Redox Regulation of Transglutaminase 2 Activity*

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Transglutaminase 2 (TG2) in the extracellular matrix is largely inactive but is transiently activated upon certain types of inflammation and cell injury. The enzymatic activity of extracellular TG2 thus appears to be tightly regulated. As TG2 is known to be sensitive to changes in the redox environment, inactivation through oxidation presents a plausible mechanism. Using mass spectrometry, we have identified a redox-sensitive cysteine triad consisting of Cys230, Cys370, and Cys371 that is involved in oxidative inactivation of TG2. Within this triad, Cys370 was found to participate in disulphide bonds with both Cys230 and its neighbor, Cys371. Notably, Ca2+ was found to protect against formation of these disulphide bonds. To investigate the role of each cysteine residue, we created alanine mutants and found that Cys230 appears to promote oxidation and inactivation of TG2 by facilitating formation of Cys370–Cys371 through formation of the Cys230–Cys370 disulphide bond. Although vicinal disulphide pairs are found in several transglutaminase isoforms, Cys230 is unique for TG2, suggesting that this residue acts as an isoform-specific redox sensor. Our findings suggest that oxidation is likely to influence the amount of active TG2 present in the extracellular environment.

Human transglutaminase 2 (TG2) modifies protein- or peptide-bound glutamine residues by either cross-linking their reactive carboxamide side chains to primary amines or by deamidation, converting glutamine residues to glutamate (1, 2). Ca2+ is required for this catalytic activity and induces conformational changes in the enzyme, which arrange the active-site residues for catalysis, including Cys277 (3–6). As TG2 is abundantly expressed in both the intracellular and extracellular environments of many tissues, its catalytic activity must be tightly regulated to avoid excess modification of cellular and tissue components. GDP and GTP act as allosteric inhibitors by inducing a closed conformation in which the active site is buried (6–8). Low Ca2+ concentration and high GTP/GDP concentration in the cytosol typically prevent TG2 activation within cells. Despite conditions in the extracellular milieu that favor activation, extracellular TG2 also appears to be predominantly inactive under normal conditions but can be activated by certain types of inflammation and cell injury (9).

The catalytic activity of TG2 is implicated in the pathogenesis of several human diseases, including celiac disease (10). Celiac disease is caused by an aberrant immune response to proline- and glutamine-rich peptides from dietary gluten in the small intestine of genetically predisposed individuals (11). In the celiac immune response, the enzymatic activity of TG2 is crucial, as TG2-mediated deamidation of gluten peptides increases their T-cell antigenicity (12, 13).

In contrast to our knowledge of the mechanistic basis for TG2 inactivity in the intracellular environment, the mechanisms underlying regulation of TG2 activity in the extracellular compartment remain unclear. TG2 harbors no disulphide bonds in its native state, which is unusual for enzymes in the extracellular environment (14). Previous studies have shown that TG2 is susceptible to oxidation, resulting in inactivation (19–22). Thus, modulation of enzymatic activity through oxidation presents a plausible mechanism for regulation of extracellular TG2 activity. We have investigated the events underlying oxidative inactivation of TG2 and report the identification of a redox-sensitive cysteine triad consisting of Cys230, Cys370, and Cys371. Within this triad, Cys230 appears to set the threshold for intramolecular disulphide bond formation and thereby inactivation. Oxidation was influenced by the presence of Ca2+ and substrate, suggesting that the local environment can modulate and fine-tune oxidative inactivation of TG2 in the extracellular milieu.

 EXPERIMENTAL PROCEDURES

TG2 Expression and Purification—Human wild-type (WT) TG23 and mutants C230A, C370A, and C371A were expressed...
in *Escherichia coli* BL21(DE3) cells as N-terminally His<sub>6</sub>-tagged proteins (pET-28a, Novagen, Madison, WI). Expression of TG2 was induced by isopropyl-β-D-thiogalactopyranoside or OnEx Solution 1–3 (Novagen), followed by overnight incubation at 24 °C for the WT enzyme and 12–18 °C for mutants. The recombinant protein was purified by nickel-nitri-

 tổnic acid affinity chromatography and anion exchange chromatography before dialysis and storage at −70 °C. The human active-site TG2 mutant C277S, expressed in a baculovirus system and purified to homogeneity by Ni<sup>2+</sup> affinity chromatography, was a gift from Eckart Mummert (Phadia GmbH, Freiburg, Germany).

