Interaction of cysteine proteinases with recombinant kininogen domain 2, expressed in *Escherichia coli*

Karin Ylinenjärvi[a], Thomas W. Prasthofer[b,**], Nancy C. Martin[b], Ingemar Björk[a,*]

[a]*Department of Veterinary Medical Chemistry, Swedish University of Agricultural Sciences, Uppsala Biomedical Center, Box 575, S-751 23 Uppsala, Sweden*

[b]*Department of Medical and Physiological Chemistry, Uppsala University, Uppsala Biomedical Center, Box 575, S-751 23 Uppsala, Sweden*

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Abstract The calpain-binding domain 2 of the kininogens, the major plasma inhibitors of cysteine proteinases, was expressed in *Escherichia coli*. Expression of soluble protein was optimal at 15°C and was augmented by growing the bacteria in sorbitol and betaine. The recombinant domain showed high affinity (K_i 0.3–1 nM) for cathepsin L and papain, and a somewhat lower affinity (K_i ~15 nM) for calpain. The binding to cathepsin H was substantially weaker, and no inhibition of actinidin and cathepsin B was detected. The affinity for cathepsin L was comparable to that reported for the domain isolated from plasma L-kininogen, whereas the affinities for papain and calpain were about tenfold lower. The latter difference may be due to the recombinant domain being nonglycosylated.

Key words: Kininogen; Domain; Cysteine proteinase; Cysteine proteinase inhibitor; Cystatin; Bacterial expression

1. Introduction

The low-molecular-weight (L-) and high-molecular-weight (H-) kininogens are the major plasma inhibitors of cysteine proteinases [1–4]. In addition, both kininogens serve as precursors of kinins, and kininogen also participates in the contact phase of blood clotting [5–7]. The regions of the two kininogens that constitute the heavy chain after excision of the kinin segment are identical and contain three domains homologous to low-molecular-weight tissue inhibitors of cysteine proteinases, cystatins [1,8]. However, the N-terminal domain, domain 1, lacks the residues important for proteinase binding in cystatins [9,10] and thus has no detectable inhibitory activity [8]. Both domains 2 and 3 inhibit papain-like cysteine proteinases, but domain 2 is unique in that it also inactivates calpain [8]. The two inhibitory domains have been isolated by proteolytic cleavage of plasma L-kininogen [8]. Kininogen domain 3 has also been expressed in *E. coli*, but no expression of domain 2 was obtained in the system used [11].

To enable characterization of structure–function relationships in kininogen domain 2, in particular the background for calpain inhibition, we have developed an expression system for this domain in *E. coli*. The isolated domain was highly active, but had slightly lower affinities for some proteinases than those reported for the domain obtained by cleavage of L-kininogen. This difference may be due to the recombinant domain lacking oligosaccharide side chains.

*Corresponding author. Fax: (46) (18) 550762.
E-mail: Ingemar.Bjork@bmc.uu.se

***Present address:* Albany College of Pharmacy, 106 New Scotland Avenue, Albany, NY 12208-3492, USA.

Abbreviations: AMC, 7-amido-4-methylcoumarin; DTT, dithiothreitol; Omp A, outer membrane protein A of *E. coli*; PCR, polymerase chain reaction; Suc, succinyl; Z, benzyloxycarbonyl.

2. Materials and methods

2.1. Proteinases

Papain (EC 3.4.22.2), actinidin (EC 3.4.22.14) and the inactive papain, derivative, *S*-(methylthio)papain, were obtained as described earlier [12–14]. Recombinant rat cathepsin B (EC 3.4.22.1), expressed in yeast [15], was a gift from Dr. John S. Mort, Shriners Hospital, Montreal, Canada. Cathepsin H (EC 3.4.22.16) and cathepsin L (EC 3.4.22.15), purified from bovine and sheep liver, respectively [16,17], were donated by Dr. Robert W. Mason, Alfred I. duPont Institute, Wilmington, DE, USA. *m*-Calpain (EC 3.4.22.17), isolated from rabbit lung [18], was a gift from Dr. Jan-Olof Karlsson, University of Göteborg, Sweden.

