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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

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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.

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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.

Results and discussion

Design and production of potential transglutaminase isopeptidase substrate for anisotropy assay

To develop a peptide–protein-based isopeptidase assay, the first step was to select proper substrate components for the production of a substance that contains only one N^{ε}-(γ-glutamyl)lysine isopeptide bond and the substrates have a significant difference in their size to provide sufficient signal for the detection of anisotropy change. In previous publications, fluorescein-labeled FLpepT26 peptide was an effective TG2-specific amine acceptor substrate [15] that could be incorporated into the amine donor BSA and recently into S100A4 monitored by fluorescence polarization assay [18]. Both FLpepT26–BSA and FLpepT26–S100A4 as N^{ε}-(γ-glutamyl) lysine crosslinked products were considered as substrates for isopeptidase activity measurements. However, BSA contains several surface-exposed Lys residues compared with S100A4, which has only one TG2-reactive Lys residue (Lys100) and, unlike BSA, does not contain transglutaminase-reactive glutamine residues [18], making S100A4 an ideal candidate to serve as a substrate in an isopeptidase reaction. To compensate for the size difference between S100A4 (11.5 kDa) and BSA, and to get higher anisotropy change and better fluorescence polarization signal in the designed assay, S100A4 was expressed with GST tag. GST alone did not show explicit activity either as an amine donor (in fluorescence anisotropy assay with FLpepT26 peptide; see Fig. S1A in online supplementary material) or as an amine acceptor (in dansyl–cadaverine incorporation assay; see Fig. S1B). In the transamidase reaction, the K_m values for S100A4 (GST) and BSA were comparable to earlier observations [15,18], whereas V_{max} was approximately 10 times higher in the case of S100A4 (GST) as amine donor (Fig. S1C), suggesting that S100A4 (GST) could be a very potent Lys donor to produce an isopeptidase protein substrate with a single isopeptide bond.

The crosslinking reaction was carried out on a large scale as described in Materials and Methods to produce the FLpepT26–S100A4 (GST) containing the N^{ε}-(γ-glutamyl)lysine bond. The reaction was stopped by the addition of 10 mM EDTA (final concentration) to prevent unwanted further modification of the crosslinked molecules during their separation from free FLpepT26 peptide, and the buffer was replaced by Mops buffer (pH 6.8) for optimal isopeptidase activity [19] (see scheme in Fig. 1A).

The efficiency of the separation was checked on SDS–PAGE, confirming the presence of the crosslinked, fluorescently labeled FLpepT26–S100A4 (GST) molecule at approximately 42 kDa molecular weight in the gel (Fig. 1B) and the disappearance of the FLpepT26 peptide that has 1885 Da molecular weight but generally appears in the gel as a 10-kDa diffuse band.

Validation of potential FLpepT26–S100A4 (GST) as isopeptidase substrate for TG2

The prepared product was tested to check whether it can be used as a substrate for TG2 isopeptidase activity measurement. The result of the cleavage of the isopeptide bond could be visualized by SDS–PAGE (Fig. 1B). The acceptance of FLpepT26–S100A4 (GST) by TG2 as isopeptidase substrate can be easily followed, with the amount of the substrate decreasing while an increasing amount of the released fluorescein-conjugated peptide appears during the enzymatic reaction.

To further confirm that isopeptidase activity occurred, the product of the isopeptidase reaction was applied to a Jupiter 300 C5 RP–HPLC column (Phenomenex) and the peak corresponding to the deamidated FLpepT26 was further analyzed by HPLC–ESI–MS/MS (tandem mass spectrometry). The results showed that the released peptide was a single compound with a molecular mass of 1884.9 Da and a slightly higher retention time compared with FLpepT26 (1886.1 Da) (see Figs. S2 and S3 in supplementary material). Both peptides were subjected to CID to confirm the location of the mass difference (Fig. S4). MS/MS sequencing of the triply protonated parent ions (m/z 629.3 and 629.7) resulted in a complete set of mainly *b*- and *y*-type fragment ions (fragmentation pathway is presented in Fig. S4). The identified peptide fragments correlate with the sequence of FLpepT26, confirming the reliability of the analysis. Based on the mass differences of the N-terminal peptide fragments, the Gln-to-Glu transformation in the released peptide was unambiguously confirmed (see Table S1 in supplementary material).

