The active-site residue Cys-29 is responsible for the neutral-pH inactivation and the refolding barrier of human cathepsin B

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Abstract Human cathepsin B, the most abundant lysosomal cysteine protease, has been implicated in a variety of important physiological and pathological processes. It has been known for a long time that like other lysosomal cysteine proteases, cathepsin B becomes inactivated and undergoes irreversible denaturation at neutral or alkaline pH. However, the mechanism of this denaturation process remains mostly unknown up to this day. In the present work, nuclear magnetic resonance spectroscopy was used to characterize the molecular origin of the neutral-pH inactivation and the refolding barrier of human cathepsin B. Two forms of human cathepsin B, the native form with Cys-29 at the active site and a mutant with Cys-29 replaced by Ala, were shown to have well-folded structures at the active and slightly acidic condition of pH 5. Surprisingly, while the native cathepsin B irreversibly unfolds at pH 7.5, the C29A mutant was found to maintain a stable three-dimensional structure at neutral pH conditions. In addition, replacement of Cys-29 by Ala renders the process of the urea denaturation of human cathepsin B completely reversible, in contrast to the opposite behavior of the wild-type cathepsin B. These results are very surprising in that replacement of one single residue, the active-site Cys-29, can eliminate the neutral-pH denaturation and the refolding barrier. We speculate that this finding may have important implications in understanding the process of pH-triggered inactivation commonly observed for most lysosomal cysteine proteases.

Key words: Nuclear magnetic resonance; Human cathepsin B; Irreversible neutral-pH inactivation; Refolding barrier

1. Introduction

Cysteine proteases represent a major component of the lysosomal proteolytic repertoire and play an important role in intracellular protein degradation [1,2]. Of them, cathepsin B is the most abundant one which has also been implicated in a variety of physiological and pathological processes, such as general intracellular protein turnover [3], bone resorption [4], cartilage proteoglycan breakdown [5], antigen processing [6] and the malignant progression of tumors [7]. Like other papain-like cysteine proteases, cathepsin B is synthesized as an inactive proenzyme with an N-terminal 62-residue proregion and a 254-residue protease domain. This proenzyme is efficiently sorted to the lysosome and is converted into the single-chain form of the mature enzyme by limited proteolysis. The mature cathepsin B is stable and optimally active at slightly acidic pH but becomes inactivated under neutral or slightly alkaline conditions because of irreversible denaturation [8–11]. This neutral-pH inactivation was thought to be one of the major mechanisms of removing unwanted cysteine proteases in the extracellular space [2,8,12]. Therefore, functional cathepsin B in the extracellular space needs to be stored as a non-covalent complex with its own propeptide fragments [12].

Surprisingly, when returned to the native condition at pH 4–6, the neutral-pH-inactivated cathepsin B was found to form an inactive and partially folded intermediate isolated from the active state [8–11]. This observation implies that a large barrier may exist between the inactivated intermediate and the active state, therefore raising the possibility that cathepsin B could be one more example that its folding is under kinetic control, as observed for two well-studied proteases, the α-lytic protease [13] and subtilisin [14]. In previous studies, it has been demonstrated that the propeptide of the α-lytic protease or subtilisin is absolutely required for regeneration of the active proteases and that the absence of the propeptide will trap these enzymes in the 'molten globule'-like, inactive intermediate state [13,14].

Since the inactivation at neutral pH is common to all lysosomal cysteine proteases, understanding of the inactivation mechanism of cathepsin B will provide a molecular basis to clarify functions of lysosomal cysteine proteases in vivo and shed light on the nature of the refolding barrier separating the native state and the partially folded intermediate. However, in contrast to extensive biochemical characterizations [1,15], the structural details of the irreversible inactivation of cathepsin B have been examined in only a few studies and only by use of circular dichroism (CD) spectroscopy [8,9]. Therefore, we have carried out a nuclear magnetic resonance (NMR) study to explore the molecular mechanism of the neutral-pH inactivation and the refolding barrier which causes the irreversible inactivation. Our results reveal that replacement of the active-site Cys-29 can eliminate the irreversible inactivation and the refolding barrier of human cathepsin B.