**Inhibitor Synthesis and Preparative Inhibition of TG2**—The active-site inhibitor Ac-P(6-diazo-5-oxo-L-norleucine)LPF-NH<sub>2</sub> was synthesized and used to inactivate human TG2 as described previously (15).

**TG2 Deamidation Kinetics**—Kinetic parameters for de-



amidation of glutamine side chains in peptide substrates were measured using modifications of a method described previously (16). The components were as follows: 1) a concentrated buffer consisting of 1 M MOPS, 5 mM EDTA, and 50 mM α-ketoglutarate (pH 7.2); 2) 100 mM CaCl<sub>2</sub>; 3) 300 mM benzoylloxycarbonyl-Gln-Gly (Sigma) prepared as a solution of the sodium salt by titration with sodium hydroxide and diluted to 10× stocks of the desired reaction concentrations; 4) 50 mM NADH; and 5) lyophilized ammonium-free glutamate dehydrogenase (Biozyme Laboratories) dissolved to 0.5 units/µl in 200 mM MOPS, 1 mM EDTA, and 10 mM α-keto-



glutarate (pH 7.2) and clarified by centrifugation. Each reaction contained 20 µl of component 1, varying amounts of component 2, 10 µl of component 3, 2.5 µl of component 4, and an appropriate amount of water to reach 91.8 µl. To this mixture was added 7.2 µl of component 5. The resulting mixture was pre-equilibrated for 30 min at room temperature, and 97 µl was added to a microtiter plate well. To initiate the deamidation reaction, typically 3 µl of WT or mutant TG2 stock solution was added to each well. Absorbance was monitored at 340 nm and 30 °C using a Molecular Devices SpectraMax Plus 384 microplate reader. Slopes were calculated from the linear regions of each trace, typically from 1600 to 3600 s. Ca<sup>2+</sup> concentrations reported are those of CaCl<sub>2</sub> added minus the EDTA concentration in the final reaction buffer. For a given experiment, the data for wild-



type TG2 and all mutants were collected simultaneously to facilitate direct comparison. Rate constants were calculated using Origin 6.0.

**Sample Oxidation and In-gel Digestion for Mass Spectromet-



ic Analysis**—Controlled oxidation was performed by treat-



ment of WT or mutant TG2 with varying ratios of GSH and GSSG in N<sub>2</sub>-flushed 100 mM Tris-HCl (pH 7.2) and 0.5 mM EDTA. Typically, 0.1 or 0.05 µg/µl enzyme was incubated with 2 mM GSSG and 0.24, 0.45, 1.25, or 2.4 mM GSH in the presence or absence of synthetic peptide substrate (DQ2-α-I, QLQPFPQPQQLPY; or DQ2-α-II, PQPQLYPQPQQLPY-NH<sub>2</sub>) and 0, 1, 2, 5, or 10 mM CaCl<sub>2</sub>. Oxidation was performed at 30 or 37 °C and stopped at the indicated time points by precipitation of TG2 using trichloroacetic acid to a final concentration of 10% (w/v), followed by a 10-min centrifugation at 4 °C and precipi-



tation two times in 100% ice-cold acetone. Precipitated protein was resuspended and alkylated with 25 mM iodoacetamide (IAM; Sigma) in 1% SDS and 50 mM Tris-HCl (pH 8.0) at room temperature with shaking in the dark for 1 h, followed by non-



reducing SDS-PAGE (12% Tris-HCl Ready Gel, Bio-Rad) and Coomassie Blue staining. Coomassie Blue-stained bands were cut out, destained, and washed with H<sub>2</sub>O, 1:1 H<sub>2</sub>O<sub>2</sub>/acetonitrile, and 100% acetonitrile (three times for 20 min each). Gel pieces were either directly digested with trypsin (see below) or reduced with 10 mM D-l-dithiothreitol (DTT; Sigma) and alkylated with 55 mM iodoacetic acid (IAA; Sigma), followed by washing and overnight trypsin digestion at 37 °C.