2.2. Construction of expression vector

cDNA was synthesized from human liver total RNA [19] with oligo-$d(T)_{16}$ as primer (GeneAmp RNA PCR kit; Perkin-Elmer Cetus). A segment of the kininogen cDNA from nucleotides 427 to 852, containing the sequence coding for domain 2 [1], was amplified by PCR (GeneAmp). The product had the expected sequence [1,20], except for a T→C substitution at position 578, leading to a Met→Thr replacement. The T of the published sequence was restored by site-directed mutagenesis (Altered Sites Mutagenesis System; Promega). A further PCR amplification of this cDNA fragment created a *Hin*dIII cleavage site by G→C and G→T substitutions at nucleotides 451 and 453, respectively, and a stop codon by a G→T substitution at nucleotide 837, following which a *Sal*I cleavage site was introduced. The PCR product was ligated into the expression vector (pFLAG-1; International Biotechnologies) and the correct sequence of the construct verified.

2.3. Expression and purification

E. coli, strain UT5600, transformed with the construct were grown at 37°C in LB medium [21], containing 0.4% (v/v) glycerol, 50 μg/ml ampicillin, 1 M sorbitol and 2.5 mM betaine [22], to an absorbance at 600 nm of 0.5–1. Expression was induced by adding isopropyl β-D-thiogalacto-pyranoside to 0.5 mM, and the bacteria were grown at 15°C for another 14–16 h. The cells were sonicated on ice in the presence of 1 mM EDTA and 1.5 mM of the serine proteinase inhibitor, 4-(2-aminoethyl)-benzenesulfonyl fluoride (Pentapharm). The lysate was applied to a 5-ml HiTrap *N*-hydroxysuccinimide-activated column (Pharmacia Biotech), containing ~6 mg *S*-(carboxymethyl)papain/ml gel, and the bound protein was eluted at pH 11.0. The fusion protein was digested for 24 h at 37°C with enterokinase (Biozyme) at an amount of 2 μg/mg protein in 0.05 M Tris-HCl, 0.1 M NaCl, 2 mM EDTA, 10% (v/v) glycerol, pH 8.0. Domain 2 was isolated by gel chromatography on a Superdex 16/60 column (Pharmacia Biotech).

2.4. Protein analyses

SDS-PAGE was done on 16.5% (w/v) gels with the Tricine buffer system [23]. N-terminal sequences were determined as described by Björk et al. [24]. Molecular mass was analyzed in a Kratos Kompact

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MALDI III instrument with α-cyano-4-hydroxycinnamic acid as matrix. Fluorescence emission spectra were measured as reported previously [12,25].

2.5. Proteinase binding

Titrations of active papain or *S*-(methylthio)papain with domain 2 for determination of apparent binding stoichiometries were monitored by fluorescence [12,13]. Binding stoichiometries were also determined by titrating 20 nM papain with increasing concentrations of inhibitor and assaying residual activity with the substrate, carbobenzoxy-L-phenylalanyl-L-arginine *p*-nitroanilide (200 μM; Bachem), in the presence of 0.5 mM dithiothreitol at pH 7.4.

Inhibition constants and association rate constants for the interactions of domain 2 with cysteine proteinases were measured at 25°C [8,15,26].

2.6. Protein concentrations

Concentrations of domain 2 were determined by absorbtion measurements at 280 nm with the use of a calculated molar absorption coefficient [27] of 12,400 $M^{-1} \cdot cm^{-1}$ and a relative molecular mass of 14,500 [1].