2. Materials and methods

2.1. Protein expression

Recombinant and deglycosylated wild-type human cathepsin B, a mutant of human cathepsin B with Cys-29 replaced by Ala, and rat cathepsin B with Cys-29 replaced by Ser were expressed in the methylotrophic yeast *Pichia pastoris* as α-factor fusion constructs of the proenzymes in the pPIC9 vector and purified from the culture supernatant using procedures published previously [12,16]. The ^{15}N-labelled

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wild-type cathepsin B and the C29A mutant were obtained by growing the yeast in minimal media BMG and BMM (Invitrogen) with $^{15}NH_4SO_4$ as the sole nitrogen source. Autoprocessing of the wild-type human cathepsin B occurs during the expression step where the conditioned medium became acidic as a result of cell growth. The mature wild-type human cathepsin B was inhibited by adding methylmethane thiosulfonate (MMTS) into the supernatant of the cell culture [17]. The processing of the human procathepsin B mutant was achieved by incubating the proenzyme first with catalytic amounts of the wild-type cathepsin B (~0.5 μg of active cathepsin B for 1 mg of the C29A procathepsin B) followed by treatment with agarose-immobilized pepsin. The mature C29A cathepsin B mutant was purified by use of a SP-Sepharose column [17]. The C29S rat cathepsin B mutant was prepared and purified as described previously [18].

2.2. Preparation of neutral-pH-denatured cathepsin B

The neutral-pH-inactivated sample of the wild-type human cathepsin B (MMTS-inhibited form) was prepared by diluting ~100 μl of a sample of cathepsin B (~0.3 mM, pH 5.0) into 20 ml of 50 mM phosphate buffer at pH 7.5. The diluted cathepsin B was split into two parts with equal volumes: one was concentrated by centrifugation to 400 μl, giving an NMR sample at pH 7.5. The other was kept at pH 7.5 for 1 h, then the pH was adjusted to pH 5.0 and concentrated to 400 μl. The enzymatic activity was monitored by the proteolytic cleavage of the substrate Z-Phe-Arg-AMC [16]. The NMR samples of the two mutants (human C29A and rat C29S) at pH 7.5 were prepared following the same protocol at similar protein concentrations. Active human cathepsin B was generated by adding dithiothreitol (DTT) to the MMTS-inhibited cathepsin B sample at pH 5 to give a DTT concentration of 4 mM. Excess MMTS and DTT in the sample were removed by passing through a Sephadex LH-20 gel filtration column. The obtained MMTS-free active cathepsin B was diluted to a very low concentration (~0.4 μM) in the pH 7.5 phosphate buffer. After 1 h at 4°C, the pH of the sample was adjusted back to 5 and excess MMTS was added to the sample. The NMR sample was prepared by concentrating the diluted sample of cathepsin B to 400 μl.

2.3. Preparation of urea-denatured and refolded cathepsin B

Aliquots of 200 μl of each protein (wild-type and the C29A mutant, ~0.3 mM) were diluted into 40 ml of 50 mM acetate buffer at pH 5.0 in the presence of 8.05 M urea. The samples were split into two parts: one was concentrated to 400 μl to give an NMR sample in the presence of 8 M urea, the other was subjected to dialysis against the same acetate buffer without urea. The dialyzed samples were then concentrated to 400 μl.

2.4. NMR experiments

One-dimensional ^1H NMR experiments were collected using the WATERGATE pulse sequence [19] on a Bruker Avance-500 or Avance-800 NMR spectrometer. Proton two-dimensional Overhauser effect (NOESY) spectra were acquired at 800 MHz with phase-sensitive detection by time-proportional phase incrementation [20]. ^1H-^{15}N heteronuclear single-quantum correlation (HSQC) spectra were acquired at 500 or 800 MHz using a standard pulse sequence [21]. Spectral processing, display and analysis were performed using the XwinNMR software package supplied with the spectrometer system.

