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Delineating functionally important regions and residues in the cathepsin B propeptide for inhibitory activity

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Abstract Synthetic peptides derived from the proregion of rat cathepsin B were used to identify functionally important regions and residues for cathepsin B inhibition. Successive 5 amino acid deletions of a 56 amino acid propeptide from both the N- and C-termini has allowed the identification of two regions important for inhibitory activity: the NTTWQ (residues 21p–25p) and CGTVL (42p–46p) regions. Alanine scanning of residues within these two regions indicates that Trp-24p and Cys-42p contribute strongly to inhibition, their replacement by Ala resulting in 160- and 140-fold increases in K_i, respectively.

Key words: Cysteine protease; Propeptide; Inhibition

1. Introduction

Cysteine proteases play an important role in several physiological processes such as intracellular protein degradation [1], antigen processing [2] and bone resorption [3]. However, under conditions where they become deregulated, cysteine proteases are also implicated in a number of degradative and invasive pathological conditions, e.g., arthritis [4,5], tumor invasion and metastasis [6,7] and muscular dystrophy [8]. A number of potent inhibitors of cysteine proteases are available. However, because of the diversity of cysteine proteases and their contribution to normal physiological processes, cysteine protease inhibitors should preferably be enzyme selective in order to be considered for potential therapeutic applications. Designing inhibitors that are highly selective has proven to be a difficult task for the cathepsin class of cysteine proteases due to their broad substrate specificity [9]. Some success has been obtained using E-64 analogs developed for the specific inhibition of cathepsin B [10]. In this case selectivity is achieved through interaction with the 'occluding loop' of cathepsin B, a structural feature particular to this enzyme [11,12]. Selective inhibition of cathepsin B has also been observed with a 56 amino acid peptide derived from the proregion of the enzyme [13]. This propeptide is a potent inhibitor of cathepsin B with a K_i of 0.4 nM, a value 14000-fold better than that for inhibition of papain [13]. This peptide is the most highly specific inhibitor of cathepsin B available so far. Similarly, a cathepsin L propeptide was also shown to be selective for its parent enzyme cathepsin L [14]. Clearly, in addition to being potent inhibitors, the propeptides of cysteine proteases also contain features ensuring that inhibition is highly selective for the proteases from which they originate.

In this paper we report a detailed systematic study aimed at defining functionally important regions or residues for the inhibition of cathepsin B by its propeptide. To achieve this, we have used N- and C-terminal truncated synthetic peptides, coupled to alanine-scanning on identified important regions.

2. Materials and methods

2.1. Materials

The peptides were prepared by standard Fmoc chemistry on an Applied Biosystems 431A solid-phase synthesizer. The crude peptides were purified to homogeneity by HPLC on a Vydak C18 preparative column (22×250 mm) using a linear 0–70% CH_3CN gradient (containing 0.1% TFA). Identity of the peptides was confirmed using electrospray mass spectrometric analysis on a SCIEX API III spectrometer and amino acid analysis on a Beckman model 6300 amino-acid analyzer. Peptide purity was assessed by HPLC on a Vydak C18 (4.6×250 mm) column using the same gradient as for the purification procedure.

The substrate CBZ-Phe-Arg-MCA (carbobenzoxy-L-phenylalanyl-L-arginine 4-methylcoumarinyl-7-amide hydrochloride) was purchased from IAF Biochem International Inc., Laval, Canada. Recombinant cathepsin B expressed in yeast [15] was purified from the culture supernatant as described previously [13]. The enzyme was stored in the PDS-inactived form (1.5 mM 2-PDS, 50 mM sodium formate, pH 4.0) and activated by diluting stock enzyme into phosphate buffer (pH 6.0) containing 1 mM DTT for 15 min on ice.

2.2. Methods

Cathepsin B activity was measured using the substrate CBZ-Phe-Arg-MCA as described previously [16]. All measurements were performed at 25°C in 0.1 M phosphate buffer (pH 6.0) containing 1 mM EDTA, 1 mM DTT, 0.025% Brij-35 and 3% DMSO. The dissociation constants K_i were determined from $1/v$ vs. [I] plots [17] obtained by measuring the initial rate of substrate hydrolysis (v) in the presence of varying concentrations of inhibitor and at a substrate concentration of 20 μM, well below the K_M value. For some inhibitors non-linearity in the initial portion of the progress curves indicated the presence of a 'slow inhibition' process and the data was analyzed as described previously [13].