**Non-denaturing PAGE**—After preincubation of proteins as indicated in the figure legends, samples were diluted in native Laemmli sample buffer without SDS and reducing agents and separated on 4–20% Tris-HCl Ready Gel using Tris/glycine running buffer adjusted to pH 8.5 at 4 °C. Gels were run at 125 V for 1 h at 4 °C.

**Identification of Disulfide Bridges and Quantification of Cys-



teine Oxidation by Mass Spectrometry**—Trypsin-digested sam-



ples were analyzed by mass spectrometry (MALDI-ToFToF or Nano-LC-QToF system, Bruker Daltonics). Peptides harboring cysteine residues were identified by protein data base searches using the search engine Mascot (17) and by manual sequencing. Oxidation of cysteine residues was quantified by calculating the average mass shift of the isotopic envelope of peptides from control and oxidized samples alkylated with IAM and IAA. The relative oxidation of Cys<sup>370</sup> and Cys<sup>371</sup> was quantified as follows. The signal at m/z 1481.8 corresponds to the tryptic peptide in which both Cys<sup>370</sup> and Cys<sup>371</sup> were present in a reduced state and therefore were labeled with IAM (m/z 1482.8 derives from the tryptic peptide with one of the cysteine residues participating in disulfide bond for-



mation. That cysteine residue was thus labeled with IAA instead of IAM (+57 Da, +57 Da). The signal at m/z 1482.8 derives from the tryptic peptide with one of the cysteine residues participating in disulfide bond for-



mation. That cysteine residue was thus labeled with IAA instead of IAM (+57 Da, +57 Da). In addition, this signal harbor-



ated the 13C isotopic contribution from the peak at m/z 1481.8 (78% of the intensity at m/z 1481.8). The signal at m/z 1483.8 consisted of oxidized Cys<sup>370</sup> and Cys<sup>371</sup> both labeled with IAA (+58 Da, +58 Da) plus the contribution coming from the iso-



topic envelope of the signals at m/z 1481.8 (43%) and m/z 1482.8 (78%). The isotope contribution from neighboring peaks was subtracted, and relative intensities were calculated as the intensity of one peak divided by the sum of all three peaks. This corresponds to the percentage of reduced and oxidized Cys<sup>370</sup> and Cys<sup>371</sup> present.

**Determination of Enzymatic Activity of Oxidized Samples**—Synthetic peptide substrates and Ca<sup>2+</sup> were added to give final concentrations of 20–100 µM peptide, 5 mM Ca<sup>2+</sup>, and 0.1 or 0.05 µg/µl enzyme and then incubated at 37 °C. Ali-



quots were removed at the indicated time points. Deamida-



tion of DQ2-α-I or DQ2-α-II was quantified by MALDI-TOF mass spectrometry.

Transamidation of FITC-DQ2-α-II<sub>58</sub> (fluorescein isothio-



cyanate-aminohexanoic acid-PQPELPYPQPQQLPY) by 5-bioti-



namidopentylamine (5-BP) was quantified as described previ-



ously (18) by micellar electrokinetic chromatography capillary electrophoresis (Agilent) using laser-induced fluorescence detection (Picolyl, Toulouse, France). Briefly, samples were
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diluted in running buffer (64 mM borate (pH 9.3) and 20 mM SDS) and run from the anode to the cathode (20 kV, positive mode), separating native peptide and deamidated and transamidated product.

Determination of Enzymatic Activity of Fibronectin-bound TG2—Micrtotiter 96-well plates were coated overnight with human fibronectin (25 μg/ml in 50 mM bicarbonate buffer (pH 9.6); Roche Applied Science). Thereafter, TG2 (5 and 2.5 μg/ml in Tris-buffered saline with 0.1% Tween 20 and 1 mM DTT) was immobilized for 1 h at 37 °C, followed by incubation with 1 mM GSSG or 1 mM DTT for 1 h at 37 °C. Enzymatic activity was measured by addition of 40 μM 5-BP and 10 mM CaCl2. After extensive washing, incorporated 5-BP was detected with streptavidin-alkaline phosphatase. Absorption was measured at 405 nm after the addition of 1.5 mg/ml alkaline phosphatase substrate (Sigma) in 100 mM diethanolamine (pH 8.9). To verify that TG2 remained immobilized during oxidative conditions, one of the GSSG-treated samples was incubated with biotin-CUB7402 (Neomarkers) instead of 5-BP.

