Biochimica et Biophysica Acta 1830 (2013) 4790–4799

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Conformational mobility of active and E-64-inhibited actinidin

Milica M. Grozdanović [a], Branko J. Drakulić [b], Marija Gavrović-Jankulović [a,*]

[a] Faculty of Chemistry University of Belgrade, Department of Biochemistry, Studentski trg 16, 11000 Belgrade, Serbia
[b] Department of Chemistry-IChTM, University of Belgrade, Njegoševa 12, 11000 Belgrade, Serbia

ARTICLE INFO

Article history:
Received 25 February 2013
Received in revised form 12 June 2013
Accepted 13 June 2013
Available online 23 June 2013

Keywords:
Actinidin
E-64
Molecular dynamics
Cysteine protease

ABSTRACT

Background: Actinidin, a protease from kiwifruit, belongs to the C1 family of cysteine proteases. Cysteine proteases were found to be involved in many disease states and are valid therapeutic targets. Actinidin has a wide pH activity range and wide substrate specificity, which makes it a good model system for studying enzyme–substrate interactions.
Methods: The influence of inhibitor (E-64) binding on the conformation of actinidin was examined by 2D PAGE, circular dichroism (CD) spectroscopy, hydrophobic ligand binding assay, and molecular dynamics simulations.
Results: Significant differences were observed in electrophoretic mobility of proteolytically active and E-64-inhibited actinidin. CD spectrometry and hydrophobic ligand binding assay revealed a difference in conformation between active and inhibited actinidin. Molecular dynamics simulations showed that a loop defined by amino-acid residues 88–104 had greater conformational mobility in the inhibited enzyme than in the active one. During MD simulations, the covalently bound inhibitor was found to change its conformation from extended to folded, with the guanidino moiety approaching the carboxylate.
Conclusions: Conformational mobility of actinidin changes upon binding of the inhibitor, leading to a sequence of events that enables water and ions to protrude into a newly formed cavity of the inhibited enzyme. Drastic conformational mobility of E-64, a common inhibitor of cysteine proteases found in many crystal structures stored in PDB, was also observed.
General significance: The analysis of structural changes which occur upon binding of an inhibitor to a cysteine protease provides a valuable starting point for the future design of therapeutic agents.

1. Introduction

Cysteine proteases exert important roles in many biochemical processes and have been implicated in the development and progression of various disease states, such as cardiovascular [1,2], pulmonary [3], and inherited genetic diseases [4], as well as cancer [5]. In addition, cysteine proteases have been considered as potential targets for antiviral [6] and antimalarial therapies [7]. In order to develop novel compounds which would act against these biochemical targets, it is necessary to perform their detailed structural and biochemical characterization.

Cysteine proteases occur ubiquitously in living organisms and the MEROPS database lists 91 families of cysteine proteases, which have been further grouped into 9 clans. The papain-like cysteine proteases, classified as the "C1 family", are the most predominant cysteine proteases and include the mammalian cathepsins [8].

Actinidin (EC 3.4.22.14) is a C1 family cysteine protease from kiwifruit which shows sequence homology with cysteine proteases such as papain, chymopapain, ficin, and stem and fruit bromelain. It is the most abundant protein of kiwifruit (*Actinidia deliciosa*), accumulated to very high concentrations in the fruit, where it constitutes up to 60% of soluble protein [9]. Actinidin has a wide pH activity range (4–10) and wide substrate specificity, which makes it a suitable model for studying enzyme–substrate interactions [10].

Actinidin is encoded as a pre-proprotein, possessing an N-terminal signal sequence, as well as C- and N-terminal propeptides. It is synthesized as a zymogen and later processed upon secretion or sequestration in the cell [9]. The mature form of actinidin consists of 220 amino acid residues, including seven cysteines, out of which six are involved in the formation of three disulphide bridges, while the seventh is located inside the active site [11]. The polypeptide chain folds into two domains. The domain that contains residues 19–115 and 214–218 is mainly comprised of α-helices, and the domain that contains residues 1–18 and 116–213 is organized into regions of twisted β-sheets [11–14]. The protein is folded in such a manner that a cleft is settled between the domains (Fig. 1) and the N- and C-terminal ends cross over from the first domain into the second, and vice versa, acting as "belts" which stabilize the tertiary structure of the enzyme. Amino-acid residues CYS25 and HIS162 (papain numbering) located at both sides of the interdomain cleft constitute the catalytic ion pair [11,12].