3. Results and discussion

The expression vector was constructed to express residues 136 to 263 of the kininogen sequence [1,20], although with Val-136 replaced by Leu to allow in-frame cloning into the vector. The recombinant domain 2 is thus slightly longer than that isolated by cleavage of L-kininogen, which comprised residues 141 to 262 [8]. The N-terminus of the domain was fused to a FLAG sequence [28], containing an enterokinase cleavage site, followed N-terminally by the Omp A signal sequence. The latter sequence was designed to transport the fusion protein to the periplasmic space, with concomittant cleavage of this sequence.

Expression of soluble kininogen domain 2 increased at lower temperatures, 15°C being optimal. The amounts were also increased by growing the bacteria in sorbitol and betaine, conditions reported to augment expression of soluble forms of other recombinant proteins in *E. coli* [22]. Lysis of whole cells by sonication gave the highest yield of the domain. N-terminal analyses showed that the signal peptide had been cleaved from the protein isolated in this manner. Purification by affinity chromatography on immobilized papain was found to give a higher yield than affinity chromatography on a matrix-linked antibody against the FLAG sequence. The latter sequence was removed from the fusion protein by cleavage with enterokinase (Fig. 1). About 0.25 mg of purified domain 2 was obtained per liter of bacterial culture.

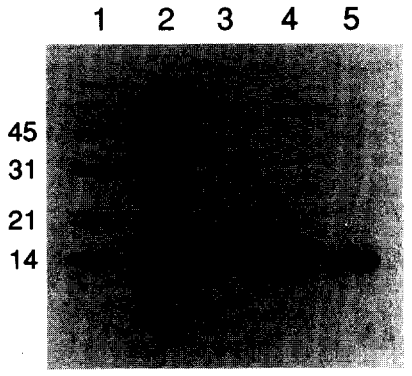

Fig. 1. SDS-PAGE of kininogen domain 2 under reducing conditions. Lane 1, standards with the molecular masses in KDa indicated. Lane 2, bacterial lysate. Lane 3, eluate from matrix-linked papain. Lane 4, eluate from matrix-linked papain, digested with enterokinase. Lane 5, kininogen domain 2 after final purification by gel chromatography.

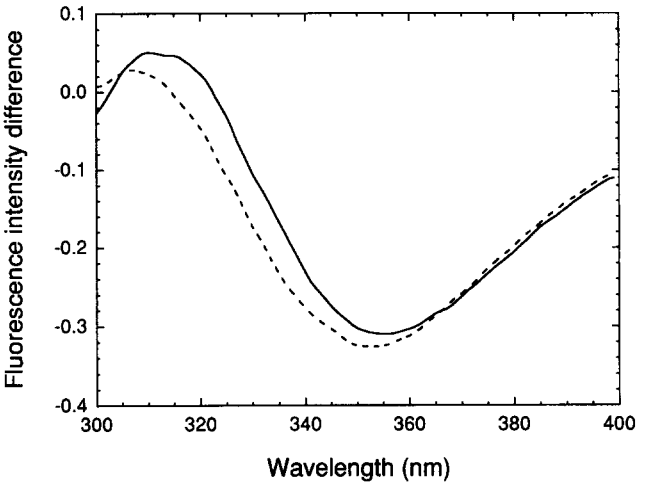

Fig. 2. Fluorescence-emission difference spectra between complexes of recombinant human kininogen domain 2 (—) or cystatin C (---) with papain and the free proteins. The spectra were calculated from separately measured corrected emission spectra [25]. The concentration of papain was 1 μM and that of active kininogen domain 2 or cystatin C 1.2 μM.

The isolated domain 2 showed only one band in SDS-PAGE (Fig. 1). It had the expected N-terminal sequence [1,20] and a molecular mass, as determined by mass spectroscopy, of 14,521 Da, in good agreement with the calculated mass of 14,537 Da. Titrations of active papain or *S*-(methylthio)papain with the isolated domain gave apparent inhibitor to enzyme binding stoichiometries of 1.4–1.7, indicating that the preparations contained 60–70% inhibitorily active protein. In all further analyses, calculations were based on concentrations of active inhibitor.

