Activity-regulating structural changes and autoantibody epitopes in transglutaminase 2 assessed by hydrogen/deuterium exchange

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ABSTRACT

The multifunctional enzyme transglutaminase 2 (TG2) is the target of autoantibodies in the gluten-sensitive enteropathy celiac disease. In addition, the enzyme is responsible for deamidation of gluten peptides, which are subsequently targeted by T cells. In order to understand the regulation of TG2 activity as well as the enzyme’s role as an autoantigen in celiac disease, we have addressed structural properties of TG2 in solution using hydrogen/deuterium exchange monitored by mass spectrometry. We demonstrate that Ca\textsuperscript{2+} binding, which is necessary for TG2 activity, induces structural changes in the catalytic core domain of the enzyme. Cysteine oxidation was found to abolish these changes, suggesting a mechanism whereby disulfide bond formation inactivates the enzyme. Further, by using TG2-specific human monoclonal antibodies generated from intestinal plasma cells of celiac disease patients, we observed that binding of TG2 by autoantibodies can induce structural changes that could be relevant for the pathogenesis. Detailed mapping of two of the main epitopes targeted by celiac disease autoantibodies revealed that they are located adjacent to each other in the N-terminal part of the TG2 molecule.
SIGNIFICANCE STATEMENT

The enzyme transglutaminase 2 (TG2) is the target of autoantibodies characteristic of the gluten-sensitive enteropathy celiac disease, and intact enzyme activity seems to be required for the disease-causing immune response. TG2 activity is regulated through conformational changes. Ca$^{2+}$ binding is required for enzyme activity, whereas oxidation inactivates the enzyme. Using hydrogen/deuterium exchange monitored by mass spectrometry, we have studied differences between active and inactive forms of TG2 in solution and found that oxidation prevents Ca$^{2+}$-induced structural changes. Further, we have characterized the TG2-binding of a panel of monoclonal autoantibodies derived from disease lesion plasma cells. Autoantibody binding affected the structure of TG2, and mapping of the targeted epitopes suggests a possible mechanism for the induction of the autoimmune response.
INTRODUCTION

Celiac disease is a gluten-sensitive enteropathy occurring in genetically predisposed individuals and is driven by CD4\(^+\) T cells reactive to peptides derived from dietary cereal gluten proteins (1). The disease is tightly associated with the production of autoantibodies against the enzyme transglutaminase 2 (TG2) (2), although it is unclear whether the autoantibodies play a pathogenic role. As other members of the transglutaminase enzyme family, TG2 catalyzes the Ca\(^{2+}\)-dependent crosslinking of proteins through the formation of N\(^\epsilon\)(\(\gamma\)-glutamyl)lysine isopeptide bonds in a reaction known as transamidation (3). Alternatively, a small-molecule primary amine or water can substitute for lysine in the reaction. When water is used as a substrate, glutamine is converted into glutamic acid, and the reaction is termed deamidation. Gluten peptides are rich in glutamine residues that can be targeted by TG2, and TG2-mediated gluten deamidation plays a central role in the activation of gluten-reactive T cells in celiac disease (4). Thus, knowing how TG2 activity is regulated is key to our understanding of the initiation of the immune response.

TG2 is ubiquitously expressed and is produced in the cytosol of human cells. A substantial amount of the enzyme, however, is exported to the extracellular environment through an unconventional mechanism (5). Once outside the cell, TG2 is in a Ca\(^{2+}\)-rich environment supportive of transamidation/deamidation, and the extracellular enzyme is believed to be involved in crosslinking of matrix proteins (6, 7). In the cytosol, on the other hand, TG2 presumably works as GTPase (8). Binding of GTP/GDP inhibits transamidation/deamidation (9, 10). Thus, a high intracellular concentration of GTP in combination with a low Ca\(^{2+}\) concentration prevents transglutaminase activity in the cytosol under normal physiological conditions (10, 11).
Depending on the binding of effectors, TG2 can adopt two distinct conformations (10, 12, 13). Crystal structures of the enzyme in a complex with GDP or GTP reveal a “closed” conformation, in which the two C-terminal $\beta$-barrel domains are folded in on the catalytic core domain and cover the active site (14, 15). Conversely, when a peptide inhibitor was irreversibly attached to the active site cysteine, the enzyme adopted an “open” conformation, where the four structural domains were aligned to give an extended structure (16). The closed and open conformations are thought to reflect intra- and extracellular TG2, respectively. Crystallization of TG2 demonstrated that the open conformation can form a vicinal disulfide bond (16), which has been shown to inactivate the enzyme and presumably serves as a redox switch regulating the activity in the extracellular environment (17, 18). In its active state, TG2 binds up to six Ca$^{2+}$ ions (10, 19). The open conformation, however, was crystallized without bound Ca$^{2+}$ and, hence, does not likely reflect the true structure of the catalytically active enzyme.