Testing FLpepT26–S100A4 (GST) as a suitable substrate for kinetic measurement of isopeptidase assay

After demonstration of effective hydrolysis of FLpepT26–S100A4 by TG2, we tested its correlation with decreased anisotropy. Because the crosslinking could be followed by the increase of anisotropy [15,18], it was expected that anisotropy should be decreased over time during the isopeptidase reaction. Indeed, anisotropy signal followed an exponential decay; using linear regression based on the first phase (9–10 min) of the decrease, reaction rates were calculated in the range of 10–600 nM TG2 concentration (Fig. 1C). At lower TG2 concentration, the decrease of anisotropy was less significant and the higher noise (deviances of the single measured values from the fitted curve on the values) prevented the determination of isopeptide bond cleavage. The remaining isopeptidase activity of the enzyme used for isopeptide substrate generation was checked by comparison of the reaction rates in the presence of 5 mM calcium versus EDTA without adding any further TG2, but there was no significant difference between the slopes of the curves (Fig. 1C). At 5 μg of substrate (which corresponds to 0.5 μM concentration), we could detect isopeptidase activity at 10 nM TG2 concentration, whereas the reaction rate was linearly dependent on TG2 concentration in the 10- to 300-nM range (Fig. 1D). The commercially available, not protein-based kinetic isopeptidase activity assays, which measure deaminylation of small nonspecific peptides by TG2 at 50 μM substrate concentration (Zedira assay), have similar sensitivity with a detection limit of approximately 13 nM TG2 [20].

Next we measured the effect of increasing FLpepT26–S100A4 (GST) substrate concentration on the reaction rate at fixed TG2 level (0.3 μM TG2, 5 mM Ca^{2+}), and we observed saturation kinetics on which curve was fitted based on the Michaelis–Menten equation (Fig. 2A; coefficient of determination: $R^2 = 0.9871$). Here the activity attained a plateau phase, and an enzymatic dissociation constant (K_m) was calculated to be 53.9 ± 4.4 nM for FLpepT26–S100A4 (GST) and V_{max} was calculated to be 57.9 ± 0.9 mr/min/μM TG2 using GraphPad Prism 5. This K_m value is extremely low compared with published values for human TG2. We measured 13.3 and 54.3 μM K_m values using Zedira assay with A101 and A102 isopeptidase substrates (deaminylation activity), respectively, in which test the substrates are peptides containing an incorporated unspecific amine (quencher-labeled cadaverine) [11]. In other transglutaminase assays, when deamidation was detected by kinetic spectrophotometric assay using Cbz-Gln-Gly as substrate, the K_m value was in the mM range [21]. In the case of cleavage of isopeptide bond between crosslinked peptides, the K_m values were also in the micromolar range [12]. This demonstrates that our newly developed method based on the novel protein substrate can serve as a highly sensitive and specific reaction to detect and measure TG2 isopeptidase activity. Furthermore, the high reactivity of TG2 with isopeptide bonds shown here suggests