3. Results and discussion

3.1. Irreversible neutral-pH inactivation of wild-type human cathepsin B

Native cathepsin B has a well-defined three-dimensional structure in the crystalline state, as determined by X-ray crystallography [22]. It is also well-folded in solution at a slightly acidic pH as shown (Fig. 1a) by an HSQC spectrum of the ^{15}N-labeled wild-type human cathepsin B. The HSQC spectrum is well-dispersed in both the ^1H and ^{15}N dimensions, with a dispersion of the amide proton and nitrogen resonances larger than 5 ppm and 30 ppm, respectively. Furthermore, a large number of crosspeaks are present in the NOESY spec-

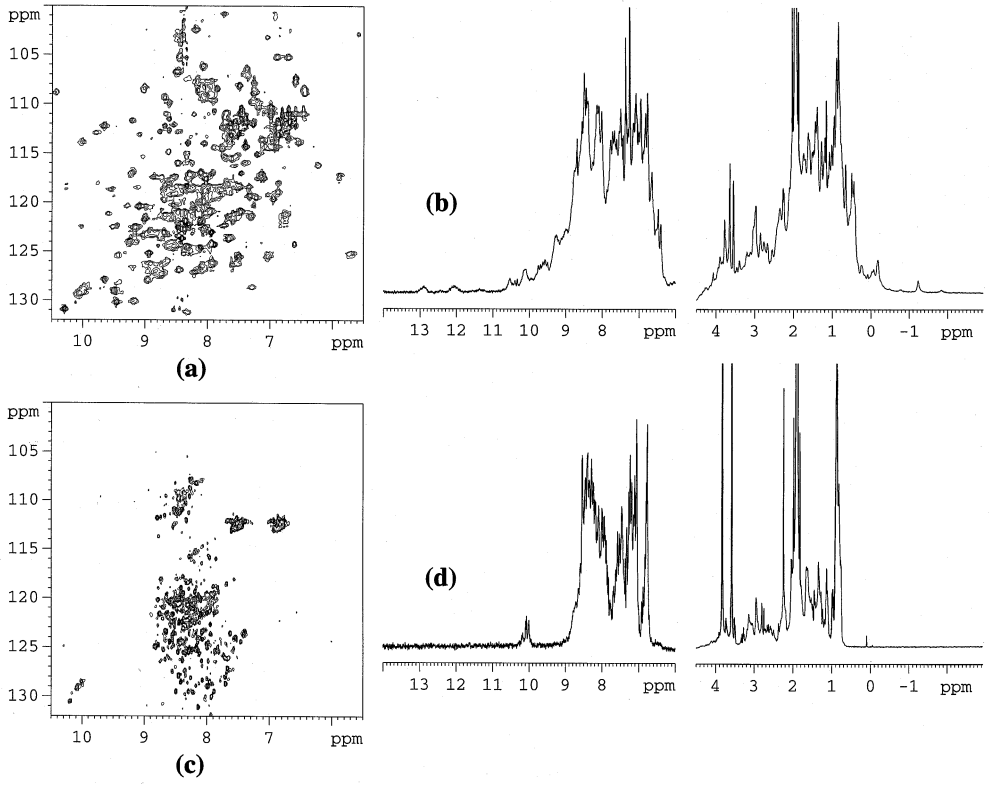

Fig. 1. The neutral-pH inactivation of the wild-type human cathepsin B. a: Two-dimensional ^1H-^{15}N HSQC spectrum of the MMTS-inhibited cathepsin B at pH 5 and 298 K. b: One-dimensional ^1H NMR spectrum of the MMTS-inhibited cathepsin B at pH 5 and 298 K. c: HSQC spectrum of the neutral-pH-inactivated cathepsin B at pH 5 and 298 K. d: One-dimensional ^1H NMR spectrum of the neutral-pH-inactivated cathepsin B at pH 5 and 298 K.

