Analytical Biochemistry 505 (2016) 36–42

Contents lists available at ScienceDirect

Analytical Biochemistry

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

Real-time kinetic method to monitor isopeptidase activity of transglutaminase 2 on protein substrate

Kiruphagaran Thangaraju [a], Beáta Biri [b], Gitta Schlosser [c], Bence Kiss [b], László Nyitray [b], László Fésüs [a, d, 1], Róbert Király [a, *, 1]

[a] Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Debrecen, 4012 Debrecen, Hungary
[b] Department of Biochemistry, Eötvös Loránd University, 1117 Budapest, Hungary
[c] MTA–ELTE Research Group of Peptide Chemistry, Hungarian Academy of Sciences–Eötvös Loránd University, 1117 Budapest, Hungary
[d] MTA–DE Stem Cell, Apoptosis, and Genomics Research Group of Hungarian Academy of Sciences, University of Debrecen, 4012 Debrecen, Hungary

ARTICLE INFO

Article history:
Received 5 January 2016
Received in revised form
19 April 2016
Accepted 19 April 2016
Available online 27 April 2016

Keywords:
Transglutaminase 2
S100A4
Isopeptidase activity
Fluorescence anisotropy

ABSTRACT

Transglutaminase 2 (TG2) is a ubiquitously expressed multifunctional protein with Ca^{2+}-dependent transamidase activity forming protease-resistant N^{ε}-(γ-glutamyl) lysine crosslinks between proteins. It can also function as an isopeptidase cleaving the previously formed crosslinks. The biological significance of this activity has not been revealed yet, mainly because of the lack of a protein-based method for its characterization. Here we report the development of a novel kinetic method for measuring isopeptidase activity of human TG2 by monitoring decrease in the fluorescence polarization of a protein substrate previously formed by crosslinking fluorescently labeled glutamine donor FLpepT26 to S100A4 at a specific lysine residue. The developed method could be applied to test mutant enzymes and compounds that influence isopeptidase activity of TG2.

Transglutaminase 2 (TG2, EC 2.3.2.13) is a ubiquitously expressed multifunctional member of transglutaminases having several catalytic activities and involved in protein–protein interactions both intra- and extracellularly [1]. It has been implicated in a variety of biological processes, including cellular differentiation, apoptosis, angiogenesis, and extracellular matrix organization, and has been linked to immunological, fibrotic, cancer, and neurodegenerative disease phenotypes [2,3]. TG2 has various catalytic activities in addition to the well-characterized transamidation that is formation of covalent bonds between protein-bound glutamine and lysine residues or primary amines; it can also work as a GTPase, a protein disulfide isomerase, and a protein kinase under specific conditions [3]. The Ca^{2+}-dependent transglutaminase activity can also mediate deamidation of glutamine residues and hydrolysis of the previously formed N^{ε}-(γ-glutamyl) lysine as well as γ-glutamylamine derivatives (isopeptidase activity); these reaction mechanisms are reviewed in Ref. [4]. The transamidase activity forms a proteinase-resistant isopeptide bond that has structural, functional, and even industrial implications, for example, in clot stabilization by the transglutaminase factor XIIIa [1], formation of cornified envelopes by transglutaminases in the skin [5], crosslinking of extracellular matrix in kidney fibrosis, and food, textile, and leather processing to improve flavor, appearance, and texture [2,6,7].

Until now, only a few reports have been published on the existence and characterization of the isopeptidase activity of TG2. The removal of the previously incorporated monoamines (deaminylation) [8–11] and the isopeptide cleavage between short peptides [12] were demonstrated measuring fluorescence intensity change or using capillary electrophoresis. On a protein level, only factor XIIIa-catalyzed isopeptidase activity has detected what can reverse the incorporation of α2-plasmin inhibitor into fibrin clots potentially regulating the fibrinolytic processes [13,14]. This raised the possibility that isopeptidase activity of TG2 could also play important roles in regulation of biological processes. However, with the lack of a proper and easily accessible assay, the full biological and pathological significance of this activity cannot be revealed.