Hydrogen/deuterium exchange monitored by mass spectrometry (HDX-MS) has proven to be a valuable tool for studying both dynamic changes in protein conformation and protein-protein interactions (20). The method utilizes the exchange of backbone amide hydrogens with deuterium when proteins are placed in a D$_2$O solution. Backbone amide hydrogens engaged in stable hydrogen bonds are protected against exchange with the solvent (21). According to the thermodynamic principle, all protecting hydrogen bonds will eventually break as the protein cycles through partially unfolded states. Therefore, over time, all backbone amides become transiently exposed to the solvent and exchange-competent. In this way, the deuterium uptake kinetics directly reflects the backbone dynamics of the protein structure. For a protein-protein complex, the binding interface will usually exhibit a decreased deuterium uptake due to having reduced backbone dynamics and being shielded from the solvent (22).
Here, we describe the use of HDX-MS to map conformational changes in TG2 induced by Ca\(^{2+}\) and GTP, and we address the structural implications of cysteine oxidation. By using a panel of TG2-specific monoclonal antibodies (mAbs) (23), we have also mapped the main epitopes targeted by celiac disease autoantibodies. Their locations might explain why one epitope seems to be favored in the response and indicate what drives the activation of autoreactive B cells in celiac disease.
RESULTS

Ca\textsuperscript{2+} ions stabilize the structure of TG2

Due to the large difference in hydrodynamic radius between open and closed TG2, the two conformational states can be separated by non-denaturing PAGE (12, 24). In agreement with earlier reports, we observed that TG2, produced recombinantly in Sf9 insect cells, adopts a closed conformation in the presence of GTP, whereas Ca\textsuperscript{2+} in combination with a specific active-site inhibitor induces an open conformation (Fig. 1A). If no effectors were added to the solutions, the protein was found to populate both conformational states, with the majority of TG2 molecules existing in the open form. Although it is possible that purified TG2 can be associated with effectors derived from the expression system, we will refer to the protein with no added effectors as “effector-free” in the following. Distinct conformational states were also observed when analyzing the intact, deuterated proteins using MS (Fig. 1B and Table S1). When labeling TG2 in the presence of GTP or inhibitor-bound TG2 (iTG2) in the presence of Ca\textsuperscript{2+}, we observed a single isotopic distribution in each mass spectrum, indicating that the proteins only populated one conformational state in solution. In the absence of externally added effectors, TG2 gave rise to two isotopic distributions when analyzed by HDX-MS, indicating that the protein adopted two states with different dynamics. The state with the lower deuterium uptake, State A, was found to mimic the GTP-bound state. However, the state displaying a greater deuterium uptake, State B, did not match the deuterium uptake kinetics of Ca\textsuperscript{2+}-bound iTG2. The Ca\textsuperscript{2+}-bound protein incorporated a smaller amount of deuterium than State B in effector-free TG2, indicating a stabilization of the protein structure. This effect could either be due to the binding of Ca\textsuperscript{2+} or the presence of an inhibitor in the active site. However, State B was found to match the deuterium uptake of iTG2 labeled in the absence of Ca\textsuperscript{2+}, indicating that the inhibitor by itself did not have a pronounced effect on the
structure of TG2 in the open conformation (Fig. S1). Hence, the observed deuterium uptake patterns show that the binding of Ca\(^{2+}\) ions has a stabilizing effect on the open form of TG2.