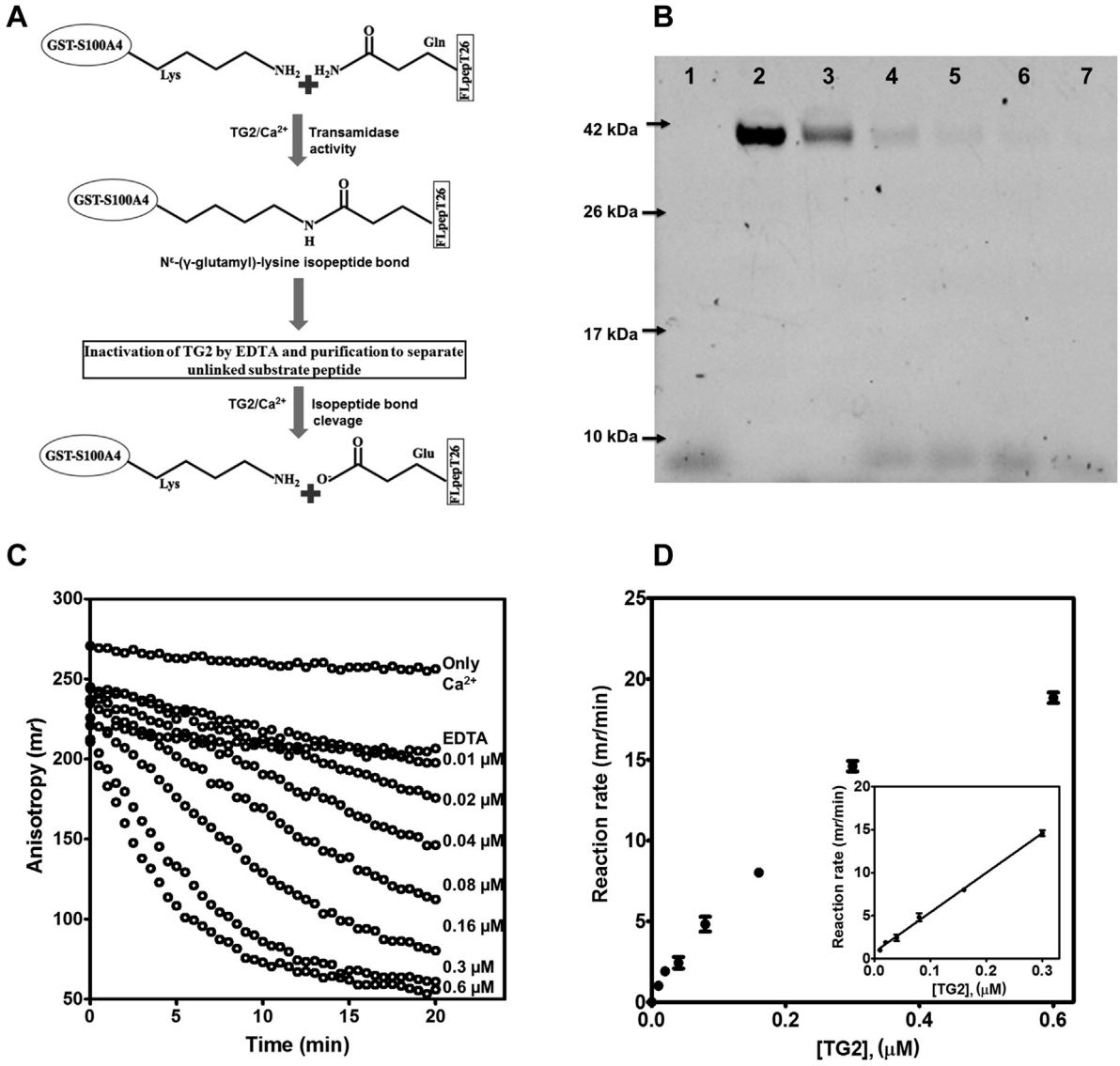

Fig.1. Design and implementation of the fluorescence anisotropy assay that monitors isopeptidase activity of TG2. (A) Work scheme: FLpepT26 peptide and S100A4 (GST) are crosslinked by TG2, and after separation of the free peptide the isopeptide containing FLpepT26–S100A4 (GST) serves as isopeptidase assay substrate. (B) Visualization of the isopeptide cleavage on FLpepT26–S100A4 (GST). The monitored kinetic reactions were performed for 90 min at 37 °C, and then products were separated by 15% SDS–PAGE and detected in a UV gel documentation system. Lane 1: representative gel image of FLpepT26 peptide (11.3 ng peptide); lane 2: separated FLpepT26–S100A4 (GST) isopeptidase substrate (8.6 μg protein); lanes 3 to 7: product of isopeptidase reactions on the substrate in the presence of 300 nM TG2 and 5 mM EDTA (lane 3) or in the presence of 5 mM Ca^{2+} and different TG2 concentrations (0.06 μM [lane 4], 0.3 μM [lane 5], 0.6 μM [lane 6], and 1.2 μM [lane 7]) (2.8 μg substrate protein). (C) Real-time monitoring of the isopeptide cleavage by measuring the anisotropy change using 5 μg of substrate protein mixture in 35 μl of reaction mix (0.5 μM FLpepT26–S100A4 (GST)), 5 mM Ca^{2+} or EDTA, and various concentrations of TG2. (D) The rate of anisotropy change shows linear correlation between 10 and 300 nM TG2 concentration. The inset shows the linear range analyzed by GraphPad Prism 5 using Pearson correlation analysis ($P < 0.0001$, $r = 0.990$). Data are presented as means ± standard deviations from three separate experiments done in triplicate.

