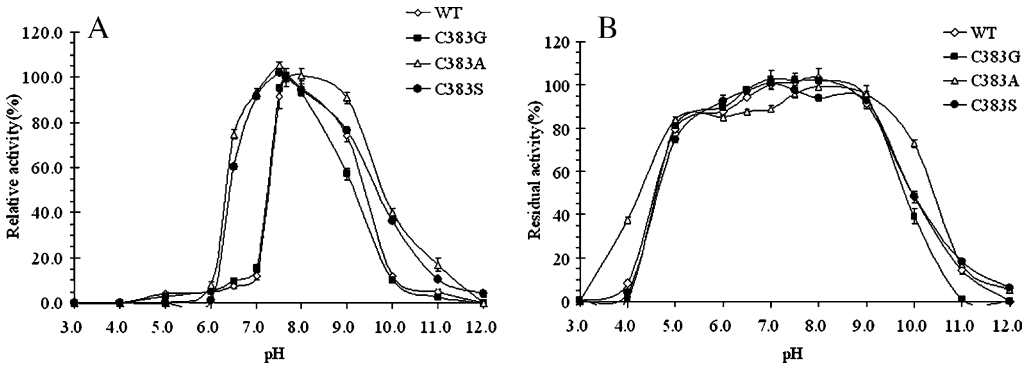


Fig. 4. (A) The effects of pH on the activity of the mutants C383G, C383A and C383S and the wild type. The activity was determined at pH levels ranging from 3.0 to 12.0 at 25 °C. The activity obtained at pH 7.65 was considered as the control (100%). (B) The effects of pH on the stability of the mutants C383G, C383A and C383S and the wild type. The pH stabilities of the mutant and wild type proteins were investigated by incubation in different buffers for 12 h at 25 °C. The residual activities were then determined at pH 7.65 and 25 °C using hippuryl-L-arginine as the substrate. The activities of the mutant and wild type proteins before incubation were considered as the control (100%).

than mutant C383G and the wild type. Both mutant C383G and the wild type exhibited only 15% activity at pH 7.0. At pH 6.0, the CPB mutants and the wild type were all inactive. At pH 9.0, mutant C383A had the highest activity, whereas mutant C383G had the lowest. The CPB mutants and the wild type all became inactive when the pH was above 11.0 (Fig. 4A).

3.3.5. pH stability

The CPB mutants and the wild type had similar stabilities when the pH ranged from 5.0 to 9.0. At pH 4.0, all mutants except mutant C383A and the wild type lost almost all of their activity. The CPB mutants and the wild type were all seen to form partial aggregations at this pH. Although there was a high accumulation of precipitated product, mutant C383A still retained approximately 40% of its activity. When the pH was above 9.0, mutants C383G and C383S and the wild type lost much of their activity, whereas mutant C383A still retained 75% activity at pH 10.0 (Fig. 4B).

4. Discussion

The proper matching of disulfide bonds plays a significant role in the process of protein folding [13]. Most disulfide bonds assist protein folding by enhancing stability through reinforcement of the correct conformation. The protein folding process consists of several steps, and disulfide bonds may be formed during every step. Disulfide mismatches that occur during the early stages greatly inhibit the ability of the protein to achieve its proper native conformation [14,15]. For this reason, the cysteine becomes special with respect to the other amino acids in a protein. The refolding process is required to turn inclusion bodies into the soluble form of a protein in vitro. The ability to perfectly refold a denatured protein is a difficult and demanding task. Recently, most studies have focused on improving refolding conditions [16]. In contrast, very little research has focused on replacing the cysteines in a protein to decrease the probability of disulfide mismatching as a method to increase the refolding efficiency.

Not all of the mutants we constructed for this study could successfully refold, even under the most optimal refolding conditions (data not shown). Only three mutants, C383G, C383A and C383S, could refold properly, but there were significant differences between their refolding efficiencies. Taken together, our data demonstrated that replacing cysteine with proper amino acids could reduce the probability of disulfide mismatches and enhance the refolding process. Mutations of this cysteine might have one or more effects on the protein: (1) altering the steric effects, such as C383R, C383E, C383L and C383Y, (2) altering the charges within the protein, such as C383R and C383E and (3) affecting side chains arrangements to becoming more hydrophilic or hydrophobic (C383S, C383A, C383L and C383G).

