FEBS Letters 353 (1994) 312–314

FEBS 14679

Inactivation of *Acacia confusa* trypsin inhibitor by site-specific mutagenesis

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Received 21 July 1994; revised version received 20 September 1994

Abstract Native *Acacia confusa* trypsin inhibitor (ACTI) contains two disulphide bonds; one is an intrachain disulphide bond (Cys^{40}–Cys^{86}), located in the A-chain, while the other is an interchain disulphide bond (Cys^{133}–Cys^{141}) connecting the A- and B-chain; the inhibitor consists of 175 amino acid residues, 136 residues in the A-chain and 39 residues in the B-chain. The putative reactive site of ACTI is located at Lys^{64}, while for all other Kunitz family trypsin inhibitors it is at Arg^{64}. When the Lys^{64} residue of ACTI was converted into Ile or Arg by site-specific mutagenesis, the K64I mutant completely lost its inhibitory activity but the K64R mutant retained most of its inhibitory activity. The C133G mutant lost its inhibitory activity while the C40G mutant did not. This suggests that the interchain disulphide bond (Cys^{133}–Cys^{141}) linking two β-strands of the six-strand β-barrel is essential for ACTI inhibitory activity, while the intrachain disulphide bond (Cys^{40}–Cys^{86}) connecting the two loops is non-essential.

Key words: Kunitz type trypsin inhibitor; Disulphide bond; Inhibitor active site

1. Introduction

Acacia confusa trypsin inhibitor (ACTI) is a Kunitz family two-chain trypsin inhibitor with a M_r of 194 kDa, having 175 amino acid residues and consisting of A- and B-chains of 136 and 39 residues, respectively [1]. The primary structure of ACTI has been elucidated [2], and has a high degree of homology to the other Kunitz family trypsin inhibitors from soybean [3], winged bean seeds [4], and *Erythrina latissima* seeds [5]. The nucleotide sequence of the cDNA for ACTI has been determined, and an extra amino acid residue, serine, was found at the junction of the A- and B-chain, which was removed by post-translational processing with specific protease(s) [6]. The reACTI, a single polypeptide chain, without post-translational processing by proteolysis is biologically active [7]. ACTI has Lys^{64} located at its reactive site, and there are two disulphide bonds, the interchain disulphide bond Cys^{133}–Cys^{141}, linking two β-strands, and the interchain disulphide bond, Cys^{40}–Cys^{86}. The ACTI cDNA has been cloned and expressed in *E. coli* and it was found that reACTI is a single polypeptide chain with a strong inhibitory effect on trypsin activity without post-translational proteolysis [7].

In the present study we used site-specific mutagenesis and cDNA expression in *E. coli* to characterize the biological properties of several ACTI mutants.

2. Materials and methods

2.1. Materials

Restriction enzymes and other reagents used in molecular biology techniques were purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden) or Promega (Madison, WI, USA). The pBS(+) plasmid was obtained from Stratagene (La Jolla, CA, USA). All other chemicals used were of analytical grade.

2.2. Site-specific mutagenesis

The plasmid containing the cDNA insert of ACTI, pBSACTI (wild-type) [7], was modified by site-specific mutagenesis, and then by PCR to generate mutated cDNA. The oligonucleotide primers synthesized to have the first 8 N-terminal amino acids of the ACTI A-chain with a *Bam*HI restriction site (primer A), the last 8 C-terminal amino acids of the ACTI B-chain with a stop codon (complement of UAA) and an *Eco*RI restriction site (primer B), and DNA fragments containing the amino acid conversions $Lys^{64} \rightarrow$ Ile, $Lys^{64} \rightarrow$ Arg, $Cys^{40} \rightarrow$ Gly and $Cys^{133} \rightarrow$ Gly (primers C, D, E and F, encoding the 6 amino acids of mutants containing a site-specific mutation, K64I, K64R, C40G and C133G, respectively), were as follows:

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Primer A 5'-AGGATCCAAAGAACTTCTGGACGCAGACGGG-3'
Primer B 5'-CTTTTCCGGGTAGCCAGTCCCATTCTTAAGG-3'
Primer C 5'-CCACCTATAATTGCAATT-3'
Primer D 5'-CCACCTAGAATTGCAATT-3'
Primer E 5'-CGAAAGCGGCCCTCTC-3'
Primer F 5'-TGTTTACGGTGAAGGT-3'
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All mutants were confirmed by sequencing the ligation products of Bluescript plasmids containing the mutated cDNA by using the dideoxynucleotide chain-termination method [8] with T7 DNA polymerase.