**Binding of Ca\(^{2+}\) or GTP stabilizes distinct regions in TG2**

To locate regions in TG2 which were impacted by effector binding, we compared the deuterium incorporation at the peptide level (local-exchange analysis) in the effector-free protein and in the Ca\(^{2+}\)-bound and GTP-bound forms. For this purpose, the labeled protein was digested with pepsin prior to MS analysis, resulting in 70.3\% sequence coverage. A complete list of identified peptides can be found in Table S2, and deuterium uptake plots for the N-terminal, core and C-terminal domains are shown in Figure S2.

Many of the peptides obtained from the catalytic core domain of TG2 incorporated more deuterium and thus appeared more dynamic in the effector-free state than in either of the effector-bound states. This indicates that both Ca\(^{2+}\) and GTP have a stabilizing effect on the structure of the core domain. When comparing the deuteration patterns for Ca\(^{2+}\)-bound iTG2 and GTP-bound TG2, we observed that the GTP-bound state exhibited less dynamic behavior in the C-terminal domains and regions of the core domain which, according to the reported crystal structures, are involved in inter-domain interactions when the enzyme adopts its closed conformation (Fig. 2A and Table S2). Other parts of the core domain, however, appeared to have a less dynamic structure in the Ca\(^{2+}\)-bound state. This was observed for peptide fragments covering residues 289-311 and 354-369. The first region comprises parts of the substrate binding pocket as well as a putative Ca\(^{2+}\)-binding site. Both Ca\(^{2+}\) binding and the presence of inhibitor in the active site are therefore plausible explanations for the observed protection. The second region contains a loop adjacent to another, high-affinity, Ca\(^{2+}\) site (19). Based on the crystal structures of the Ca\(^{2+}\)-bound forms of the homologous proteins TG3 and factor XIIIa, this loop is predicted to undergo structural changes upon Ca\(^{2+}\) binding (25, 26).
Thus, our observations with HDX-MS correspond well with previously reported models of transglutaminases.

**Disulfide bond formation in iTG2 prevents the stabilizing effects of Ca\(^{2+}\)**

Cysteine oxidation inactivates TG2 (17, 27, 28), and intramolecular disulfide bond formation presumably leads to TG2 inactivation in the extracellular environment (18, 29). In order to study the structural properties of oxidized TG2, we also performed local-exchange analysis on iTG2, which had been incubated with oxidized glutathione prior to the addition of Ca\(^{2+}\). The peptides 354-369, 355-369 and 370-378 could no longer be observed after oxidation (Table S2), indicating that Cys370 was engaged in disulfide bond formation, which prevented enzymatic cleavage of the peptide bond between residues 369 and 370. Similarly, the peptides 511-532, 517-532 and 548-555 were found to be sensitive to oxidation, strongly indicating that a bond between Cys524 and Cys554, which are in close proximity according to the crystal structures, had also formed. Local-exchange analysis revealed that oxidized iTG2 exhibited an increased deuterium uptake, compared to Ca\(^{2+}\)-bound iTG2, in peptides covering the core domain (Fig. 2B and Table S2). Interestingly, within the region 167-354, comprising most of the core domain, oxidized iTG2 instead matched the behavior of the effector-free protein (Table S2). Thus, even in the presence of Ca\(^{2+}\), oxidized iTG2 had a core domain structure resembling that of the effector-free protein.

**The N-terminal domain of TG2 is unaffected by effector binding and oxidation state**

While changes in the deuterium uptake could be observed in the C-terminal β-barrel domains as well as the catalytic core domain, when TG2 was exposed to different effectors, the peptides derived from the N-terminal β-sandwich domain exhibited deuteration patterns, which were almost identical under all the investigated conditions, including the oxidized state.
(Fig 2B and Table S2). Interestingly, epitopes recognized by a panel of TG2-specific mAbs were previously found to cluster in the N-terminal part of TG2 (30). Despite the constant dynamics of the N-terminal domain, some mAbs bind with greater affinity to the open conformation than to the closed conformation (ref. 30 and Fig. S3A). One explanation for this could be that the proximity of the C-terminal domains inhibit antibody binding to parts of the N-terminal domain when TG2 adopts the closed, GTP-bound conformation. Alternatively, core domain regions, which are close to the N-terminal domain, could take part in antibody binding. However, oxidation of TG2 prior to incubation with Ca$^{2+}$ did not affect mAb binding, indicating that prevention of Ca$^{2+}$-induced structural changes in the core domain does not compromise antibody binding (Fig. S3B).