The mutants C383R, C383E, C383L and C383Y could not refold successfully, which may be due to the large or charged side chains of the substitutions, potentially resulting in the formation of additional hydrogen bonds with other amino acids. We had constructed an expression plasmid for a truncated version of pCPB that lacked the C-terminal α-helix, but that mutants could not refold at all (data not shown). This observation indicated that the C-terminal α-helix played an important role in the refolding process. In the three-dimensional structure of rat pCPB, the α-helix comprising the amino acids from Glu155 to Tyr182 is located just next to the C-terminal α-helix that contains Cys383 (Fig. 5). Substitution of Cys383 by amino acids with large or charged side chains (C383R, C383E, C383L, C383Y) could result in the formation of new hydrogen bonds with Ile166, Ser167, Ala169 and Phe170, which all surround Cys383 (Fig. 5A, E, F, G, H). These newly formed hydrogen bonds significantly change the angle between Ile166, Ala169 and Phe170. The angles of wild type, mutants C383R, C383E, C383L, C383Y are 89.7°, 88.3°, 89.5°, 90.4° and 86.7° respectively (Fig. 5A, E, F, G, H). This variation could result in the conformation of the adjacent helix changing to some extent and in turn, directly hinder the proper refolding of the enzyme. Alanine, glycine and serine all contain short side chains that could not interact with the amino acids in the adjacent secondary structures just like cysteine of wild type (Fig. 5A–D), which could explain why mutants C383G, C383A and C383S could refold correctly.

Compared with wild type, mutants C383A and C383G had higher refolding efficiencies, whereas mutant C383S had a little lower refolding efficiency due to the similar sizes of the $-CH_2OH$ and $-CH_2SH$ side chains. In particular, the refolding efficiency of mutant C383A was increased approximately 1.58-fold, which may be due to its smaller and hydrophobic $-CH_3$ side chain versus the $-CH_2SH$ side chain of the wild type. The refolding efficiencies of the mutants C383A and C383G were quite different. One explanation for this observation could be non-polar amino acid of the position might be more suitable for refolding process. From anaylysis of Fig. 5A–D, the smaller sizes of the $-CH_3$ side chain of alanine and the –H side chain of glycine would barely affect the surroundings.

Fig. 6 highlights the specific changes in active site residues and substrate binding domains, by the distances and angles among the catalytically active zinc ion binding amino acids His161, His289, Glu164 of wild type, mutants C383G, C383A and C383S respectively. At the active site of procaboxypeptidase B the catalytically active zinc ion is penta-coordinated by the side-chains of His161, His289, a water molecule, and Glu164, the latter in a bidentate man-