Actinidin can be irreversibly inhibited by E-64 (1-(L-*trans*-epoxysuccinylleucylamino)-4-guanidinobutane), a specific cysteine protease inhibitor. E-64 is a Michael acceptor, acting as an epoxy amide. The

* Corresponding author at: Studentski trg 16, 11000 Belgrade, Serbia. Tel.: +381 11 3336 661; fax: +381 11 2184 330.
E-mail address: mgavrov@chem.bg.ac.rs (M. Gavrović-Jankulović).

0304-4165/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbagen.2013.06.015

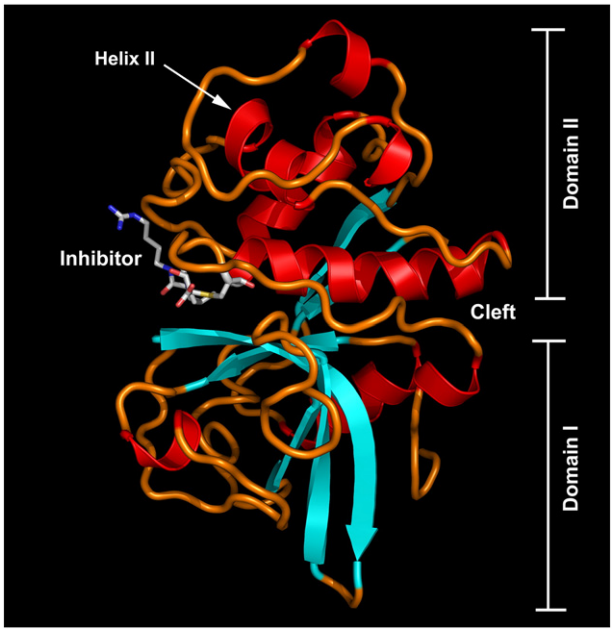

Fig. 1. Structure of actinidin and nomenclature of the domains and helices (depicted from PDB ID: 1AEC).

actinidin–E-64 complex is formed through a covalent linkage between the sulfur of the active-site cysteine 25 and the carbon atom of the oxiran moiety in position α to the carboxyl group of the inhibitor (Fig. S1 in Supplementary material). The alkylation of the active-site cysteine 25 by E-64 and similar inhibitors has been extensively studied on the quantum-mechanical level and by hybrid methods [15]. However, reports on the molecular dynamics simulation of cysteine proteases covalently inhibited with E-64, or similar inhibitors, are scarce. Bhattacharya et al. [16] used a modeled actinidin–E-64-c[1] complex, and reported 100 ps of productive run. In their simulation only the inhibitor, the amino-acid residues, and the water in a 12 Å sphere around the inhibitor were allowed to move. They observed no significant changes in the conformation of the inhibitor. Yamamoto et al. [17] reported molecular dynamics simulations of modeled papain–E-64 complexes, using ligands with both *R*- and *S*-configuration of the C2 atom covalently bound to sulfur of CYS25. Molecular dynamics simulations were carried out during 10 ps, with all of the atoms out a sphere of 10 Å around the inhibitor being fixed. The authors observed less conformational changes of the amino-acid residues in the active site of the enzyme when the C alpha atom of the inhibitor adopted an *R*-configuration than for the *S*-counterpart. Again, significant conformational mobility of the inhibitor was not reported. Both studies aimed to explain hydrogen bond networks that stabilize the inhibitor in the active site, and the short simulation times should be ascribed to limited computational power available ten or twenty years ago. Recently, fast conformational changes in a glycine rich loop of *Streptococcus pyogenes* cysteine protease SpeB were experimentally observed near the place of E-64 binding [18]. Actinidin and SpeB are two distinct enzymes; nevertheless, this shows that the covalent binding of E-64 could trigger significant local conformational changes of an enzyme.