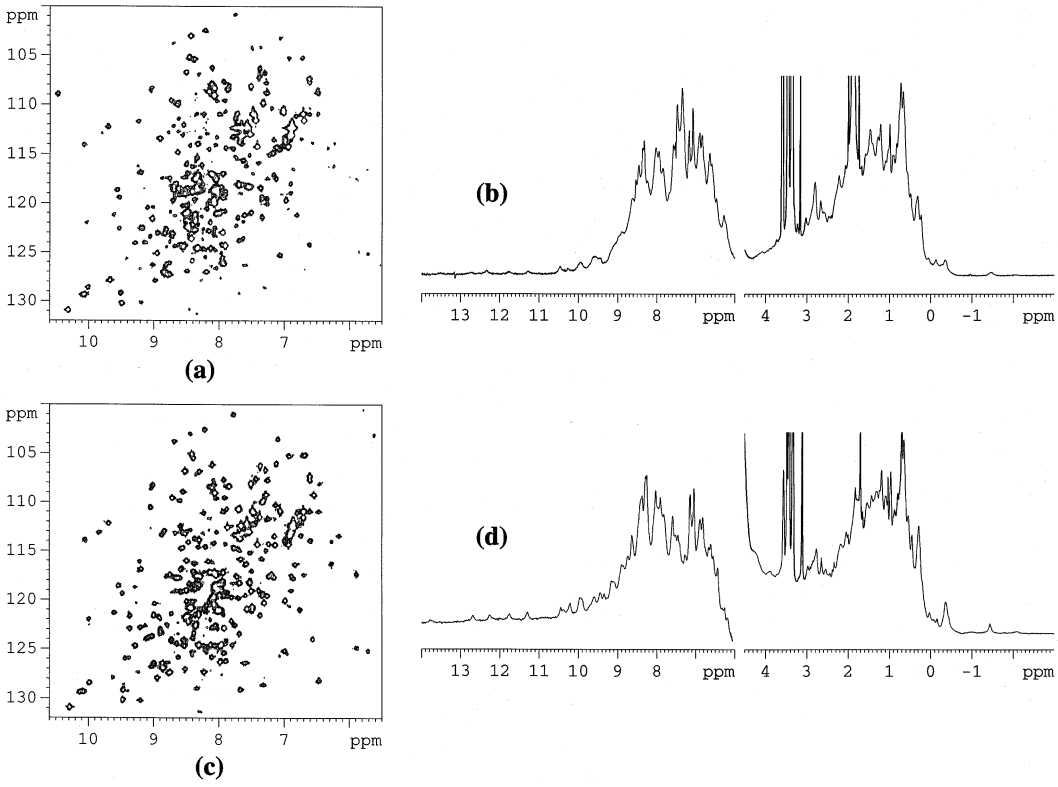

Fig. 2. The absence of the neutral-pH denaturation of the C29A mutant of human cathepsin B. a: Two-dimensional ^{1}H-^{15}N HSQC spectrum of the C29A mutant of human cathepsin B at pH 5 and 298 K. b: One-dimensional ^{1}H NMR spectrum of the C29A mutant of human cathepsin B at pH 5 and 298 K. c: ^{1}H-^{15}N HSQC spectrum of the C29A mutant at pH 7.5 and 298 K. d: One-dimensional ^{1}H NMR spectrum of the C29A mutant at pH 7.5 and 298 K.

trum of cathepsin B, indicating a tight tertiary packing within the protein (data not shown). The well-folded and well-packed properties of the native wild-type cathepsin B can also be easily visualized by the large chemical shift dispersion in a one-dimensional ^{1}H NMR spectrum (Fig. 1b). As seen in Fig. 1b, there are very downfield-shifted (at 9.55, 10.48, 11.3, 12.1 and 12.9 ppm) and very upfield-shifted proton resonances. The upfield proton resonances at −0.10, −0.4, −1.2 and −1.9 ppm are characteristic of native cathepsin B, resulting from the tight interior packing of a well-folded enzyme.

However, when wild-type cathepsin B was subjected to neutral or slightly alkaline pH, then returned back to pH 5, the protein failed to refold to the native state. The HSQC spectrum (Fig. 1c) of cathepsin B at pH 5, after treatment with pH 7.5, showed greatly reduced resonance dispersions in both spectral dimensions, i.e. only 1.5 ppm in the ^{1}H dimension and 23 ppm in the ^{15}N dimension. In parallel, the 1D proton NMR spectrum (Fig. 1d) became poorly dispersed with the disappearance of all downfield and upfield peaks, indicating clearly that wild-type cathepsin B underwent a large structural change after being subjected to pH 7.5. This inactive intermediate of the wild-type human cathepsin B is stable at pH 5 for several weeks without any detectable degradation as monitored by electrophoresis, activity assays and NMR spectroscopy, thus suggesting that there must be a large barrier isolating the partially folded intermediate from the native state. The same results were obtained with fully active human cathepsin B although only one-dimensional NMR experiments were done because of the dilute concentration and limited stability of NMR samples prepared from active cathepsin B (spectra not shown).