that its isopeptidase activity may have physiological relevance not described yet.

Considering that the added substrate in the assay is a mixture of the isopeptide bond containing FLpepT26–S100A4 (GST) and free S100A4 (GST), a significant amount of free S100A4 (GST) is present, raising the possibility that S100A4 (GST) may have influence on the isopeptidase reaction. To address this concern, the effect of S100A4 (GST) on the isopeptidase activity was analyzed using the above-mentioned Zedira assay that contains small peptide substrate and our protein substrate-based assays (see Figs. S5A and S5B, respectively, in supplementary material). The increasing S100A4 (GST) concentration results in dose-dependent increase of isopeptidase activity of TG2 in the Zedira assay; this may be explained by the reported interaction of TG2 and S100A4 [18], leading to the potential stabilization of the active conformation of the enzyme because in a previous study amine donor substrates did not influence isopeptidase activity [11]. On the other hand, in the protein-based assay, the increasing concentration of S100A4 (GST) did not lead to change of the isopeptidase activity, probably due to the relatively high ratio of S100A4 (GST) concentration in the substrate that already could be in saturation without the addition of extra S100A4 (GST). To further increase the efficiency of the developed assay, the peptide incorporation ratio should be further improved to eliminate this potential limitation factor of the method for broad applications. The effect of glycine methyl ester, which was present in the assay solutions, was also checked on the protein-based isopeptidase reaction because it was proposed to facilitate the regeneration of the enzyme from the thiol intermediate state