RESULTS

TG2 Is Reversibly Inactivated by Oxidation—TG2 is known to be sensitive to oxidative environments and will gradually lose activity upon improper handling and storage. Regulation of activity through oxidation is also likely to be relevant in vivo. This prompted us to investigate the mechanism behind oxidative inactivation of TG2. Using recombinant human TG2, we observed that prolonged handling of the enzyme in the absence of reducing agents resulted in loss of enzymatic activity (Fig. 1A). This inactivation was found to be reversible, as activity could be restored by treatment with DTT. Similar results were observed upon treatment of TG2 with oxidizing agent by using an excess of GSSG over GSH (Fig. 1B). These observations suggest that loss of enzymatic activity is accompanied by cysteine oxidation, leading to disulfide bond formation. Oxidation was also found to influence the conformation of TG2, rendering the enzyme more prone to assume an open conformation (Fig. 1C). Whereas WT TG2 migrated in a closed conformation upon incubation with GTP, oxidized TG2 was unable to assume or retain a closed conformation during native PAGE. However, treatment with DTT did partially restore this ability (Fig. 1C).

Identification of a Cysteine Triad Forming Two Disulfide Bonds upon Oxidation of TG2—TG2 has no reported disulfide bridges. Treatment of TG2 with an excess of GSSG over GSH (0.24 mM GSH and 2 mM GSSG for 3 h at 30 °C, indicated as GSSG in the figure) results in loss of enzymatic activity, which is recovered upon treatment with DTT (10 mM at room temperature for 10 min). Activity is given as percent deamidation after 90 min using 90 μM DQ2-α-II peptide and 0.05 μg/μl enzyme. TG2 conformation visualized by nondenaturing PAGE after pre-treatment with 0 mM DTT (∼DTT) or 30 mM DTT (∼DTT) for 30 min at room temperature, followed by a 1-h incubation with 500 μM GTP and 1 mM Mg2+.

WT TG2, active-site inhibitor Ac-P(6-diazo-5-oxo-L-norleucine)LPF-NH2, and TG2 oxidized by prolonged gel filtration (ox WT) are compared.

FIGURE 1. Oxidation influences activity and conformation of TG2. A, DTT treatment (20 mM DTT at 4 °C for 4 h) of TG2 oxidized by prolonged gel filtration partially recovers enzymatic activity. Enzymatic activity was measured as percent deamidation after 60 min using 20 μM DQ2-α-II peptide as substrate and 0.1 μg/μl enzyme. B, treatment of TG2 with an excess of GSSG over GSH (0.24 mM GSH and 2 mM GSSG for 3 h at 30 °C, indicated as GSSG in the figure) results in loss of enzymatic activity, which is recovered upon treatment with DTT (10 mM at room temperature for 10 min). Activity is given as percent deamidation after 90 min using 90 μM DQ2-α-II peptide and 0.05 μg/μl enzyme. C, TG2 conformation visualized by nondenaturing PAGE after pre-treatment with 0 mM DTT (∼DTT) or 30 mM DTT (∼DTT) for 30 min at room temperature, followed by a 1-h incubation with 500 μM GTP and 1 mM Mg2+.

WT TG2, active-site inhibitor Ac-P(6-diazo-5-oxo-L-norleucine)LPF-NH2-bound TG2 (iTG2), and TG2 oxidized by prolonged gel filtration (ox WT) are compared.

