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Alteration of the specificity of ecotin, an *E. coli* serine proteinase inhibitor, by site directed mutagenesis

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Abstract

The gene of ecotin, an *E. coli* proteinase inhibitor, was cloned, and by site-directed mutagenesis the active site residue of the protein, Met^{84}, was mutated to Lys, Arg and Leu. The recombinant wild-type and mutant inhibitors were overexpressed in *E. coli*, purified to homogeneity and their inhibitory effects on trypsin, chymotrypsin and elastase were compared. Of these serine proteinases trypsin is the most strongly inhibited by wild type ecotin and its mutants. According to our results the character of residue 84 of ecotin significantly but not dramatically modifies the specificity of the inhibitor.

Key words: Ecotin; Serine proteinase inhibitor; Site-directed mutagenesis

1. Introduction

The specificity of substrate-like canonical serine proteinase inhibitors, which operate according to the 'standard mechanism', is determined mostly by their P1 residue that fits well into the binding pocket of the cognate proteinase [1–4]. Trypsin inhibitors usually have Lys or Arg; chymotrypsin inhibitors Tyr, Phe, Leu or Met; while elastase inhibitors Leu, Val, Met or Ala as their P1 residue. These inhibitors are quite selective: strong trypsin inhibitors inhibit chymotrypsin or elastase weakly if at all, and vice versa, strong chymotrypsin and elastase inhibitors practically do not inhibit trypsin. On the contrary, ecotin, a 16 kDa serine proteinase inhibitor of *E. coli* has an unusually broad specificity: it is a potent inhibitor of all pancreatic serine proteinases, trypsin, chymotrypsin and elastase but does not inhibit subtilisin and the aspartyl-, sulfhydryl- and metallo-proteinases [5]. The gene of ecotin has been cloned and the reactive site P1 residue has been determined to be Met^{84} [6]. Although ecotin shows a substrate-like inhibition, on the basis of its primary structure it does not belong to the major classes of other serine proteinase inhibitors. McGrath and coworkers [6] have suggested that the broad specificity of ecotin is due to the Met^{84} P1 residue, which is well tolerated by all three pancreatic proteinases. According to earlier studies, replacement of the P1 residue of canonical inhibitors often results in the expected changes in their specificity [7–9]. In order to study the role of the Met^{84} P1 residue in the inhibition and to construct ecotin variants with higher selectivity, the Met^{84} residue was replaced by Arg, Lys and Leu via site-directed mutagenesis. Wild-type and mutant inhibitors were overexpressed in *E. coli*, purified to homogeneity and their inhibitory effects on trypsin, chymotrypsin and elastase were compared.

2. Materials and methods

2.1. Bacterial strains, vectors, enzymes and chemicals

Bovine trypsin (TPCK) and bovine chymotrypsin (TLCK) were purchased from Worthington. Porcine pancreatic elastase was obtained from Sigma. Fractogel TSK-DEAE 650 (S) was purchased from Merck. Affi-Gel 10 was acquired from Bio-Rad. Reagents for oligonucleotide synthesis and protein sequencing were obtained from Applied Biosystems. Ampli-Taq polymerase was purchased from Perkin-Elmer Cetus, T7 sequencing Kit from Pharmacia, other DNA modifying enzymes from Amersham, Boehringer and Promega. GeneClean was acquired from Bio101. *E. coli* strains BL21 (DE3), CJ236 (dut^-,ung^-) and XL1 Blue were purchased from Novagen, Bio-Rad and Stratagene, respectively. Expression vector pT7-7 was provided by Dr. Stan Tabor of Harvard Medical School, Boston. BlueScript vector was obtained from Stratagene. IPTG, *N*-benzoyl-L-arginine-*p*-nitroanilide, *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide, MUB, the fluorescent burst titrants MUGB and MUTMAC [11] were purchased from Sigma. The fluores-

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Abbreviations: PCR, polymerase chain reaction; dut, dUTPase; ung, uracil *N*-glicosylase; TPCK. *N*-tosyl-L-phenylalanine chloromethyl ketone; TLCK, *N*-*p*-tosyl-L-lysine chloromethyl ketone; IPTG, Isopropyl-β-D-thiogalactopyranoside; MUB, 4-methylumbelliferone; MUGB, 4-methylumbelliferyl-*p*-guanidinobenzoate; MUTMAC, 4-methylumbelliferyl-*p*-trimethylammonium cinnamate chloride; AMC, 7-amino-4-methylcoumarin.