**Binding of autoantibodies can induce structural changes in TG2**

The panel of TG2-specific mAbs described above was generated from small intestinal biopsies of celiac disease patients (23). Based on competitive ELISA experiments, we recently showed that 49 out of 57 mAbs could be placed into one of four distinct epitope groups (epitope 1-4, targeted by 30, 6, 11 and 2 mAbs, respectively), which correlated with mAb VH-segment usage. Some of the epitopes were partly overlapping, and they all appeared to be clustered closely together (30). In order to study antibody binding to TG2 in more detail, we used HDX-MS to identify TG2 regions with altered deuterium uptake after incubation with mAbs. Hence, mAbs targeting two non-overlapping epitopes (epitope 1 and epitope 2) were incubated with iTG2 in the presence of Ca$^{2+}$, and the resulting immune complexes were subjected to HDX-MS analysis. We selected two mAbs reacting with each epitope and chose the Ca$^{2+}$-bound enzyme as antigen, since this is assumed to represent the extracellular form of TG2, which the immune system encounters.
Digestion of labeled, mAb-bound iTG2 revealed that several peptides in all four structural domains displayed altered deuterium uptake (Fig. S4 and Table S2). Although the effect in most cases was small, the widespread changes indicate that we not only observe localized shielding of the epitope by the bound mAbs, but also an additional, allosteric effect on the TG2 structure. As the presence of an inhibitor in the active site presumably locks TG2 in an open conformation (Fig. S1), we also tested the effect of mAb binding on the effector-free enzyme by monitoring the distribution of TG2 molecules between state A and state B (Fig. 3). Interestingly, in the presence of epitope-1 mAbs the conformational equilibrium of TG2 was shifted in favor of the closed form, State A, whereas epitope-2 mAbs caused the enzyme to only populate the open conformation, State B. Although these observations were made in the absence of effectors they demonstrate the potential of antibody binding to change the TG2 structure, suggesting that celiac disease autoantibodies could have an impact on the function of the enzyme.

**Epitope mapping of anti-TG2 autoantibodies**

Three peptides in the N-terminal domain of TG2 displayed a reduction in deuterium uptake which was specific for mAbs targeting either epitope 1 or epitope 2 and was evident at all time points (Fig. 4A and Fig. S4). Thus, the peptides 5-12 and 27-40 were consistently protected more by epitope-1 than epitope-2 mAbs, whereas peptide 13-26 displayed the opposite pattern. These regions therefore represented the most likely candidates for taking part in epitope 1 and 2, respectively. We previously reported that epitope 2 can be disrupted by introduction of the triple mutation R19S E153S M659S (30). This triple mutation was first described by Simon-Vecsei et al. who showed that it inhibits the binding of serum antibodies from celiac disease patients (31). Of the residues that were mutated in the previous study, Arg19 is located within the peptide we identified as an epitope-2 candidate by HDX-MS. We
therefore tested the effect of the R19S mutation on mAb binding in ELISA using recombinant TG2 produced in *Escherichia coli*. Importantly, this protein displayed the same behavior as TG2 produced in Sf9 insect cells when analyzed by HDX-MS, indicating that the expression system does not affect the folding of the enzyme (Fig. S5). The R19S mutant showed a dramatic loss of reactivity toward epitope-2 mAbs (Fig. 4B), comparable to what can be observed for the triple mutant (Fig. S6A). The binding of epitope-1 mAbs, on the other hand, was largely unaffected, indicating that the R19S mutation selectively targets epitope 2 and, to some extent, the partly overlapping epitope 3 (Fig. 4B).