We next quantified the percentage of free versus oxidized Cys370 and Cys371 upon GSH/GSSG titration. As expected, we observed increased oxidation upon decreasing GSH/GSSG ratios (Fig. 5A). The Cys230--Cys370 disulfide bond was observed to form more readily under less oxidizing conditions (Fig. 5A, gray squares, only Cys370 oxidized) than the vicinal Cys277--Cys370 disulfide bond (white squares, both Cys277 and Cys370 oxidized). On the other hand, the vicinal disulfide bond progressively dominated under more oxidizing conditions.

Enzymatic Activity Correlates Inversely with the Oxidation of Cys277 and Cys371—We next addressed whether the presence of the identified disulfide bridges influences the enzymatic activity of TG2. Peptide substrate and Ca2+ were added to oxidized
TG2, and deamidation was quantified at various time points. The level of deamidation was found to correlate with the percentage of reduced Cys370 and Cys371 still present in the enzyme upon the addition of substrate, suggesting that both of these cysteine residues must be reduced in order for the enzyme to be active (Fig. 5B). Similar results were obtained for transamidation (supplemental Fig. S4).

**Cys230 Promotes Inactivation of TG2 and Facilitates Formation of the Vicinal Disulfide Bond between Cys370 and Cys371**

To further establish the role of the three cysteine residues, recombinant TG2 mutants C230A, C370A, and C371A were expressed. First, the enzymatic activity and conformational preference of each mutant was tested. Both the $k_{cat}$ and $K_m$ of C230A were similar to those of the WT enzyme, suggesting that the C230A mutant does not impair the catalytic cycle of TG2 (Table 1). Moreover, C230A was able to assume the closed conformation like the WT enzyme (Fig. 6A). In contrast, both C370A and C371A mutants were somewhat impaired in different ways. Substrate recognition was affected for both mutants as evidenced by increased $K_m$ values. However, although C370A remained an effective catalyst (the $k_{cat}/K_m$ was one-third that of the WT enzyme), the
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FIGURE 4. Structural basis for disulfide formation in TG2. Shown is a ribbon representation of the open (dark salmon; Protein Data Bank code 2Q3Z (15)) and closed (light blue; GDP-bound; code 1KV3 (7)) crystal structures of TG2. The vicinal disulfide loop is highlighted in red and blue for the open and closed structures, respectively. Cys230, Cys370, and Cys371 are shown as sticks, with magenta carbons for the open structure and cyan carbons for the closed structure. Red, oxygen; blue, nitrogen; yellow, sulfur.

FIGURE 5. Oxidation of Cys370 and Cys371. A, a quantification of oxidation of Cys370 and Cys371 upon GSH/GSSG titration. The x axis displays the ratio of [GSH]/[GSSG] used for oxidation calculated from their molar concentrations. The graph depicts the percentage of reduced Cys370 and Cys371 (black squares), oxidized Cys370 and reduced Cys371 (gray squares), and oxidized Cys370 and Cys371 (white squares) after a 3-h glutathione incubation at 30 °C. B, the level of enzymatic activity (white circles) correlates with the percentage of reduced Cys370 and Cys371 (black squares) present in the enzyme upon substrate addition. Activity is given as percent deamidation as measured 2 h after the addition of 100 μM DQ2-α-II peptide and 5 mM Ca2+ to glutathione-treated samples.

TABLE 1 Kinetic parameters of WT TG2 and cysteine mutants for deamination of the substrate benzoylformyl-Gln-Gly as determined using a standard coupled enzyme assay

|          | k_m (mm) | k_cat (min⁻¹) | k_cat/k_m (min⁻¹) |
|----------|----------|---------------|-------------------|
| WT       | 11.2 ± 0.6 | 11.2 ± 0.2    | 1.00 ± 0.07       |
| C370A     | >30      | ND            | 0.33 ± 0.07       |
| C371A     | >30      | ND            | 0.05 ± 0.002      |
| C230A     | 11.5 ± 0.6 | 10.3 ± 0.2    | 0.90 ± 0.07       |

ND, not determined.