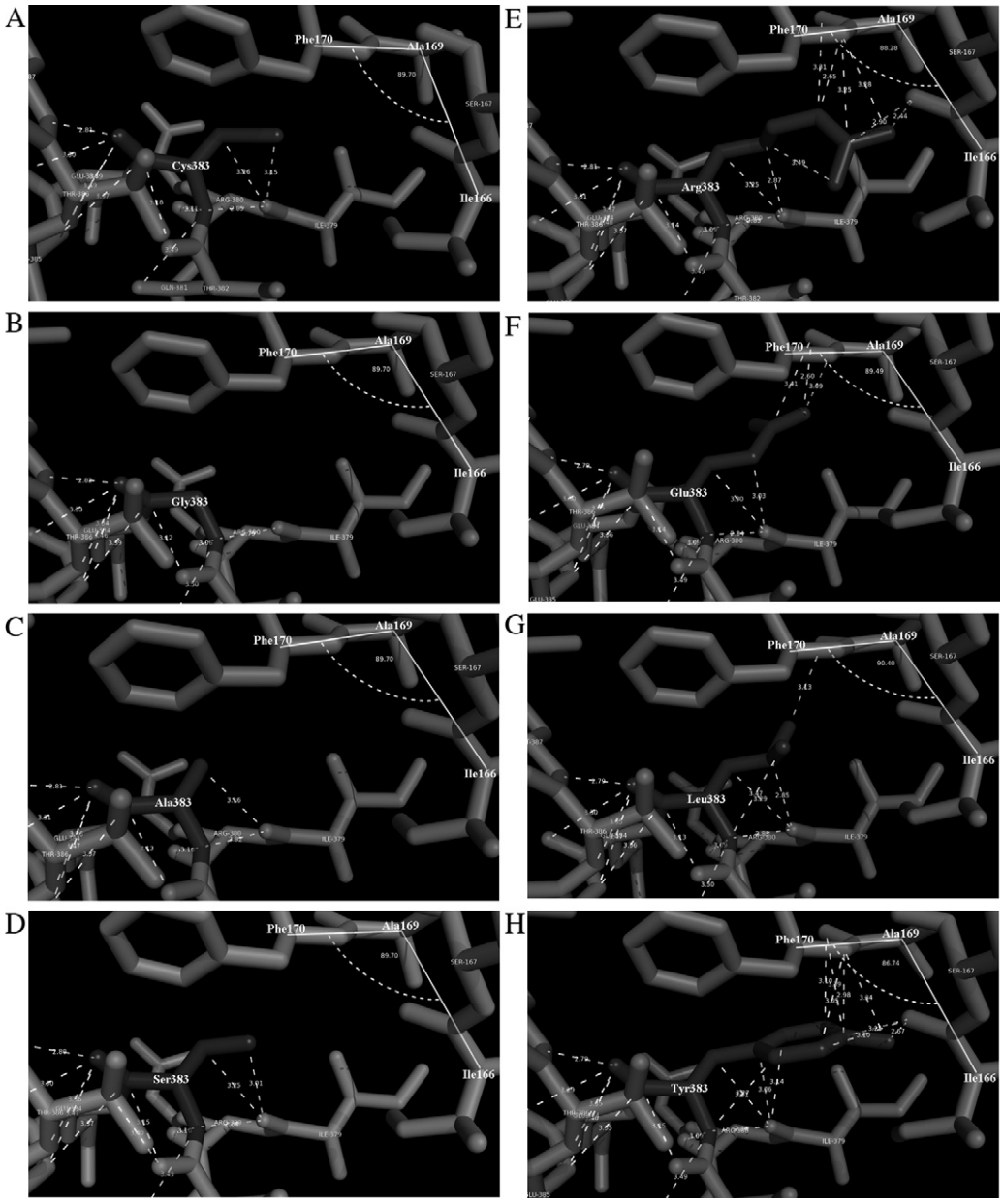


Fig. 5. Three-dimensional model showing the interactions around the mutation site in the wild type and the mutants C383G, C383A, C383S, C383R, C383E, C383L and C383Y (shown in A–H respectively). The distances (Å) of the hydrogen bonds formed by the amino acids in the mutation site are shown. The angle formed by Ile166, Ala169 and Phe170 in the adjacent helix is shown as an arc.

ner [4]. The distances among His161, His289 and Glu164 have kept the same, but the angles have changed faintly, when Cys383 has been changed by Gly, Ala, Ser (Fig. 6). The changed angles reflect the slight variation of active site, and might reflect changes of the hydrogen bond connections inside catalytic domain. Although faint changes occurred in the catalytic domain, these variations might affect the interactions between enzyme and its substrate causing the catalytic efficiency of mutants to increase. The catalytic efficiency was assessed by the value of k_{cat}/K_m. The CPB mutants (C383A, C383G and C383S) showed enhanced k_{cat}/K_m values, even though their K_m values were increased. The location of C383 is longer than 10 Å from the catalytic site (Fig. 1), but this point mutation (C383A, C383G and C383S) led to the increase in enzyme activity.