3. Results and discussion

The sequence of the propeptide previously found to be a potent inhibitor of rat cathepsin B is reported in Fig. 1. The peptide corresponds to residues 1p to 56p of the rat cathepsin B proregion. Two series of peptides corresponding to successive deletions every five residues from both N- and C-termini of this 56 amino acid peptide were synthesized and their inhibitory activity against cathepsin B assayed. The results are reported in Fig. 2a. It can be seen that successive deletions of 5 residues from both ends of the propeptide result in a progressive increase in K_i for up to 20 and 10 residue deletions in the N- and C-terminal regions, respectively. For these six peptides the K_i value does not go higher than 150 nM and they can still be considered good inhibitors of cathepsin B. Further deletions on either end cause a relatively important decrease of inhibitory activity. This is best visualized in Fig. 2b, where ΔK_i represents the ratio of K_i values for each suc-

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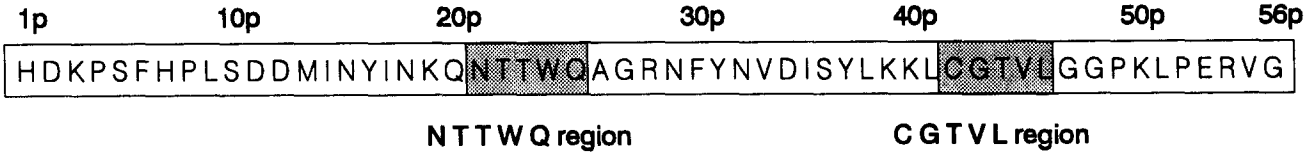

Fig. 1. Sequence of a 56 amino acid peptide corresponding to residues 1p to 56p of the rat cathepsin B proregion [20]. Propeptide numbering is based on the procathepsin B sequence starting at the N-terminal amino acid. Residues in the propeptide are identified with the suffix p. Two regions identified as being functionally important are marked by gray-shaded boxes.

cessive 5 amino acid deletion. Removal of the N-terminal fragment 21p–25p (NTTWQ) causes a 150-fold increase in K_i while removal of the C-terminal 42p–46p residues (CGTVL) leads to an even greater increase in K_i ($\Delta K_i = 625$). The resulting peptides, prcb(26p–56p) and prcb(1p–41p), display K_i values of 9.7 μM and 83 μM, respectively. Further truncations result in gradual but less important increases in K_i up to mM values.

The systematic removal of 5 amino acid fragments from both ends of the propeptide has allowed us to identify two seemingly important functional regions of the cathepsin B propeptide: the NTTWQ and CGTVL regions (Fig. 1). To evaluate the contribution of individual amino acid side chains within these regions, 10 synthetic peptides were prepared where residues 21p–25p and 42p–46p were individually replaced by an alanine. The peptide template used for this study is prcb(21p–56p) which is shorter than the 56 amino acid peptide by 20 residues yet it still displays good inhibitory activity ($K_i = 65$ nM). The results are illustrated in Fig. 3. For the NTTWQ region (Fig. 3a) it can be seen that the tryptophan residue at position 24p contributes the most to inhibitory activity, replacement of Trp-24p by Ala causing a 160-fold increase in K_i. Cys-42p in the CGTVL region is also an important residue, its replacement by Ala causing a 140-fold increase in K_i. Amongst the remaining residues in these two regions, replacement of Gly-43p and Leu-46p has a moderate 14-fold effect on inhibition while other replacements have less significant effects.

The results obtained with the truncated propeptides indicate that part of the N- and C-terminal regions are not essential for inhibition and it delimits the most important region of the propeptide to residues 21p to 46p. A similar but less systematic study of the interaction between cathepsin L and its propeptide also indicated that 20 and 15 amino acids could be removed from the N- and C-terminal parts of the propeptide without destroying the inhibitory activity [14]. Recently, the structure of human procathepsin B has been solved [18,19]. Assuming the propeptide interacts with cathepsin B in the same manner as the proregion in procathepsin B, the availability of the data presented in this work allows the functional evaluation of structural features evidenced by the proenzyme structure. The first 10 amino acids at the N-terminus of procathepsin B were found to be disordered and mobile in the crystal and other than the covalent link to the N-terminus of the mature enzyme the C-terminal region beyond residue 48p makes little contact with the enzyme. Accordingly, deletion of these residues does not greatly affect the inhibitory activity of the propeptide. As shown by the X-ray structure residues 11p–20p form a three-turn α-helix and N-terminal deletions up to residue 16p cause a progressive increase in K_i, indicating that this region contributes to the inhibitory activity. It is perhaps somewhat surprising, however, to find that peptide prcb(21p–56p), where the residues forming the α-helix are absent, is still a good inhibitor of cathepsin B with a K_i of 65 nM. It can be seen in Fig. 2a that the 5 amino acid deletion from residue 16p to 21p actually causes a small 2.4-fold decrease in K_i. It was found that N-terminal acetylation of prcb(21p–56p) resulted in a 30-fold increase in K_i, to a value of 2.0 μM, and it is possible that the free N-terminus present in prcb(21p–56p) could interact with the enzyme and in part compensate for the loss of the α-helix.