In order to confirm the location of epitope 1, we constructed TG2 variants harboring triple mutations in either of the two candidate regions identified by HDX-MS (Fig. S6B). Both triple mutations affected binding of epitope-1 mAbs, suggesting that both regions contribute to the epitope. To further dissect epitope 1, we assessed the binding of mAbs to TG2 containing single-amino acid mutations in the two identified regions. Glu8 in the first region and Lys30 in the second region were found to be the most important residues for binding. Mutation of each of these residues selectively abolished most of the binding for epitope-1 mAbs (Fig. 4B).

We have previously provided evidence that epitope 1 overlaps with a binding site for fibronectin (30). The fibronectin binding site is known to be in the N-terminal domain of TG2 and could consist of several separate stretches of amino acids (32-34). In one study, Asp94 and Asp97 were shown to be important for the interaction (33). However, we observed no compromising effect of the previously described D94A and D97A mutations on the binding of epitope-1 mAbs (Fig. 4B and Fig. S6C). Interestingly, most epitope-3 mAbs lost reactivity upon introduction of the D94A mutation (Fig. 4B), suggesting that epitope 3, which does not overlap with the fibronectin binding site (30), is located around Asp94.
Since the epitope groups, we have assigned, reflect targeted surface areas rather than specific interactions, not all mAbs in each group showed the same loss of reactivity toward the TG2 mutants (Fig. 4B). Thus, for some antibodies, other residues than the ones identified are expected to be important for binding. We cannot rule out that neighboring residues in the core domain are involved, but our results indicate that the main epitopes targeted by celiac disease autoantibodies are clustered in the N-terminal domain of TG2 with the four residues reported in Figure 4 collectively being important for binding most of the antibodies. The introduced mutations selectively disrupted individual epitopes and did not affect the overall conformation of TG2 as judged by non-denaturing PAGE analysis (Fig. S7A). Interestingly, for a number of mAbs the effect of the mutations was decreased, if binding was assessed in the presence of Ca$^{2+}$ using inhibitor-bound TG2 as antigen (Fig. S7B). Thus, Ca$^{2+}$ binding to TG2 can shield the effect of introduced mutations, suggesting that there could be one or more Ca$^{2+}$ binding sites in the vicinity of the antibody binding sites.
DISCUSSION

TG2 plays a central role in celiac disease, both through its enzymatic activity, which leads to gluten deamidation, and as the target of autoantibodies. Conformational changes in TG2 control the enzyme’s activity and are also important for autoantibody binding. Describing the different structural states of TG2 and knowing how the enzyme conformation is regulated are therefore relevant for our understanding of celiac disease pathogenesis.

Studies performed in mice have shown that extracellular TG2 exists predominantly in a catalytically inactive form under normal conditions but can become activated through inflammation or in the presence of a reducing agent (18, 29). The latter observation suggests that the enzyme is inactivated by oxidation in the extracellular environment. In this regard, Cys370 in TG2 has been implicated in disulfide bond formation with its neighbor Cys371 or, alternatively, with Cys230 (16, 17, 35). Based on the disappearance of signals from the mass spectrum of TG2 following oxidation, we could confirm the formation of these disulfide bonds. In addition, we detected a previously undescribed disulfide bond between Cys524 and Cys554 (Table S2).