The binding of domain 2 to papain was accompanied by fluorescence changes (Fig. 2) highly similar to those observed for the binding of human cystatin C to the enzyme [25]. This finding indicates that the kininogen domain interacts with papain in a similar manner as the homologous inhibitor, cystatin C.

Kininogen domain 2 showed the highest affinity for papain and cathepsin L of the six cysteine proteinases investigated (Table 1) and a somewhat lower affinity for calpain. The binding to cathepsin H was substantially weaker, and no inhibition of actinidin and cathepsin B was detected. The affinity of the recombinant domain 2 for cathepsin L was comparable to that reported previously for the domain isolated by proteolytic cleavage of plasma L-kininogen, whereas the affinities of the recombinant domain for papain and calpain were about tenfold lower [8]. This discrepancy may be partly due to differences in the methodology and enzyme preparations used. The fact that the recombinant domain is not glycosylated may also have affected the binding to certain target proteinases. Association rate constants were measured only for the high-affinity enzymes, papain and cathepsin L, revealing rapid binding (Table 1).

Comparison of the data in this work with the inhibitory

Table 1
Inhibition constants (K_i) and association rate constants (k_{ass}) for the binding of recombinant kininogen domain 2 to target proteinases at 25°C

Proteinase	K_i (nM)	$10^{-6} \cdot k_{ass}$ ($M^{-1} \cdot s^{-1}$)
Papain	1.0 ± 0.1 (n = 5)	2.9 ± 0.1 (n = 13)
Actinidin	≥ 1000	N.D.
Cathepsin B	≥ 1000	N.D.
Cathepsin H	110 ± 17 (n = 5)	N.D.
Cathepsin L	0.3 ± 0.03 (n = 13)	3.1 ± 0.3 (n = 6)
Calpain	17 ± 2 (n = 5)	N.D.

The values are given with their standard errors and with the number of measurements in parentheses. The buffers and substrates used in the analyses were: for papain and actinidin 0.05 M Tris-HCl, 0.1 M NaCl, 100 μM EDTA, 0.5 mM DTT, 3% (v/v) acetonitrile, pH 7.4, and 20 μM Z-Phe-Arg-AMC; for cathepsin B 0.05 M MES-NaOH, 0.1 M NaCl, 100 μM EDTA, 0.5 mM DTT, 0.1% (w/v) poly(ethylene glycol), pH 6.0, and 20 μM Z-Arg-Arg-AMC; for cathepsin H 0.1 M sodium phosphate, 1 mM EDTA, 1 mM DTT, pH 6.0, and 10 μM H-Arg-AMC; for cathepsin L 0.1 M sodium acetate, 1 mM EDTA, 1 mM DTT, 0.005% (w/v) Brij 35, pH 5.5, and 5 μM Z-Phe-Arg-AMC; for calpain 0.05 M Tris-HCl, 0.1 M NaCl, 2 mM DTT, 5 mM $CaCl_2$, 0.1% (w/v) Triton X-100, pH 7.4, and 1 mM Suc-Leu-Tyr-AMC. The K_m values used for correction of the apparent K_i and k_{ass}values for substrate competition have been reported elsewhere [16,17,32,33]. N.D., not determined.

properties reported previously for recombinant kininogen domain 3 and for domain 3 isolated from plasma L-kininogen [8,11] indicates that kininogen inhibition of the physiological papain-like target enzymes, cathepsin B and L, is predominantly effected by domain 3. In particular, cathepsin B is inhibited only by the latter domain, although with moderate affinity. The inhibition of cathepsin H by domain 2 presumably is too weak to be of appreciable physiological importance; like cathepsins B and L, this enzyme may be inhibited more strongly by domain 3, but such data are lacking. Although domain 2 may contribute to kininogen inhibition of cathepsin L, the predominant role of this domain in kininogen function instead appears to be to inhibit calpain [8,29–31].

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