2.3. Construction of expression plasmids

The primers were used for PCR on the Bluescript plasmids (mutants) to generate ACTI-encoding DNA fragments for subcloning. DNA fragments encoding 176 amino acids of ACTI (wild-type and mutants) flanked by *Eco*RI and *Bam*HI sites, were ligated into pGEX-2T with T4 DNA ligase. The desired expression vector was identified by colony hybridization and the orientation of each insert was confirmed by nucleotide sequencing. These expression plasmids were designated pGAEX-2T (wild-type), pGM1EX-2T(K64I), pGM2EX-2T(K64R), pGM3EX-2T(C40G) and pGM4EX-2T(C133G), respectively.

2.4. Transformation and purification of GST fusion proteins

The *E. coli* TG1 cells were transfected with wild-type and mutant plasmids by $CaCl_2$-mediated transformation. The culture cells at a cell density of 4×10^8 cells/ml were induced with 0.5 mM isopropyl β-D-thiogalactopyranoside for 3 h and lysed on ice by mild sonication after addition of Triton X-100 to 1%. The fusion proteins were purified by affinity chromatography with a glutathione-Sepharose 4B column [1,9,10]. The purified fusion proteins were cleaved from the GST leader peptide with thrombin (1:50 w/w) and the reaction products were purified with a FPLC Mono Q column (1.6 × 50 mm) which was pre-equilibrated with 50 mM Tris-HCl buffer (pH 8.0) and then eluted with a 0–0.3 M NaCl gradient in the same buffer.

2.5. Analysis of expressed proteins

The purified reACTI (wild-type), K64I mutant, K64R mutant, C40G mutant and C133G mutant were subjected to SDS-PAGE [11]. The N-terminal amino acid sequence of reACTI was analyzed directly with an ABI 477A sequencer and ABI 120A analyzer using about 1 nmol

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Abbreviations: ACTI, *Acacia confusa* trypsin inhibitor; L-BAPNA, N^{α}-benzoyl-L-arginine-4-nitro-anilide hydrochloride; BTEE, *N*-benzoyl-L-tyrosine ethyl ester; re, recombinant; GST, glutathione-*S*-transferase; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; M_r, relative molecular mass.

SSDI 0014-5793(94)01066-8

of reACTI. The protein was quantitatively determined by the method of Lowry [12].

2.6. Inhibition of trypsin

The trypsin inhibitory activity of mutants and wild-type ACTI was measured by incubating the recombinant ACTIs with trypsin in 1 ml of 0.1 M Tris-HCl buffer, pH 8.0, containing 0.01 M $CaCl_2$ for 5 min at 37°C. Residual trypsin activity was determined by adding 10 μl N^{α}-benzoyl-L-arginine-4-nitro-anilide hydrochloride (L-BAPNA) (50 mg/ml in DMSO) at 37°C. After 20 min incubation, the reaction was stopped by adding 0.5 ml 10% acetic acid. The degree of inhibition was determined by measuring the optical density at 410 nm.

3. Results

3.1. Construction of mutant ACTI cDNA

The primary structure of ACTI was elucidated recently. It contains two disulphide bonds, Cys^{40}–Cys^{86} and Cys^{133}–Cys^{141}, and the putative reactive site of ACTI was found to be Lys^{64} [3]. Two mutant plasmids, pGM1EX-2T to produce mutant K64I mutant, and pGM2EX-2T to produce mutant K64R mutant, were prepared to study the effects of these mutations on trypsin inhibitory activity. Two other mutant plasmids, pGM3EX-2T for the C40G mutant, and pGM4EX-2T for the C133G mutant, were also constructed to investigate the role of the disulphide bond on trypsin inhibitory activity. The mutations of ACTI by site-specific mutagenesis were confirmed by nucleotide sequence analysis of mutant plasmids.

3.2. Purification of mutant ACTIs

The reGST-ACTI fusion proteins were obtained by the expression of mutant plasmids in *E. coli* TG1 cells. The fusion proteins were purified from the *E. coli* lysate by affinity chromatography with a glutathione-Sepharose 4B column. The purified fusion proteins were then treated with thrombin and followed by purifying with a Mono Q column. Two peaks were obtained and the first peak eluted with 0.06 M NaCl was identified as reACTI. The homogeneity of purified mutant reACTIs was analyzed by SDS-PAGE and the results are summarized in Fig. 1. The N-terminal amino acid sequence of mutant reACTIs was determined with an automated sequencer, and the results indicated that mutant reACTIs have the same N-terminal amino acid sequence (about 18 amino acid residues) as that of native ACTI (data not shown). The K64I, K64R and C40G mutants had the same electrophoretic mobility as that of native ACTI, while the C133G mutant exhibited slower mobility than that of reduced reACTI.