3.2. Molecular determinant of the neutral-pH denaturation

To identify the interactions responsible for the neutral-pH inactivation, we evaluated the pH dependence of a single mutant of human cathepsin B and a mutant of rat cathepsin B by use of NMR spectroscopy. The mutant of rat cathepsin B, with the active-site residue Cys-29 replaced by serine, has been shown to adopt the same three-dimensional structure as the wild-type cathepsin B and display specific interactions with the cathepsin B propeptide and subfragments [18,23]. Although the three-dimensional structure of the C29A mutant of human cathepsin B is not yet available, this mutant had similar binding properties to the cathepsin B propeptides as the C29S rat cathepsin B (unpublished observations). As seen from Fig. 2a, the ^{1}H-^{15}N HSQC spectrum of ^{15}N-labeled human cathepsin B (C29A) is as well-dispersed as that of the wild-type enzyme at pH 5 (Fig. 1a), indicating the lack of significant conformational changes induced by the C29A mutation. In addition, the 2D ^{1}H NOESY spectrum of the C29A mutant of human cathepsin B at pH 5 contains a large number of NOE crosspeaks which are similar to those for wild-type human cathepsin B (spectrum not shown), indicating that the mutant cathepsin B also has a well-folded three-dimensional structure with tight tertiary packing. This tertiary folding can also be seen from its well-dispersed 1D proton NMR spectrum at pH 5 (Fig. 2b). The C29A mutant of human

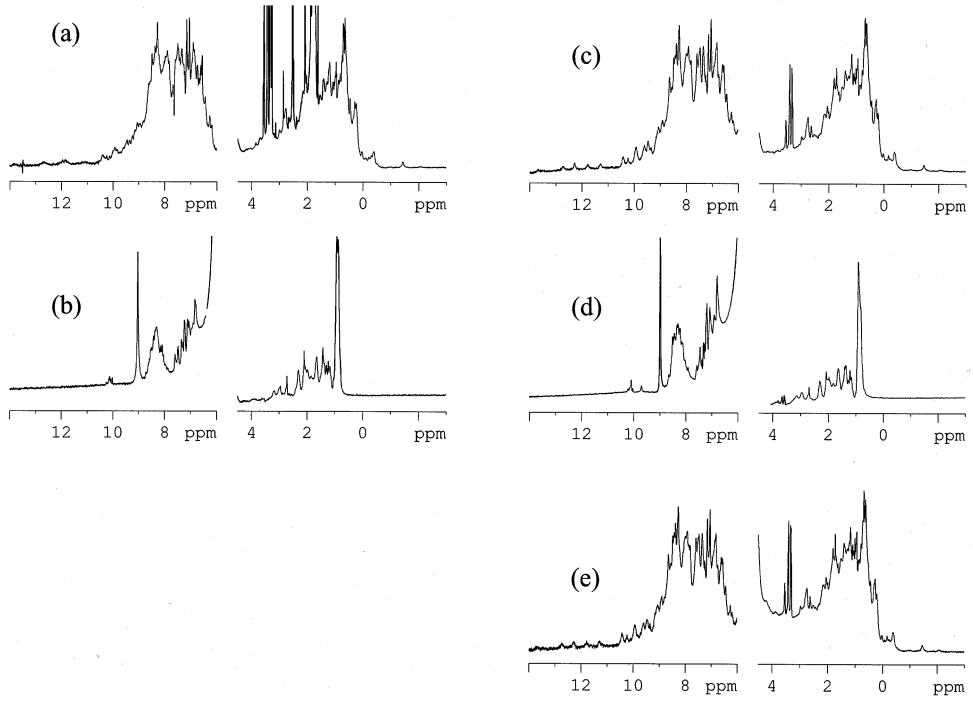

Fig. 3. The reversible denaturation with 8 M urea of the C29A mutant of the human cathepsin B. a: One-dimensional ^{1}H NMR spectrum of the wild-type human cathepsin B at pH 5 and 298 K. b: One-dimensional ^{1}H NMR spectrum of the wild-type human cathepsin B in the presence of 8 M urea at pH 5 and 298 K. c: One-dimensional ^{1}H NMR spectrum of the C29A mutant at pH 5 and 298 K. d: One-dimensional ^{1}H NMR spectrum of the C29A mutant in the presence of 8 M urea and at pH 5 and 298 K. e: One-dimensional ^{1}H NMR spectrum of the C29A mutant at pH 5 and 298 K after dialysis to remove urea.

cathepsin B has a 1D proton spectrum very similar to that of the wild-type human cathepsin B (Fig. 1b). The proton NMR spectrum (not shown) of the C29S rat cathepsin B shows only minor differences from that of the C29A human cathepsin B, even though almost 20% of the residues in rat cathepsin B are different from human cathepsin B [24]. For example, it still has dramatically downfield-shifted and upfield-shifted proton resonances, indicating similar three-dimensional structures of the mutant human and rat enzymes as can be inferred from the similar crystal structures of the corresponding active enzymes [22,25].