Treatment of C230A with an excess of GSSG over GSH at 37 °C resulted in oxidation and loss of enzymatic activity (data not shown). However, C230A was less susceptible to oxidation (Fig. 6C) and inactivation (data not shown) than the WT enzyme. This difference in inactivation kinetics was even more pronounced when oxidation was performed at 30 °C. After a 3-h incubation, WT TG2 was extensively oxidized and almost completely inactivated, whereas C230A retained activity and was minimally oxidized (Fig. 6, D and E). Additionally, the enzymatic activity of WT TG2 could be recovered by incubation with DTT prior to substrate addition, whereas this made little difference for C230A (Fig. 6, D and E). This suggests that Cys230 facilitates formation of the vicinal disulfide bond between Cys370 and Cys371 and thereby inactivation of TG2. This role for Cys230 is supported by direct observation of the Cys230–Cys370 disulfide bond formed in the C371A mutant (supplemental Fig. S5). Despite lacking the ability to form the aforementioned disulfide bridges, C370A was inactivated upon oxidation with GSSG (data not shown). Moreover, the closed form of C370A was more susceptible to opening by oxidation by GSSG than the WT enzyme (data not shown). This could be due to glutathionylation of Cys230, which was found to occur very rapidly for the C370A mutant (supplemental Fig. S6) but was not observed for the WT enzyme. Steric hindrance due to this modification is likely to affect both activity and conformation. Notably, no oxidation of Cys371 was observed in the C370A mutant.

Calcium Protects against Oxidation and Inactivation—Hitherto, all oxidation experiments were performed in the absence of Ca2+. The effect of calcium on oxidation is challenging to address, as TG2 is prone to exert extensive self-cross-linking. To circumvent this problem, Ca2+ titration was performed in the presence of saturating amounts of peptide substrate. In these experiments, increasing amounts of Ca2+ appeared to protect against oxidation (Fig. 7A). As the lack of oxidation could be due to binding of Ca2+ and/or catalytic turnover of the enzyme, Ca2+ titration was also tested in the absence of substrate using the active-site TG2 mutant C277S (Fig. 7B). Again, Ca2+ clearly protected against oxidation with a dramatic change at ~1–3 mM Ca2+, which is close to the K_m of Ca2+ (16). The presence of saturating amounts of substrate in the absence of Ca2+ also had a slight protective effect against oxidation (data not shown).

Fibronectin-bound TG2 Is Inactivated by Oxidation—To address whether TG2 associated with fibronectin is also susceptible to oxidation, TG2 was immobilized on fibronectin-coated enzyme-linked immunosorbent assay plates, followed by oxidation with GSSG. Enzymatic activity was then assessed by incubation with 5-BP and Ca2+, followed by streptavidin-alkaline phosphatase to detect incorporated 5-BP. Treatment with 1 mM GSSG completely abrogated enzymatic activity, demonstrating that fibronectin-associated TG2 is also susceptible to oxidation (Fig. 8).

DISCUSSION

We have identified a redox-sensitive cysteine triad consisting of Cys230, Cys370, and Cys371 that is involved in oxidative inac-
that oxidative inactivation of TG2 can be modulated by the local environment in vivo.

Folk and colleagues reported reversible disulfide bond formation upon treatment of TG2 with various oxidizing reagents (19–21). The observed oxidation was independent of the active-site Cys277, indicating the presence of additional redox-sensitive cysteine residues within the enzyme that could not be identified at the time. We have demonstrated that oxidative loss of enzymatic activity correlates with oxidation of three cysteine residues, Cys230, Cys370, and Cys371. These residues are in close proximity in the crystal structures of both the closed and open conformations of TG2 (7, 15). Within this triad, Cys230 participates in two disulfide bonds with either Cys370 or its neighbor, Cys371. The vicinal disulfide bond was observed previously in the open crystal structure (15), but also, in the crystal structure, Cys230 is observed to be within bonding distance of Cys370, providing structural support for our findings. Cys230–Cys370 appears to form under less oxidizing conditions than Cys370–Cys371, which is progressively formed upon increasingly oxidizing conditions, suggesting the mechanism of inactivation depicted in Fig. 9. Mutation of Cys230 to alanine rendered the enzyme less susceptible to inactivation by oxidation with reduced formation of Cys370–Cys371. Thus, the formation of Cys230–Cys370 is observed to facilitate subsequent formation of the vicinal disulfide bond, indicating that Cys230 is acting as a redox sensor that initiates oxidation and inactivation of TG2. Although vicinal cysteine pairs are found in several transglutaminase isoforms (TG1, TG4, TG5, and TG7), Cys230 is unique for TG2. It therefore appears that Cys230 allows an isoform-specific mechanism to regulate transglutaminase activity under oxidizing conditions.