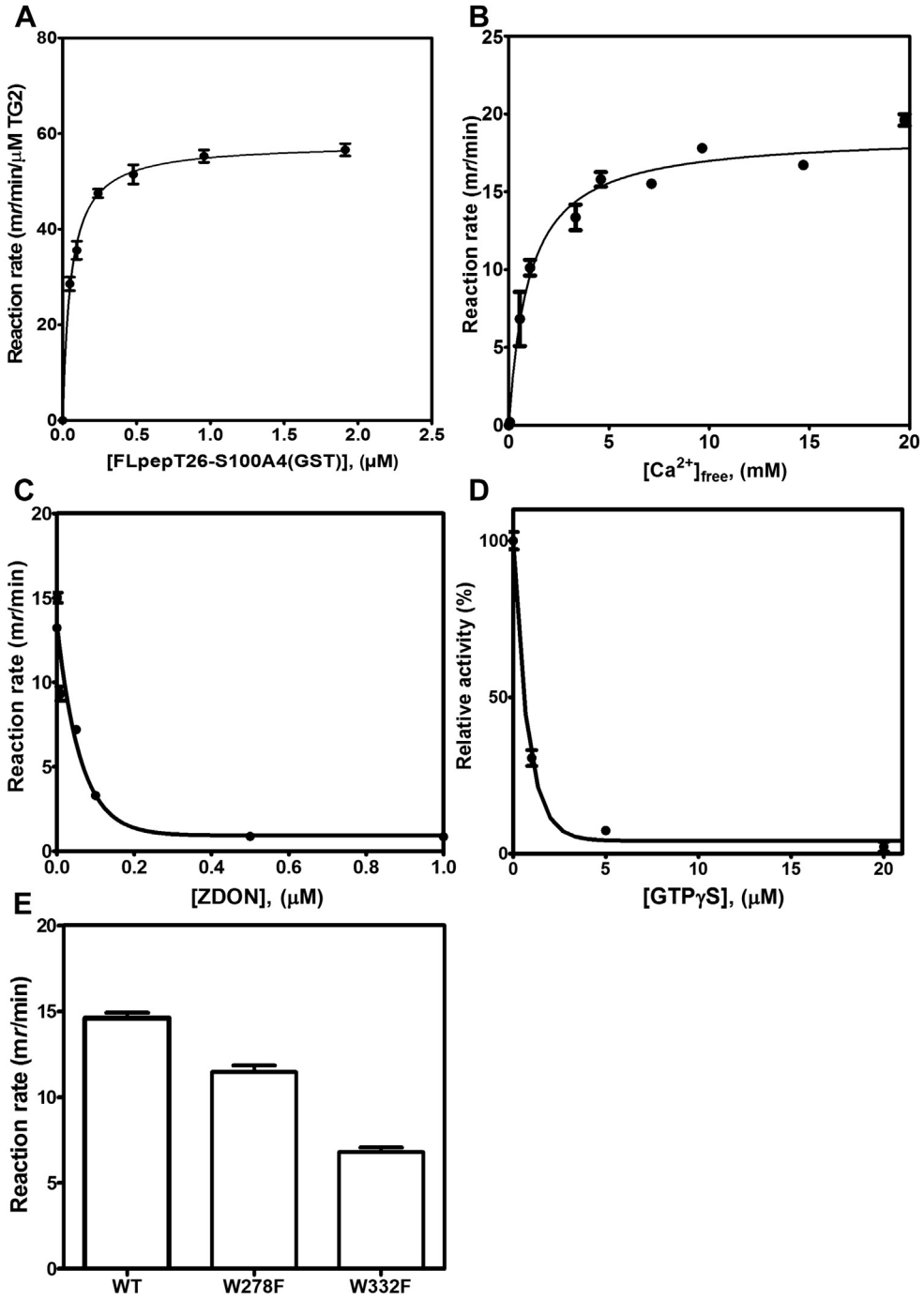

Fig.2. Characterization of the newly developed protein-based isopeptidase assay. (A–D) Determination of the Michaelis constant (A), the Ca^{2+} dependence (B), the inhibitory effect of ZDON (C), and GTPγS (D). ZDON inhibitor was tested after 5 min of preincubation in the presence of 5 mM Ca^{2+}, 0.3 μM TG2, and 5 μg of substrate, whereas in the case of GTPγS dependence the Ca^{2+} concentration was 2 mM. (E) Mutants were also tested in the presence of 5 mM Ca^{2+} and at 300 nM TG2 concentration. In all negative control experiments, 5 mM EDTA was present instead of Ca^{2+}. Data are presented as means ± standard deviations from two separate experiments done in triplicate.

during the isopeptidase reaction (aminolysis) [20]. However, fluorescence polarization was comparable in both the presence and absence of glycine methyl ester (data not shown), in concordance with our earlier observations [11], and the mass spectrometry (MS) analysis has not identified transamidated peptide product in the applied assay conditions, suggesting that transamidation does not have a significant rate in TG2-catalyzed reaction (see Fig. S4 in supplementary material) [9].

To further characterize the kinetics of the protein–peptide-based isopeptidase reaction, Ca^{2+} dependency was also tested because TG2 undergoes a large conformational change during its activation by Ca^{2+} [22] that could influence substrate accessibility to the active site (Fig. 2B). In general, the crosslinking and deaminylation activity of wild-type TG2 reaches the maximum reaction rate in the presence of approximately 1.5 mM Ca^{2+} concentration [23]. However, in the case of FLpepT26–S100A4 (GST) cleavage,

4–5 mM Ca^{2+} concentration was needed to saturate the reaction rate (WT EC_{50} = 0.96 ± 0.17 mM, calculated by fitting a dose–response curve hypothesizing standard slope), suggesting that isopeptidase activity may have a more important role in the extracellular matrix, where Ca^{2+} concentration is higher than in the cytosol.