Surprisingly, when the active-site residue Cys-29 was replaced, the resulting mutants no longer underwent the process of the neutral-pH denaturation. As shown in Fig. 2c, the ^{1}H-^{15}N HSQC spectrum of ^{15}N-labeled human cathepsin B (C29A) is also well-dispersed at pH 7.5, indicating that no structural denaturation occurred at this pH. Furthermore, when the C29A cathepsin B at pH 7.5 was returned to pH 5, the protein produced a ^{1}H-^{15}N HSQC spectrum (not shown) identical to the original one at pH 5 before the increase of pH (Fig. 2a). The 2D ^{1}H NOESY spectrum of the C29A mutant at pH 7.5 also contains as many NOE cross-peaks as at pH 5 (spectra not shown), thus indicating convincingly that the C29A mutant is still well-folded at pH 7.5 with no neutral-pH denaturation occurring. The intactness of the mutant cathepsin B at pH 7.5 can also be seen in the 1D proton spectrum at pH 7.5 (Fig. 2d), which is as well dispersed as that at pH 5 (Fig. 2b). Similarly, the 1D proton spectrum of rat C29S at pH 7.5 is almost superimposable with that at pH 5 (data not shown), again indicating that no neutral-pH denaturation occurs in the C29S mutant. Further experiments indicated that even no denaturation was detected by NMR for the C29A mutant at pH 8.2 (data not shown). Therefore, it can be concluded that the active-site residue Cys-29 is responsible for the neutral-pH inactivation of cathepsin B.

3.3. Origin of the refolding barrier of human cathepsin B

The irreversible denaturation of cathepsin B implies that a refolding barrier may exist between the native state and the intermediate. In an attempt to identify the molecular origin of the refolding barrier of cathepsin B, we compared the unfolding–refolding behavior of human wild-type cathepsin B and the C29A mutant subjected to chemical denaturants. Since the C29A mutant cannot be denatured by neutral-pH conditions, we denatured both proteins with 8 M urea, a classic and strong chemical denaturing condition. As seen in Fig. 3a, the starting state, the native state of wild-type cathepsin B at pH 5, is well-folded. However, in the presence of 8 M urea, the protein is severely denatured (Fig. 3b) and gives a poorly dispersed NMR spectrum. All downfield and upfield resonances disappeared, typical of an unfolded protein. Due to the loss of the chemical shift dispersion of the methyl groups, a strong and broad peak appears at $\sim$0.80 ppm.

The denatured sample of the wild-type cathepsin B was subjected to refolding by dialysis against 50 mM acetate buffer at pH 5. However, even at a very low concentration (0.1 μM), protein aggregation was observed and a large amount of precipitate was formed during refolding. After removing the precipitate, the obtained NMR sample is so dilute that high-quality NMR spectra could not be obtained (spectra not shown). This result is in agreement with the previous finding