It is commonly regarded that, under the appropriate conditions, all polypeptides bind SDS in a constant weight ratio (1.4 g SDS per gram of polypeptide) and migrate solely on the basis of their mass and not of their net charge or structure [19]. However, there have been numerous reports on the appearance of higher bands for monomeric proteins in non-reducing SDS-PAGE than in reducing conditions, and this has generally been attributed to the existence of nonlinear species due to unbroken intrachain disulfide bonds [20–22]. The molecular mass of actinidin has been determined as approximately 23.5 kDa by amino-acid sequencing and mass spectrometry analysis [23,24]. However, there have been reports of varying electrophoretic mobility for actinidin in reducing SDS-PAGE, with a range of molecular weights between 22 and 30 kDa being described [25–27]. In a previous study the connection between the anomalous behavior of actinidin in reducing SDS-PAGE and the activity of the enzyme was identified [24]. Actinidin inhibited by thermal treatment, or by a cocktail of protease inhibitors, traveled as a higher band of approximately 30 kDa, while the active enzyme revealed a molecular weight of 22 kDa in reducing SDS-PAGE.

In this study, it was demonstrated by isoelectric focusing and 2D PAGE that the net charge of proteolytically active and E-64 inhibited actinidin remains the same, yet these two forms have different gel migration rates. Further on, based on results from CD spectroscopy and hydrophobic ligand binding experiments, it was proposed that this anomalous behavior is due to conformational changes that occur upon binding of the inhibitor. Results of the molecular dynamics (MD) simulations indeed show that significant differences in conformational mobility exist between the inhibited and uninhibited enzyme. In addition, significant conformational mobility of the inhibitor molecule was also observed. Simulations revealed that the folding of the inhibitor molecule probably triggered conformational mobility of the inhibited enzyme. Similar conformational mobility was not observed in the active enzyme. To the best of our knowledge, no similar reports on differences that occur in electrophoretic and conformational mobility upon binding of an inhibitor to a cysteine protease can be found in literature.

2. Material and methods

2.1. Purification of actinidin

Actinidin was isolated from 200 g of fresh kiwifruit (*A. deliciosa*, Hayward cv) bought at a local market. A total extract of soluble proteins was prepared by homogenizing the pulp of peeled kiwifruit in 400 mL of 50 mM sodium-citrate buffer, pH 4.5, followed by extraction of the proteins for 2 h at 4 °C. The obtained extract was centrifuged (3500 ×*g*, 30 min) and dialyzed overnight against the extraction buffer. The extract was applied onto an SP-Sephadex C-50 column (150 mm × 27 mm) (GE healthcare, Uppsala Sweden) pre-equilibrated in the extraction buffer. The unbound fraction (400 mL) was dialyzed for 48 h against a 50 mM Tris–HCl buffer, pH 8.0, with several buffer changes. Following this, the sample was applied onto a QAE-Sephadex A-50 ion exchange column (100 mm × 27 mm) pre-equilibrated with 50 mM Tris–HCl buffer, pH 8.0, and the column was subsequently eluted with a salt gradient (0 M to 1 M NaCl). The collected fractions were analyzed by SDS-PAGE, and those containing purified actinidin were pooled and concentrated to a final concentration of 1 mg mL^{-1}, as determined by Bradford assay [28].

2.2. Inhibition of actinidin

For preparation of the E-64–actinidin complex, purified actinidin was mixed with an equimolar amount of E-64 (AppliChem, GmbH, Darmstadt, Germany) and incubated for 45 min. Unbound inhibitor was removed by overnight dialysis.

2.3. Isoelectric focusing and 2D-PAGE zymography

Isoelectric focusing (IEF) was performed in a 5% (w/v) polyacrylamide gel to which 2.4% of ampholytes were added (pH 3.5–10, GE Healthcare), according to the procedure described by Bollag et al. [29]. A volume of 25 μL each of 1 mg mL^{-1} samples of actinidin and E-64–actinidin complex were applied onto the gel in native conditions.

[1] In E-64-c the *n*-butyl-guanidino moiety of E-64 is changed to an *iso*-amyl moiety.