The mechanism by which TG2 is inactivated by cysteine oxidation is not clearly understood, but based on the negative effect of Ca\(^{2+}\) on the rate of oxidation (17, 27), it has been proposed that structural changes induced by disulfide bond formation interferes with Ca\(^{2+}\) binding and, thus, activation of the enzyme (17). Our data support a model in which oxidation negatively affects Ca\(^{2+}\) binding, since the core domain of oxidized TG2 remains dynamic in the presence of Ca\(^{2+}\), exhibiting a HDX pattern similar to effector-free TG2. This suggests that disulfide bond formation prevents the Ca\(^{2+}\)-induced structural changes that are necessary for TG2 activation. Interestingly, Cys370 and Cys371 are situated in a loop which displayed decreased dynamics in the Ca\(^{2+}\)-bound protein. Thus, it is likely that structural changes in this loop play a key role in regulating the enzymatic activity.
In the absence of effectors, we observed that the binding of TG2 by mAbs induced large-scale conformational changes in the enzyme (Fig. 3). In the presence of Ca^{2+}, mAb binding also appeared to cause structural changes in TG2, as several regions outside of the epitope displayed altered deuterium uptake upon mAb binding. TG2-reactive autoantibodies produced in celiac disease could therefore influence TG2 function. Indications of this have previously been observed by the addition of celiac disease antibodies to cell culture systems (36-41). The mechanisms underlying the various biological effects reported are not well understood, though. Direct interference with enzymatic activity is not expected to play a role, as it has previously been shown that the TG2-reactive mAbs used in this study do not inhibit TG2-mediated transamidation/deamidation (23). However, TG2 has been implicated in a number of processes that do not depend on its catalytic activity, but rather on interactions with components of the extracellular matrix and the plasma membrane (42-45). It is therefore possible that TG2-reactive autoantibodies can play a pathogenic role in celiac disease by inducing conformational changes that interfere with the binding between TG2 and its interaction partners. Another intriguing possibility is that antibody binding renders the enzyme more or less susceptible to the formation of intramolecular disulfide bonds.

It was previously suggested that the transamidation activity of TG2 is directly involved in the activation of autoreactive B cells in celiac disease through the formation of isopeptide-linked B-cell receptors (BCRs) of the IgD isotype on the surface of TG2-targeting naïve cells (23). B cells that bind TG2 in a way that allows the enzyme to be catalytically active would then become selectively activated, and this should be reflected in the location of the epitopes. Recently, we showed that epitope 1 is recognized by more than half of the mAbs cloned from TG2-specific plasma cells in the celiac disease lesion (30). The majority of these mAbs used the VH5-51 gene segment, which is dominating both in the mAb panel used here (23) and in an earlier reported panel of TG2-reactive antibody fragments obtained from phage display
libraries (46). The overrepresentation of VH5-51 antibodies is most likely related to the location of the epitope they recognize. Thus, a possible explanation for the VH5-51 preference is that TG2 bound by an epitope 1-targeting BCR is oriented in a way that optimizes catalytic formation of isopeptide crosslinks between BCRs on the cell surface. In this regard, it is noteworthy that the residues we have identified to be part of epitope 1 lie on an axis, which includes the active site (Fig. 4A). The location of epitope 1 thereby predicts that the active site will face toward the BCR upon binding and implies that BCR crosslinking can happen efficiently. The location of epitope 2, on the other hand, suggests that the active site will point in a different direction with respect to the BCR upon binding. This could be a reason why epitope 2 is only recognized by 10% of the mAbs in the TG2-specific panel (30) and thus appears to be targeted less frequently than epitope 1 by plasma cells in the celiac lesion.

In conclusion, our data demonstrate the use of HDX-MS for studying conformational changes and mapping of epitopes in TG2. Binding of Ca\(^{2+}\) and GTP was associated with stabilization of distinct regions in the catalytic core and C-terminal domains of the enzyme. Notably, oxidation prevented the Ca\(^{2+}\)-induced changes. Binding of mAbs derived from celiac disease patients resulted in pronounced allosteric effects on TG2 structure, suggesting a process whereby celiac disease autoantibodies could alter the function of TG2. Finally, mapping of two of the main epitopes revealed that they are located close to each other in the N-terminal domain of TG2. We propose that the location of the preferentially targeted epitope 1 indirectly supports the enzymatic activity of BCR-bound TG2 by positioning the enzyme in an orientation that is optimal for crosslinking of BCRs on the cell surface. This effect could be instrumental for the activation of TG2-reactive B cells in celiac disease.
MATERIALS AND METHODS

Proteins. Recombinant human TG2 was either obtained from Phadia as purified protein produced in Sf9 insect cells or expressed in E. coli and purified as previously described (17). TG2 mutants were produced in E. coli. Mutations were introduced in the TG2 sequence using the QuickChange Site-directed Mutagenesis Kit (Stratagene) or by PCR amplification followed by subcloning into the pET-28a vector (Novagen) between the NdeI and HindIII sites. All mutations were verified by DNA sequencing (GATC Biotech). TG2-specific human mAbs were produced essentially as previously described (23, 47). Briefly, IgG1 and Igκ expression vectors containing the cloned heavy and light chain variable regions were co-transfected into HEK293 cells. Six days after transfection, antibodies were purified from the cell supernatants on Protein G sepharose (GE Healthcare). The TG2-specific mouse mAb CUB7402 was obtained from NeoMarkers.