The alanine scanning experiments can be used to evaluate the functional contribution of individual side chains to inhibition. The aromatic side chain of Trp-24p in NTTWQ is positioned in a depression on the surface of cathepsin B occupied by two aromatic side chains (Tyr-183 and Tyr-188). Replacement of Trp-24p by an alanine confirms that this interaction,

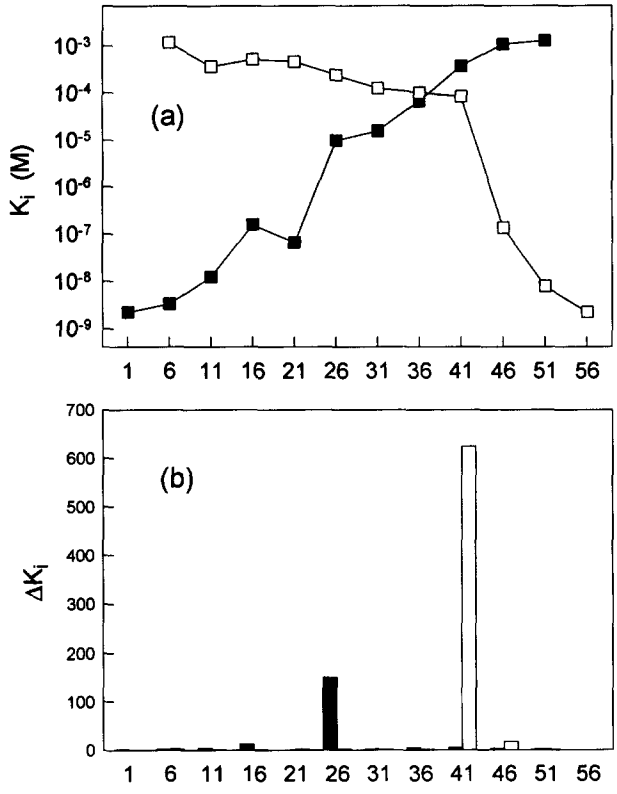

Fig. 2. Influence of N- and C-terminal truncations on the inhibitory activity of the cathepsin B propeptide. (a) K_i as a function of the N- or C-terminal residue. The effect of N-terminal deletions is represented by the black squares for peptides with an intact C-terminal residue (56p) and varying N-terminal residues indicated on the x-axis. Similarly, C-terminal deletions are represented by white squares for peptides starting with residue 1p and ending at the position indicated on the x-axis. (b) Relative effect of succesive 5 amino acid deletions. ΔK_i represents the ratio of K_i values for two successive peptides differing by a 5 residue deletion (e.g., K_i for prcb(11p–56p)/K_i for prcb(6p–56p)).

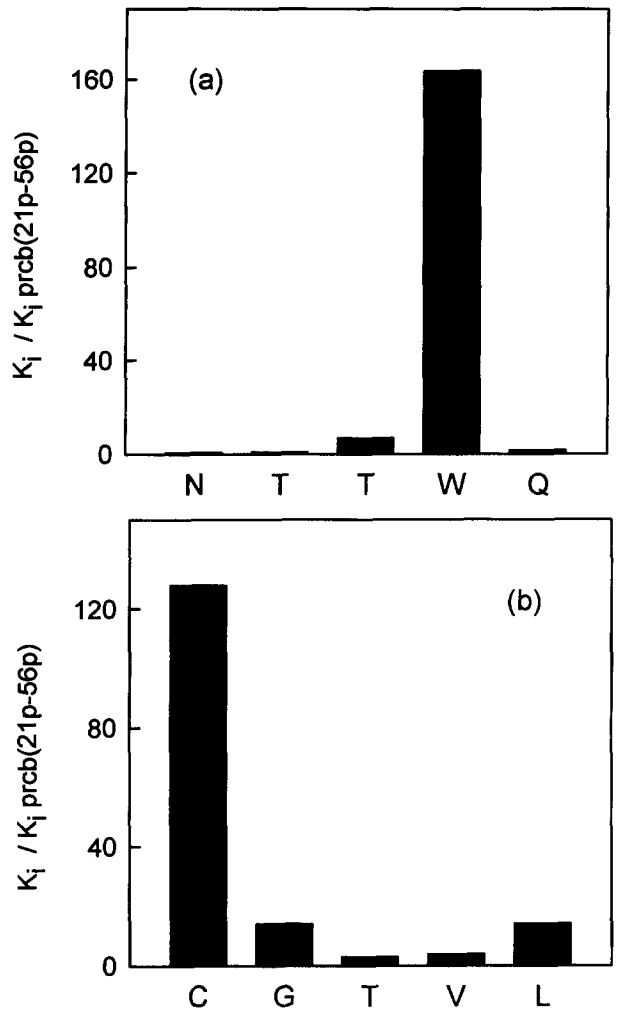

Fig. 3. Alanine scan of the NTTWQ (a) and CGTVL (b) regions. The results are reported as the ratio of K_i for the Ala derivative over K_i for peptide prcb(21p–56p).

even though it is located far from the active site, contributes significantly to inhibition. The CGTVL region also found to be important for inhibition is located at the active site of cathepsin B. This peptide stretch adopts an extended conformation but binds to the active site in a direction opposite to that expected for a substrate, therefore preventing hydrolysis. The side chain of residue Cys-42p occupies the S_1' subsite and interacts with many hydrophobic residues. Gly-43p penetrates deep into the active site and is in close contact to Gly-27. Replacement of Gly-23p by an alanine cause a 14-fold increase in K_i, and it is expected from the structure that substitution by residues with larger side chains would not be tolerated in this position. Further work will involve functional characterization of residues in the central region of the propeptide which, based on the procathepsin B structure, are suspected to be functionally important for inhibition of cathepsin B.

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