We studied the properties of those CPB mutants that could successfully refold in vitro and compared them with those of the wild type. Mutants C383G, C383A and C383S were more stable at higher temperatures than the wild type. Because changes in the native conformation are usually associated with a lower thermal stability [17], the high thermal stability of these Cys383 mutations in rat pCPB is quite interesting. Another related interesting phenomenon noted here is that, despite their higher thermal stability, the rate of catalytic activity of the CPB mutants did not increase as much as that of the wild type as the temperature increased. Based on the observed K_m values, we can propose that the affinity between the substrate and the catalytic domain of the wild type protein is much higher than those of the CPB mutants. As temperature rises, the collisions of molecules, including the enzyme and the substrate, in the system intensifies, and an enzyme that has a higher affinity could catalyze the substrate more effectively than an enzyme with a weaker affinity [18].

Although the optimal pH levels were the same for the CPB mutants and the wild type, their activities were different at different pH levels. For mutants C383A and C383S, the catalytic

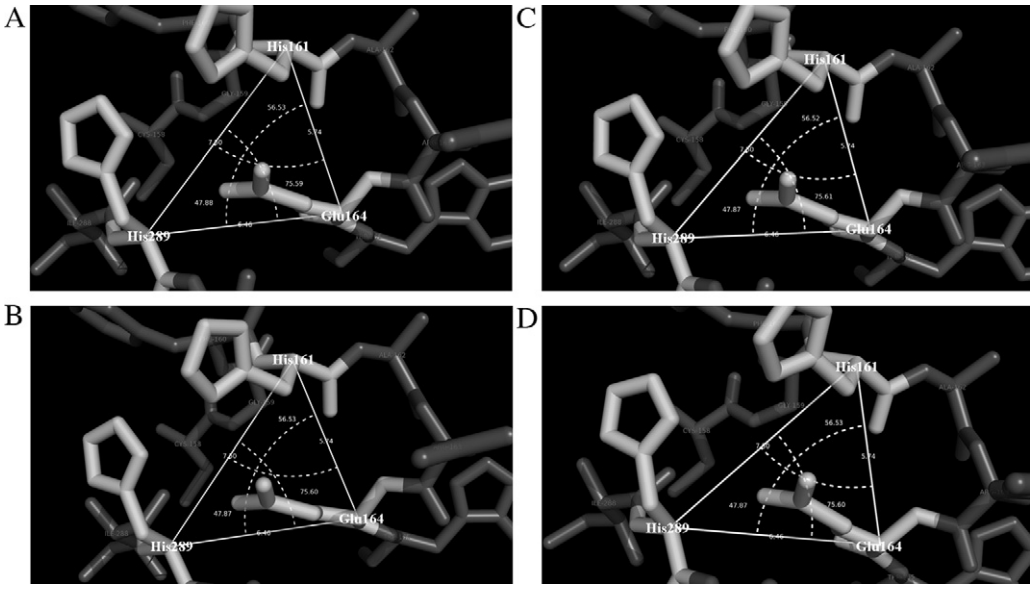


Fig. 6. The distances and angles among the catalytically active zinc ion binding amino acids His161, His289, Glu164. (A–D) Refer to wild type, C383G, C383A and C383S respectively.

domain could perform well in an even wider pH range. In contrast, substitution with glycine might result in a charge distribution in the catalytic domain that is more sensitive to pH.

The pH stabilities of the CPB mutants and the wild type were also investigated. All the CPB mutants except C383A and the wild type performed similarly. The mutant C383A was more stable in a wider range of pH. Substitution with alanine might form a more hydrophobic interior microenvironment, potentially forming a better balance in an unfavorable pH environment. Because Cys383 is located within the interior of the enzyme, the mechanism by which its substitution with different amino acids resulted in a change in the surface charge distribution deserves further study.

In conclusion, the identity of the amino acid selected to replace cysteine is quite important. To increase the refolding efficiency of the enzyme, the potential effects of the substituted amino acid on the protein itself should be seriously considered in addition to its ability to reduce the probability of disulfide mismatches. The replacement of cysteines within a protein with particular amino acids to increase the refolding efficiency is an effective method to improve the renaturation of IBs. The site-directed mutagenesis of Cys383 in rat procarboxypeptidase B not only increased its refolding efficiency, but also improved its catalytic efficiency. This method could provide a new way to improve the renaturation process of other IBs that contain disulfide bonds.

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