TG2 modifications. In order to avoid auto-crosslinking of TG2 in the presence of Ca\(^{2+}\), the active site cysteine was irreversibly bound to the substrate-mimicking peptide Ac-P(DON)LPF-NH\(_2\) (where DON is the electrophilic amino acid 6-diazo-5-oxo-L-norleucine, ref. 16) (Zedira). TG2 was preincubated with 0.5 mM of the inhibitor for 30 min at room temperature before the reaction was started by the addition of CaCl\(_2\) to a final concentration of 5 mM. After 20 min incubation, excess free inhibitor was removed by size-exclusion chromatography. To obtain the oxidized enzyme, iTG2 was incubated with 2 mM oxidized glutathione in the absence of Ca\(^{2+}\) for 3 h at 30°C.

Deuterium labeling. All exchange reactions were performed at 25°C in tris-buffered saline (TBS) with or without 5 mM CaCl\(_2\). Buffer made with D\(_2\)O was added to 90% (v/v) to initiate labeling at a final TG2 concentration of 0.06 mg/ml. After 10, 100 and 1000 s, samples were
collected and pH lowered to 2.5 by the addition of formic acid. The samples were then immediately snap-frozen in liquid nitrogen and stored at -80°C until analyzed (SI Methods). Undeuterated control samples were treated in the same way. To study effector-bound TG2, the enzyme was pre-treated at 2.6 mg/ml with 1 mM GTP or 5 mM CaCl₂. The effect of antibody binding was assessed by incubating TG2 or iTG2 with a two-fold molar excess (by antigen binding sites) of TG2-specific mAbs for at least 30 min at room temperature prior to labeling. Triplicate labelings were performed and analyzed for samples which did not contain antibodies. For antibody-interaction studies, a single labeling was performed, and each sample was analyzed twice.

Non-denaturing PAGE. The preparations of TG2 and iTG2 that were used for deuterium labeling and antibody binding studies were also analyzed by non-denaturing PAGE as previously reported (12, 24). The enzyme was either left untreated or incubated with 1 mM GTP or 5 mM CaCl₂ for 30 min at room temperature prior to loading of the gel. Electrophoresis was performed at 125 V for 75 min using ice-cold running buffer and with the gel chamber submerged in an ice bath.

ELISA assays. Wild-type or mutant TG2 produced in E. coli was coated in microtiter plates at 3 µg/ml in TBS. Incubations with mAbs were done at 37°C in TBS with 0.1% (v/v) Tween20, followed by detection of bound antibody with alkaline phosphatase-conjugated rabbit anti-human IgG or goat anti-mouse IgG (Abcam). After addition of phosphatase substrate, absorbance was measured at 405 nm in a microplate reader (Thermo Scientific), and saturation binding curves were produced by non-linear regression analysis of the OD values. In experiments where only a single mAb concentration was used, this was picked to fall within the linear range of the assay based on initial titration of each mAb. In cases where Ca²⁺
was present, 5 mM CaCl$_2$ was included during coating of iTG2 and incubation with antibodies. TG2 produced in Sf9 insect cells was used to compare the recognition of open and closed or oxidized and reduced TG2 by mAbs. Open, Ca$^{2+}$-bound iTG2 was generated as described above. Closed, GTP-bound TG2 was generated by incubating the enzyme with 1 mM GTP prior to coating and keeping the GTP concentration at 50 µM during coating and antibody incubations. Oxidized iTG2 was generated prior to coating and compared to non-oxidized iTG2 in the presence of 5 mM CaCl$_2$.