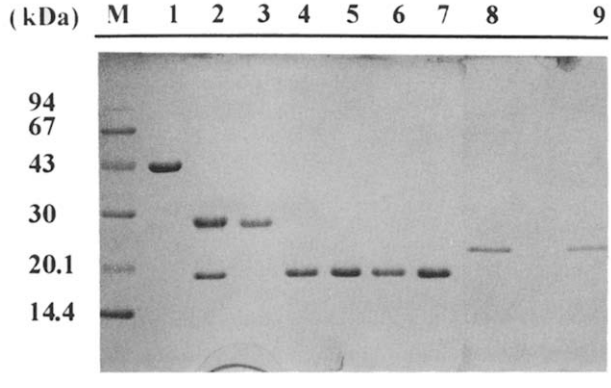

Fig. 1. SDS-PAGE in 12% polyacrylamide showing the expression and purification of reACTI and its mutants. Lane 1–8 were run without reduction. Lane 1, the fusion protein, reGST-ACTI, was purified on a glutathione-Sepharose 4B column. Lane 2, reGST-ACTI was endoproteolytically cleaved with thrombin. Lane 3, purified reGST. Lane 4, purified reACTI (wild-type). Lane 5, purified K64I mutant. Lane 6, purified K64R mutant. Lane 7, purified C40G mutant. Lane 8, purified C133G mutant. Lane 9, reACTI treated with 5% β-mercaptoethanol in 50 mM Tris-HCl buffer, pH 8.0, at 37°C for 1 h.

3.3. Trypsin inhibitory activity of mutant ACTIs

The IC_{50} of native and reACTI trypsin inhibitory activity was shown to be identical. The effects of various missense mutants of ACTI on the trypsin inhibitory activity were shown in Fig. 2. The results showed that the single amino acid change of the active site, $Lys^{64} \rightarrow Arg$, does not significantly affect the inhibitory activity of the K64R mutant, while the substitution $Lys^{64} \rightarrow Ile$, the K64I mutant, almost abolishes the trypsin inhibitory activity. No detectable inhibitory activity was found in the C133G mutant, and no significant inhibitory activity change was observed in the C40G mutant. The K_i values with trypsin of the mutants, K64R, C40G, C133G, K64I, and wild-type reACTI were determined to be 1.024, 0.934, 29793, 27432 and 0.274 nM, respectively.

4. Discussion

The primary structures of many Kunitz family trypsin inhibitors have been elucidated, and most of them have an Arg residue at the reactive site [4,5,13]. However, ACTI has Lys^{64} at its putative reactive site. Site-specific mutagenesis was carried out to confirm the reactive site of ACTI. The $Lys^{64} \rightarrow Ile$ substitution resulted in the complete loss of trypsin inhibitory activity, while with the conservative $Lys^{64} \rightarrow Arg$ substitution there was no significant change in inhibitory activity. This suggests that either Arg or Lys at the reactive site is essential for the Kunitz family trypsin inhibitor activity.

There are two disulphide bonds Cys^{40}–Cys^{86} and Cys^{133}–Cys^{141}, in ACTI, which are conserved in soybean trypsin inhibitor [13], winged bean trypsin inhibitor [4,14], *Erythrinus caffra* trypsin inhibitor [15], and *Adenanthera pavonina* trypsin inhibitor [16]. A $Cys \rightarrow Gly$ exchange by site-specific mutagenesis causes the disruption of the disulphide bond of the mutant protein: thus it may induce an unfolding or a conformational change of protein structure and affect its biological activity. The single amino acid substitution $Cys^{133} \rightarrow Gly$ resulted in the complete loss of inhibitory activity, while the $Cys^{40} \rightarrow Gly$ substitution did not. This suggests that the disulphide bond Cys^{133}–Cys^{141} is essential for ACTI inhibitory activity, but that Cys^{40}–Cys^{86} is not. The conformational change in mutant C133G is also apparent from its electrophoretic mobility, since the mutant C133G loses its interchain disulphide bond.

It was shown previously that the single chain of reACTI is as biologically active as the two-chain native ACTH [7]. Disruption of the interchain disulphide bond (Cys^{133}–Cys^{141}) causes the dissociation of two polypeptide chains of native ACTI but not of reACTI since reACTI is a single polypeptide chain without post-translational processing by proteolysis. This suggests that there is refolding of the molecule and functioning of native disulphide bonds. For the C133G mutant ($Cys^{133} \rightarrow Gly$), non-native intramolecular disulphide bonds Cys^{141}–Cys^{40} and Cys^{141}–Cys^{86} may have formed [17,18].