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cent turnover substrates *N*-succinyl-Ala-Ala-Pro-Arg-AMC and *N*-succinyl-Ala-Ala-Pro-Phe-AMC were synthesized as described previously [12]. All other chemicals were reagent grade or the best commercially available.

2.2. Cloning of the ecotin gene

The standard techniques of molecular cloning were performed essentially as described [13,14]. *E. coli* genomic DNA was prepared as template for polymerase chain reaction. PCR primer oligonucleotides were designed using the sequence of ecotin gene [6]. Primer sequences were:

5′-GCGAGAATTCTACCTGCAGTATTGTTTGCC-3′

corresponding to base 76 to 99, and

5′-ACGAGGATCCTAAGACGGCCAGCGTCGC-3′

corresponding to base 639 to 656. These two oligonucleotides allowed us to amplify the DNA coding ecotin, including its signal peptide. The signal peptide was slightly modified for correct frame fitting to the expression vector but this did not seem to affect the transport processes. *Eco*RI (first) and *Bam*HI (second) restriction sites are underlined. Primers at the 5′ ends were extended by four additional nucleotides for efficient cutting by the restriction enzymes. 20 ng of template DNA, 100 pmol of each primer, 100 nmol of dNTP-s and 1 U of Taq polymerase were in 50 μl final volume. 30 PCR cycles were carried out as follows: denaturation for 50 s at 94°C, annealing for 90 s at 60°C and extension for 60 s at 72°C. An aliquot was run on a 0.8% agarose gel. Amplified DNA was collected and purified by chloroform extraction and ethanol precipitation, digested with *Eco*RI and *Bam*HI. The sample was loaded on a 0.8% agarose gel and the 580 base DNA fragment was isolated from the gel by GeneClean. The isolated fragment was ligated into the precut pT7-7 vector. The gene was sequenced, and found to be identical with the previously published result [6]. IPTG induced overexpression was verified by electrophoresis on SDS-PAGE [15].

2.3. Site-directed mutagenesis

The oligonucleotides for mutagenesis were:

1. 5′-CCCCGGTGTCGACGCGGATGGCCT-3′
2. 5′-CCCCGGTGTCGACGAAGATGGCCT-3′
3. 5′-CCCCGGTGTCGACGCTGATGGCCT-3′

In place of the Met^{84} codon (ATG) Arg (CGG), Lys (AAG) and Leu codons (CTG) were synthesized (bold letters). An extra silent mutation was designed in order to form a new *Sal*I restriction site (underlined) to facilitate screening. The fragment containing the ecotin gene was cut out from pT7-7 vector with *Eco*RI and *Hin*dIII, and ligated into the BlueScript vector. Uracil containing template DNA for in vitro mutagenesis was prepared by using CJ236 (dut^-,ung^-) *E. coli* strain, and the mutated strand was selected in XL1 Blue (dut^+,ung^+) strain as described by Kunkel [16]. Mutant genes were ligated back into the pT7-7 expression vector and were sequenced before use.

2.4. Protein purification

E. coli BL21(DE3) culture carrying the pT7-7 vector was grown in LB medium containing ampicillin. When the culture reached A_{600} 0.8 it was induced with 0.5 mM IPTG. Cells were harvested 8 h after induction. Recombinant wild-type and mutant ecotins were purified from *E. coli* as described previously [6] with some modifications. After isolation of the periplasmic protein fraction and intensive dialysis against 1 mM HCl, precipitated proteins were removed by centrifugation (20,000 × *g* for 10 min) and the pH was adjusted to 8.0 with Tris base. The sample was centrifuged as before and the supernatant was loaded onto a TSK-DEAE 650 (S) column (1.0 cm × 15 cm) equilibrated with 10 mM Tris-HCl pH 8.0, 1 mM $MgCl_2$ buffer. The flow-through fraction was collected and the pH was adjusted to 4.5 with 1 N HCl. This sample was loaded onto an Affi-Gel 10 trypsin affinity column. Ecotin was eluted with 50 mM HCl and on the basis of SDS-PAGE and native nondenaturing PAGE it was proved to be homogenous.

2.5. Enzyme and inhibitor standardization

Trypsin and chymotrypsin stock solutions were made by dissolving protein in 1 mM HCl containing 20 mM $CaCl_2$. Elastase stock solution was made in acetate buffer pH 4.5 containing 20 mM $CaCl_2$. Concentration of trypsin and chymotrypsin was determined by active-site titration [11]. Wild-type and mutant ecotin samples were standardized by titration with trypsin at 10^{-7} M final concentration using 10^{-4} M *N*-benzoyl-L-arginine-*p*-nitroanilide. The wild-type inhibitor was then used for titration of elastase using 10^{-4} M *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide as substrate.