Intracellularly, TG2 activity is regulated reciprocally by Ca^{2+} and nucleotides [3]. The inhibitory effect of GTPγS, a stable GTP compound, was also tested on isopeptidase activity of TG2 (Fig. 2D). GTPγS had similar inhibitory effect on isopeptidase activity of TG2 in the protein-based assay as in the Zedira assay (with A102 substrate) [11].

To see the importance of the substrate quality, crosslinked BSA–FLpepT26 was also produced and tested in the isopeptidase assay, but no reaction could be detected either by monitoring polarization change or by SDS–PAGE analysis (data not shown). This provides further evidence for the importance of the applied protein substrate containing the isopeptide bond to detect specific isopeptide cleavage. The active lysine S100A4 residue, involved in crosslinking, is localized in the C-terminal tail of S100A4 that could be easily accessible for the active site of TG2.

Potential applications of newly developed kinetic isopeptidase assay

There is increasing interest from the pharmacological industry to regulate transglutaminase activity by inhibitors, particularly in neurodegenerative diseases and kidney and pulmonary fibrosis, and to prevent thrombosis [3]. However, the effects of newly developed inhibitors were always tested on the crosslinking activity of TG2, and there has been no comparison of their effect on isopeptidase activity of TG2, particularly in a kinetic protein substrate assay that has not existed so far. A commercially available, cell-permeable, irreversible active site-directed inhibitor of TG2, the ZDON (Zedira [24]), was tested in our newly developed method (Fig. 2C). Approximately 0.3 μM ZDON was enough for complete inhibition of the isopeptidase activity, which is in good correlation with the expected value in the case of an irreversible inhibitor in the presence of 0.3 μM TG2, although the apparent IC_{50} value was 30.7 nM (calculated by fitting a dose–response curve hypothesizing standard slope), which is lower than a reported one in an in vitro transamidation assay [24].

Enzyme assays are useful to characterize the effect of mutations on activity. In our recent study, two special mutants were produced [11]. One is deficient in transamidase activity but has higher isopeptidase activity compared with the wild type (isopeptidase mutant W332F), and another one has opposite characteristics (transamidase mutant W278F). Interestingly, in the protein-based assay, both mutants demonstrated lower activity than wild-type TG2 (46.6 and 78.6%, respectively; Fig. 2E). Moreover, the W332F mutant showed lower activity, whereas the W278F mutant showed higher activity, compared with the Zedira deaminylation assay [11]. The larger size of the substrate may mask the earlier observed differential effect of these mutations [11], suggesting that steric features have a strong control on the TG2 isopeptidase activity.

For more detailed study of isopeptidase activity and its biological significance, the replacement of the substrate components with other TG2-specific substrates could provide further mechanistic details about the isopeptidase reaction. Results of such studies can lead to pharmacological approaches to accelerate the removal of pathologically accumulated, N^{ε}-(γ-glutamyl)lysine crosslinked proteins from fibrotic tissues or in the case of neurodegenerative disorders. These bonds otherwise are catabolized slowly, requiring degradation by proteases to yield γ-glutamyl-ε-lysine isodipeptide that is released into the circulation to be cleaved by γ-glutamylamine cyclotransferase in the intestine and kidney [25,26].

Conclusions

We have developed a kinetic method to follow isopeptidase activity of TG2 in real time using a crosslinked protein substrate in the presence and absence of different effectors/inhibitors. Reversible covalent modifications of proteins have crucial regulatory functions in cells and tissues like those mediated by ubiquitin ligases and deubiquitinases or kinases and phosphatases. Unlike phosphorylation and ubiquitination, there are no enzymes in nature to reverse transglutaminase-mediated protein crosslinking. Instead, transglutaminases themselves, such as TG2, possess hydrolytic activity to cleave the N^{ε}-(γ-glutamyl)lysine bonds that they produce. The biological and regulatory significance of this reaction, as well as the potential use of the isopeptidase activity of transglutaminases in pathological conditions, requires further investigations. We believe that the presented assay can serve as a useful tool for those kinds of studies.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ab.2016.04.012.

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