In addition to inhibition of enzymatic activity, oxidation also affects the conformation of TG2, rendering the enzyme more susceptible to assuming an open conformation as resolved by native PAGE. This can be reversed by treatment with DTT,
indicating that disulfide bond formation impedes flexibility in the hinge of the enzyme. In line with this, we observed that the C370A mutant, which cannot form the two identified disulfide bonds, migrated in a closed conformation irrespective of the presence of reducing agents, whereas reducing agents were clearly beneficial for the WT enzyme. On the other hand, the C371A mutant migrated only in an open conformation, which might be due to stable Cys\(^{370}\)–Cys\(^{370}\) bond formation.

The transglutaminase activity of TG2 is strictly Ca\(^{2+}\)-dependent. It is known that binding of Ca\(^{2+}\) ions induces conformational changes in the enzyme that expose the active site (4, 5). The presence of Ca\(^{2+}\) also influences oxidation of TG2, although there are conflicting reports about this in the literature (19, 20). We observed that Ca\(^{2+}\) protected against formation of the Cys\(^{230}\)–Cys\(^{370}\) and Cys\(^{370}\)–Cys\(^{371}\) disulfide bonds. Interestingly, Cys\(^{230}\) resides within one of the proposed Ca\(^{2+}\)-binding sites of TG2 (23). Occupancy of this site by Ca\(^{2+}\) could thus prevent formation of the Cys\(^{230}\)–Cys\(^{370}\) bond. Conversely, once this bond is formed, it would impede binding of Ca\(^{2+}\), resulting in loss of enzymatic activity. Notably, the Cys\(^{370}\)–Cys\(^{371}\) bond was found to induce significant changes in the peptide backbone conformation, which might influence overall binding of Ca\(^{2+}\) to TG2 (15).

FIGURE 7. Effect of Ca\(^{2+}\) on oxidation. A, effect of Ca\(^{2+}\) on oxidation of WT TG2 (30 min at 37 °C with 0.24 mM GSH and 2 mM GSSG) in the presence of 250 μM DQ2-α-Ⅱ peptide. A short incubation time was chosen to avoid loss of enzyme upon self-cross-linking. B, effect of Ca\(^{2+}\) on oxidation of baculovirus-produced active-site TG2 mutant C277S (3 h at 30 °C with 0.24 mM GSH and 2 mM GSSG). The graphs depict the percentage of reduced Cys\(^{370}\) and Cys\(^{371}\) (black squares), oxidized Cys\(^{370}\) and reduced Cys\(^{371}\) (gray squares), and oxidized Cys\(^{370}\) and Cys\(^{371}\) (white squares) as observed by MALDI-TOF mass spectrometry.

FIGURE 8. TG2 immobilized on fibronectin is inactivated by oxidation. Incorporation of 40 μM 5-BP by fibronectin-immobilized TG2 in the presence of 10 mM CaCl\(_2\) was measured after incubation with 0 mM GSSG or 1 mM GSSG. TG2 was still bound to fibronectin after GSSG treatment as detected by monoclonal antibody CUB7402.

FIGURE 9. Schematic depiction of oxidation in the redox-sensitive cysteine triad of TG2. Our data suggest that oxidation of TG2 and the accompanying inactivation are initiated by formation of the Cys\(^{230}\)–Cys\(^{370}\) disulfide bond (2), which precedes and facilitates formation of the vicinal Cys\(^{370}\)–Cys\(^{371}\) disulfide bond (3). Oxidation and inactivation are reversible in vitro by use of reducing agents, whereas this might be mediated by unknown factors in vivo.
some yet unknown mechanism keeps the enzyme active in an oxidative extracellular environment. Further studies are required to address this issue.

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