2.6. Measurement of equilibrium dissociation constant (K_i)

K_i values were determined by the method of Green and Work [17] as modified by Empie and Laskowski [18]. A constant amount of enzyme was incubated with incremental amounts of inhibitor. After reaching equilibrium the quantity of the free enzyme was determined with the appropriate substrate. All measurements were carried out in 50 mM Tris-HCl, 20 mM $CaCl_2$ and 0.005% (w/v) Triton X-100, pH 8.3. Substrates (10^{-4} M) were: *N*-succinyl-Ala-Ala-Pro-Arg-AMC for trypsin, *N*-succinyl-Ala-Ala-Pro-Phe-AMC for chymotrypsin and *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide for elastase. The production of AMC was measured at excitation and emission wavelengths 366 and 440 nm, respectively, by a SPEX FluoroMax spectrofluorometer. The production of *p*-nitroaniline was measured at a wavelength of 410 nm as a function of time by a Shimadzu UV-2101PC spectrophotometer.

Initial elastase and chymotrypsin concentrations were within a factor of 10 of the K_i. When the equilibrium dissociation of the trypsin-inhibitor complexes was studied, however, no reliable enzyme assay was feasible at an initial trypsin concentration lower than 10^{-11} M. The free trypsin concentrations in mixtures with equimolar amounts of ecotin mutants Met^{84}Leu and Met^{84}Arg were 2.3×10^{-12} M and 9.5×10^{-13} M, respectively. These concentrations allowed us to determine fairly accurate K_i values for these complexes. On the other hand, the concentrations of the remaining free enzyme in the identically prepared equilibrium mixtures with wild-type ecotin and ecotin mutant Met^{84}Lys were lower than 5×10^{-13} M, too low and variable values to determine accurate K_i values for these complexes. Thus, on the basis of these assay results we can at least estimate the K_i values of wild type ecotin and mutant Met^{84}Lys to be comparable and lower than 10^{-13} M.

3. Results and discussion

The Met^{84} active site residue of ecotin was replaced with Arg, Lys and Leu residues. Wild type and mutant

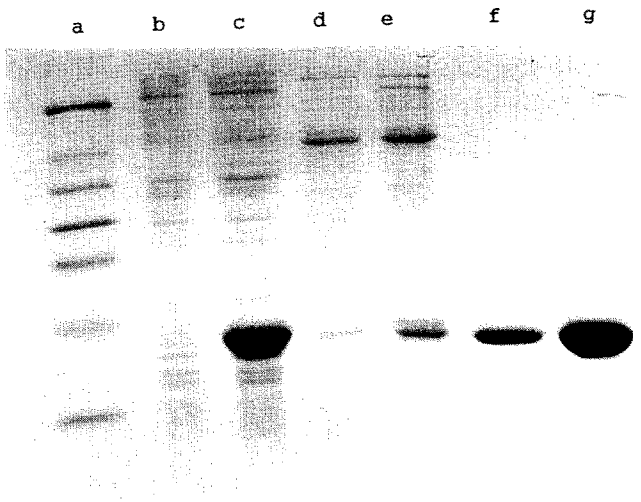

Fig. 1. Purification of ecotin from *E. coli*. 1–50 μg protein samples were subjected to 15% SDS-PAGE. Molecular weight standard (a), *E. coli* homogenate before (b), and after induction (c), periplasmic fraction before (d), and after induction (e), ecotin purified on a TSK-DEAE column (f), ecotin further purified by affinity chromatography.