Abbreviations used: TG2, transglutaminase 2; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; UV, ultraviolet; ESI, electrospray ionization; HPLC, high-performance liquid chromatography; CID, collision-induced dissociation; MS/MS, tandem mass spectrometry; MS, mass spectrometry.

* Corresponding author.
E-mail address: kiralyr@med.unideb.hu (R. Király).

[1] These authors contributed equally to this study.

http://dx.doi.org/10.1016/j.ab.2016.04.012
0003-2697/

A real-time fluorescence polarization assay has been published [15] to measure transamidase activity of TG2 during crosslinking a fluorescently labeled TG2-specific dodecapeptide, FLpepT26, into bovine serum albumin (BSA), resulting in higher anisotropy of the enzymatically crosslinked product. We hypothesized that in the case of a proper lysine donor substrate, after the forward reaction the cleavage of the isopeptide bond by TG2 also leads to anisotropy change that could be monitored using the same biophysical feature used in proteinase and deubiquitinating assays [16,17].

Here we report the development of a kinetic fluorescent polarization-based assay to follow isopeptidase activity of TG2 on a novel crosslinked protein–peptide substrate. By enzymatic crosslinking of the fluorescently labeled FLpepT26 dodecapeptide and S100A4, a recently characterized specific amine donor of TG2 [18], the purified product was subsequently used as a substrate to demonstrate the cleavage of the isopeptide bond and follow this activity by measuring fluorescence polarization in real time.

Materials and methods

Materials

All materials were purchased from Sigma (St. Louis, MO, USA) unless otherwise indicated. The FLpepT26 peptide was obtained as published in Ref. [18]. ZDON was sold by Zedira (Darmstadt, Germany).

Expression and purification of proteins

The Val224 containing recombinant human TG2 (UniProt code: P21980) and its mutants were expressed in N-terminally $(His)_6$-tagged form (pET-30 Ek/LIC-TG2; MW = 82,745 Da) and purified by Ni-NTA affinity chromatography as described previously [11].

N-terminal GST-tagged S100A4 (pETARA–S100A4; UniProt code: P26447; MW = 39,559 Da) was expressed in Rosetta 2 (Novagen, Darmstadt, Germany). The overnight culture was inoculated in 1:20 ratio into LB medium containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol and was grown at 25 °C until the optical density reached 0.6 to 0.8 at 600 nm. The expression was induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 18 °C overnight. Cells were harvested and pellets were dissolved in buffer A (20 mM Tris–HCl [pH 7.2], 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid [EDTA], and 1 mM dithiothreitol [DTT]) containing 1% Triton X-100, 10% glycerol, 1 mM phenylmethanesulfonyl fluoride (PMSF), and protease inhibitor cocktail. After sonication, the supernatant was separated by centrifugation at 20,000 *g* (4 °C, 25 min) and loaded onto glutathione Sepharose 4B resin (GE Healthcare, UK). After extensive washing with buffer A, the S100A4 (GST) protein was eluted using 10 mM reduced glutathione in Tris–HCl (pH 8.0) and dialyzed overnight in buffer A. Protein concentration was determined by the Bradford method (Bio-Rad Protein Assay, Bio-Rad, München, Germany).