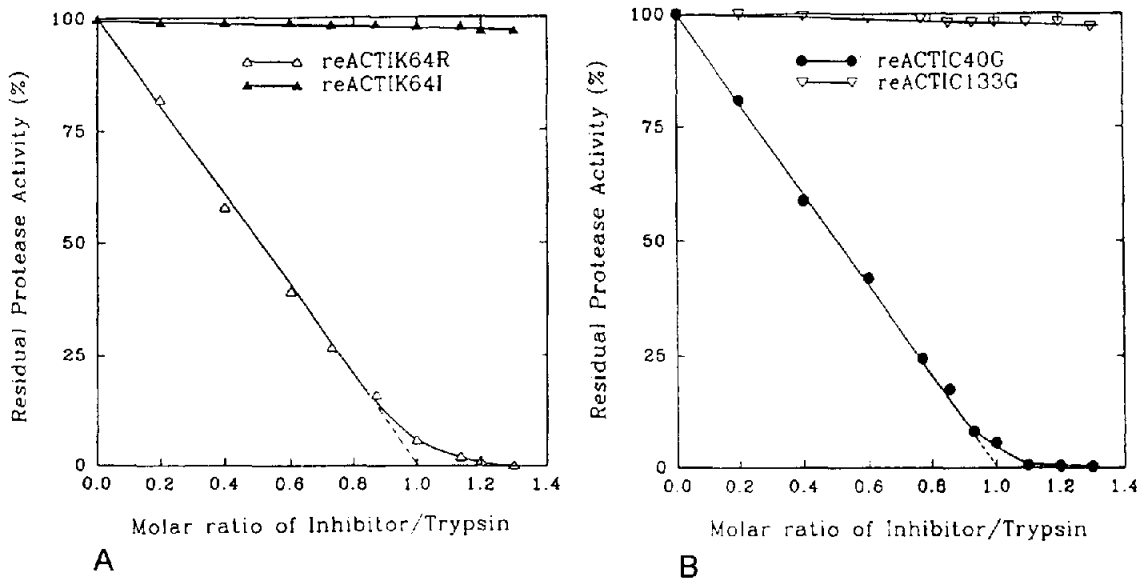

Fig. 2. Trypsin inhibitory activity of ACTI, reACTI and mutant proteins. (A) The effects of mutants K64I and K64R on trypsin activity. (B) The effects of mutants C40G and C133G on trypsin activity.

However, the similar mobility between non-reduced C133G mutant and reduced native-like reACTI observed in SDS-PAGE strongly suggests that the C133G mutant has no disulphide bonds at all, forming a non-native and inactive molecule. For the C40G mutant ($Cys^{40} \rightarrow Gly$), one may expect non-native intramolecular disulphide bonds Cys^{86}–Cys^{133} and Cys^{86}–Cys^{141} to have formed. However, the similar trypsin inhibitory activity and mobility in SDS-PAGE of the C40G mutant and reACTI suggests that proper refolding of the C40G mutant and the formation of the disulphide bond, Cys^{133}–Cys^{141}, takes place.

The X-ray crystallography of two Kunitz family trypsin inhibitors from *Erythrina caffra* seeds [19] and soybean [20,21] were elucidated. Both trypsin inhibitors have 12 antiparallel β-strands joined by several loops. The inhibitor shows approximately 3-fold symmetry about the axis of the barrels formed by β-strands. The amino acid sequence of ACTI shows a high degree of homology to those of the Kunitz family trypsin inhibitors isolated from soybean and *Erythrina caffra* seed [2]. Accordingly, the two disulphide bonds in the reACTI molecule are the disulphide bond (Cys^{40}–Cys^{86}) linking two loops and the disulphide bond (Cys^{133}–Cys^{141}) connecting two β-strands of the six strand β-barrel according to the 3D structure of soybean or *Erythrina caffra* trypsin inhibitors. The C133G mutant lost its inhibitory activity, strongly suggesting that the linkage between the two antiparallel β-strands (C1 and C2 designated by Onesti et al. [19], for *Erythrina caffra* trypsin inhibitor) is essential for maintaining the tertiary structure of ACTI and its trypsin inhibitory activity. When the intrachain disulphide bond Cys^{40}–Cys^{86} was split, the C40G mutant conserved its inhibitory activity, suggesting that the linkage between the two loops was not important for maintaining its intact structure and inhibitory activity.

Acknowledgements: This work was supported in part by Grant NSC 82-0412-B002-214 from National Science Council, ROC.

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