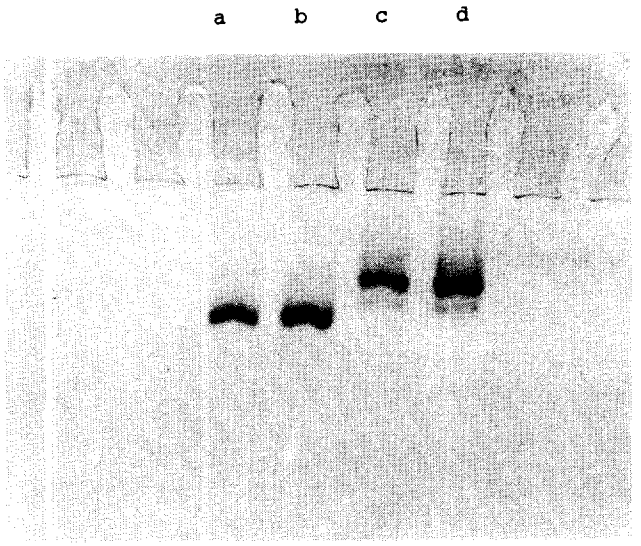

Fig. 2. Native gel electrophoresis of wild-type (Met^{84}) ecotin (a) and mutants, Met^{84}Leu (b), Met^{84}Lys (c) and Met^{84}Arg (d). Protein content of samples was 5–6 μg.

ecotin genes were inserted into the pT7-7 vector. These constructs result in the generation of high levels of ecotin (Fig. 1). The slightly altered signal peptide of ecotin directs the gene product into the periplasmic space of *E. coli*. The yield of production is about 50 mg ecotin/5 g of transformed cells that is about 500 times higher than that of the nontransformed cells. The last purification step, affinity chromatography yielded pure ecotin. Gel electrophoretic patterns (Fig. 2) show that the Met^{84}Lys and Met^{84}Arg mutants of ecotin carry an extra positive charge as compared to the wild type and the Met^{84}Leu mutant ecotin.

For the first time we have determined the K_i values of ecotin on the three pancreatic proteolytic enzymes, trypsin, chymotrypsin and elastase, known to be potently inhibited by ecotin (Table 1 and Fig. 3). Of the three serine proteinases tested, trypsin is the most strongly inhibited ($K_i < 10^{-13}$). The inhibitory effect of ecotin on chymotrypsin is about two, and on elastase is about five orders of magnitude smaller. Since canonical serine proteinase inhibitors with Met P1 residue are usually specific for chymotrypsin and/or elastase, and not for trypsin, the above finding is quite unexpected. Our results suggest that binding sites of ecotin other than the P1 site fit most

Table 1
Equilibrium dissociation constant (K_i) values of wild-type and mutant ecotin variants on trypsin, chymotrypsin and elastase

K_i (M)	Trypsin	Chymotrypsin	Elastase
Met^{84} (wild type)	$< 10^{-13}$	4.0×10^{-12}	1.3×10^{-9}
Met^{84}Leu	6.5×10^{-13}	3.6×10^{-12}	2.3×10^{-9}
Met^{84}Lys	$< 10^{-13}$	4.7×10^{-11}	0.7×10^{-7}
Met^{84}Arg	1.0×10^{-13}	3.9×10^{-11}	1.0×10^{-7}

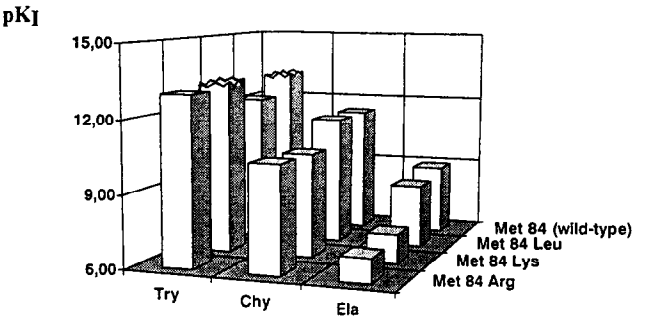

Fig. 3. Comparison of the pK_i ($-\log K_i$) values of wild-type (Met^{84}) ecotin and its mutants on trypsin (Try), chymotrypsin (Chy) and elastase (Ela).

tightly to trypsin, moderately to chymotrypsin, and least to elastase.

The Met^{84}Leu mutation of ecotin does not affect its inhibitory property on chymotrypsin and elastase but results in an increase of K_i on trypsin. Replacement of Met^{84} with the basic amino acid residues, Lys and Arg causes a significant decrease of inhibition of both chymotrypsin (one order of magnitude) and elastase (two orders of magnitude). While our equilibrium dissociation assay did not allow us to distinguish between the extremely strong inhibitory effects of wild type ecotin and mutant Met^{84}Lys on trypsin, ecotin mutant Met^{84}Arg is a less potent trypsin inhibitor than the former ones.

In general, our data show that in case of ecotin the contribution of the P1 residue to the inhibition is relatively small, suggesting that residues other than the P1 site are intimately involved in binding. Thus ecotin seems to have a more extended binding site than the other canonical inhibitors.

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