Large-scale production of crosslinked FLpepT26–S100A4 (GST)

The mixture of 5 μM FLpepT26, 12.8 μM S100A4 (GST), and 5 nM TG2 was incubated for 1 h in the presence of 5 mM Ca^{2+} in the reaction buffer (20 mM Tris–HCl, pH 7.5) containing 150 mM NaCl, 5 mM DTT, and 0.01% Tween 20. The reaction was stopped by the addition of 10 mM EDTA (final concentration) to prevent unwanted modification of the crosslinked molecules during their separation. FLpepT26–S100A4 (GST) with unmodified S100A4 (GST) was purified from the free unbound FLpepT26 peptide by a centrifugal concentrator filter (Amicon Ultra, 10 kDa, Millipore, Billerica, MA, USA). Then the buffer was replaced by 20 mM Mops buffer (pH 6.8) containing 0.5 mM EDTA, 150 mM NaCl, 5 mM DTT, and 0.01% Tween 20 because isopeptidase activity prefers slightly acidic pH [19]. Due to co-purification of FLpepT26–S100A4 (GST) and S100A4 (GST), their ratio was calculated based on the total protein concentration (determined by Bio-Rad Protein Assay) and its fluorescein content (absorption at 493 nm) using 79,600 M^{-1} cm^{-1} as molar extinction coefficient for fluorescein. In optimized conditions, the FLpepT26–S100A4 (GST) content was approximately 15% as an average in the reaction product, meaning that 5 μg of purified mixture of FLpepT26–S100A4 (GST) and S100A4 (GST) corresponds to 0.5 μM FLpepT26–S100A4 (GST) in 35 μl of the isopeptidase assay.

Preparation of samples for SDS–PAGE analysis

The reaction was stopped by adding 6 × denaturation buffer (375 mM Tris–HCl [pH 6.8], 600 mM DTT, 12% [m/v] sodium dodecyl sulfate [SDS], 60% [v/v] glycerol, and 0.06% [m/v] bromophenol blue), and the samples were boiled for 10 min. SDS–PAGE (polyacrylamide gel electrophoresis) was performed using 15% Tris–glycine gel. The fluorescence was detected immediately by an ultraviolet (UV) gel documentation system (Protein Simple, AlphaImager, HP system).

Mass spectrometric analysis of peptide after isopeptide cleavage

Electrospray ionization (ESI) mass spectrometric measurements were carried out on a Bruker Daltonics Esquire 3000 Plus (Bremen, Germany) ion trap mass spectrometer using online high-performance liquid chromatography (HPLC) coupling. HPLC separation was performed on a Jasco PU-2085 Plus HPLC system using a Supelco Ascentis C18 column (2.1 × 150 mm, 3 μm). Linear gradient elution (0 min 2% B, 3 min 2% B, 27 min 60% B) with eluent A (0.1% HCOOH in water) and eluent B (0.1% HCOOH in acetonitrile/water, 80:20, v/v) was used at a flow rate of 0.2 ml/min at ambient temperature. The HPLC system was directly coupled to the mass spectrometer. Collision-induced dissociation (CID) experiments were used for peptide sequencing.

Kinetic isopeptidase activity measurement

In a 35-μl reaction volume on 384-well untreated Polystyrene Black Microplates (Nunc, Thermo Scientific, Denmark, cat. no. 262260), 0.5 μM of the FLpepT26–S100A4 (GST) crosslinked substrate was tested in 20 mM Mops reaction buffer (pH 6.8) containing 150 mM NaCl, 6 mM glycine methyl ester, 5 mM DTT, 0.1% Tween 20, and various concentrations of TG2. The reaction was started by the addition of 5 mM $CaCl_2$ (5 mM EDTA was used as negative control) and performed at 37 °C, measuring the change in fluorescence polarization (FP) value by a Synergy H1 microplate reader (GreenFP filter cube, excitation 485 nm, emission 528 nm; BioTek, Winooski, VT, USA). The reaction rates were calculated from the initial slopes of the kinetic curves in terms of anisotropy per minute.

Data analysis

Data analysis, curve fitting, and kinetic calculations were performed by GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA) using the appropriate incorporated equations and tools mentioned where appropriate. In the case of Ca^{2+} dependence experiments, the free calcium ion concentrations were calculated using the online version of MaxChelator (WebmaxC Standard, http://www.stanford.edu/~cpatton/maxc.html) due to the EDTA content of the substrate.