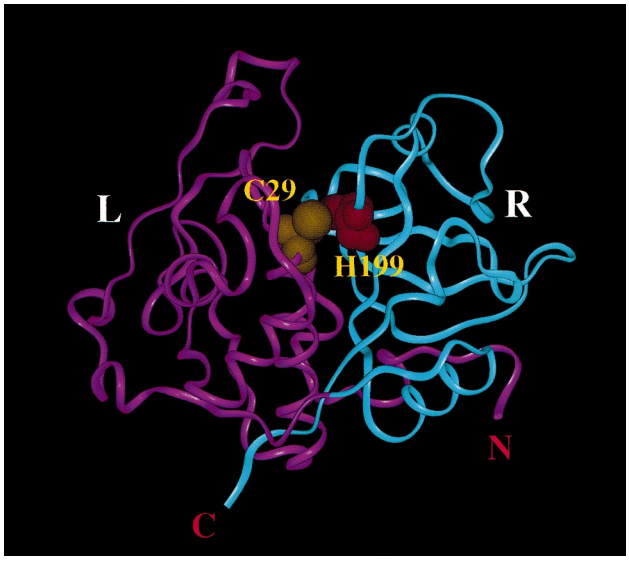

Fig. 4. The three-dimensional structure of the mature human cathepsin B, composed of two distinct subdomains, i.e. the L- (or N-terminal) and R- (or C-terminal) lobes carrying residues Cys-29 and His-199, respectively [2]. These two subdomains of cathepsin B are covalently connected only at the 'bottom' of the molecule through the polypeptide backbone along with interdigitating interactions of the N- and C-terminal residues into the other subdomain. The two structural subdomains are colored differently. The active-site residues Cys-29 and His-199 are shown in the space-filling mode with different colors, Cys-29 in yellow and His-199 in red.

that any severe denaturation, such as induced by the neutral pH or urea, was irreversible for the buffalo cathepsin B [8].

As seen in Fig. 3c, the proton NMR spectrum of the C29A mutant of human cathepsin B is also well-dispersed at pH 5. When 8 M urea was introduced, the 1D spectrum of the C29A mutant (Fig. 3d) is nearly identical to Fig. 3b, indicating that like the wild-type protein, the C29A cathepsin B was also severely unfolded under this condition. Fig. 3e shows the 1D spectrum of the C29A mutant after overnight dialysis to remove urea. This spectrum is nearly identical to that of the starting protein, suggesting that the C29A mutant can spontaneously and rapidly refold to the native state. It therefore appears that the critical residue responsible for the refolding barrier of human cathepsin B is residue Cys-29 which constitutes the Cys-29–His-199 catalytic diad of the active enzyme.

Fig. 4 presents a schematic representation of the three-dimensional structure of cathepsin B, with the active-site residues Cys-29 and His-199 emphasized in a space-filling mode. Both Cys-29 and His-199 are located at the interface between the two subdomains of cathepsin B. There has been the hypothesis that neutral pH may disrupt a salt bridge between Cys-29 and His-199 through the deprotonation of the His-199 side chain, resulting in the dissociation of the two subdomains of cathepsin B and leading to denaturation [9]. Our current results appear consistent with this hypothesis in that residue Cys-29 was found to be responsible for the irreversible denaturation of cathepsin B. However, the NMR data also imply that it cannot be the disruption of the Cys-29–His-199 salt bridge that leads to the neutral-pH denaturation since the substitution of Cys-29 with Ala in human cathepsin B and with Ser in rat cathepsin B failed to destroy the three-dimensional structure and the binding activities of cathepsin B. The irreversible neutral-pH inactivation/denaturation has been observed previously with intact cathepsin B and with Cys-29 blocked by various inhibitors [2,8–11,15], as we report here with the active enzyme and with Cys-29 blocked through disulfide bond formation with MMTS. In contrast, the C29A substitution for human cathepsin B and the C29S substitution for rat cathepsin B all result in the disappearance of both the neutral-pH and the irreversible chemical denaturation. We therefore speculate here that it may be the deprotonation of His-199 or the loss of a single positive charge alone that leads to the neutral-pH inactivation/denaturation of cathepsin B. Indeed, the active-site region of human cathepsin B is filled with seven negatively charged residues including the active-site Cys-29, but with only four neutralizing positively charged residues, including His-199 at low pH [9]. The repulsive negative charges may be compensated by some positively charged residues in the prosegment [23,26], a structural feature that may account for the neutral-pH stability of procathepsin B and non-covalent cathepsin B–propeptide complexes [12]. The replacement of Cys-29 with Ala in human cathepsin B and with Ser in rat cathepsin B eliminates one potentially destabilizing negative charge, or the side-chain $-S^-$ group, from the active site and may significantly alter the side-chain pK_a of His-199, thereby resulting in the disappearance of the neutral-pH inactivation/denaturation.

In summary, we first investigated the neutral-pH inactivation of the wild-type human cathepsin B by use of NMR spectroscopy and showed that the wild-type human cathepsin B cannot refold spontaneously to the active state after being exposed to neutral pH. We then demonstrated that replacement of a single residue, the active-site Cys-29, can eliminate the neutral-pH inactivation/denaturation and refolding barrier of cathepsin B. We suggest that our present results may have direct implications for understanding the neutral-pH inactivation and irreversible denaturation commonly observed for most lysosomal cysteine proteases.

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