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FIGURE LEGENDS

**Fig. 1.** Detection of open and closed TG2 conformations. (A) Separation of open and closed TG2 by non-denaturing PAGE. TG2 was either left untreated or incubated with GTP or Ca$^{2+}$ prior to loading. In order to avoid extensive auto-crosslinking of active TG2, the peptide inhibitor Ac-P(DON)LPF-NH$_2$ was included in the sample with Ca$^{2+}$. This treatment yields TG2 with the substrate-mimicking inhibitor bound irreversibly to the active site cysteine (iTG2) (16). (B) Deconvoluted mass spectra showing the mass of full-length TG2 or iTG2 before (solid black line) and after 10 s (dashed red line) or 1000 s (dashed blue line) incubation in 90% D$_2$O. Incubation was done in the absence of effectors or with GTP or Ca$^{2+}$ present. Covalent attachment of inhibitor peptide to the active site cysteine results in a 638 Da mass increase. The spectra are aligned accordingly, so that deuterium uptake can be compared directly for the different states.

**Fig. 2.** TG2 regions which undergo effector-induced structural changes. The relevant regions are highlighted on both the open conformation (PDB code 2Q3Z) and the closed conformation (PDB code 1KV3) crystal structure, containing bound peptide inhibitor and GDP, respectively (bound molecules are shown in red stick representation). Importantly, the open structure does not contain bound Ca$^{2+}$ ions and is therefore not identical to the protein studied here. Coloring matches the one used in Table S2. (A) Regions with lower deuterium uptake in Ca$^{2+}$-bound iTG2 compared to GTP-bound TG2 are shown in turquoise, whereas regions displaying the opposite pattern are shown in magenta. (B) Orange regions displayed increased deuterium uptake, when iTG2 had been oxidized prior to the addition of Ca$^{2+}$. Regions where the deuterium uptake was similar in all investigated states are shown in green.
**Fig. 3.** Deconvoluted mass spectra of intact effector-free TG2 before (solid black line) and after (dashed colored lines) 1000 s incubation in 90% D$_2$O. Deuterium incorporation was done in the presence or absence of TG2-reactive mAbs recognizing epitope 1 (mAbs 679-14-E06 and 693-10-B06) or epitope 2 (mAbs 693-1-F06 and 693-1-A03). Different mAbs recognizing the same epitope have the same effect on the distribution of TG2 molecules between closed (State A) and open (State B) conformations.

**Fig. 4.** Identification of epitopes targeted by TG2-specific autoantibodies. (A) Mapping of epitopes using HDX-MS. Regions protected from deuterium incorporation are shown on the open TG2 structure (PDB code 2Q3Z) with a peptide inhibitor (red stick representation) attached to the active-site cysteine residue. iTG2 was incubated in 90% D$_2$O containing 5 mM CaCl$_2$ for 10 s, 100 s or 1000 s in the presence or absence of mAb and digested with pepsin before analysis. Regions that were protected by mAbs recognizing epitope 1 (mAbs 679-14-E06 and 693-10-B06) but not by mAbs recognizing epitope 2 (mAbs 693-1-F06 and 693-1-A03) are shown in blue, whereas regions with the opposite pattern are shown in green. Residues that were found to be important for mAb binding are shown in red stick representation. (B) Confirmation of epitopes by mutational analysis. The reactivity of 53 mAbs to wild-type (WT) and mutant TG2 was tested in ELISA. Signals obtained with the indicated mutants are given relative to the signals obtained with WT TG2. The majority of the mAbs could be placed into one of three main epitope groups (epitope 1-3) based on their ability to compete with each other for binding (30). Two mAbs have been assigned to a minor epitope (epitope 4, open circles) and five mAbs presumably target other epitopes. Red bars indicate medians.
**A**

Image shows a gel with lanes labeled 1, 2, and 3. Lane 1 is labeled "Effector-free TG2," Lane 2 is labeled "TG2 + GTP," and Lane 3 is labeled "iTG2 + Ca^{2+}.

**B**

Graphs show relative intensity plots for different states:
- **Effector-free TG2**
- **TG2 + GTP**
- **iTG2 + Ca^{2+}**

Peak positions are indicated with Da units for different time points: Undeuterated, 10 s exchange-in, and 1000 s exchange-in.
TG2 + GTP
Closed conformation

Protected when GTP-bound
Regions w/o coverage

Protected when Ca²⁺-bound
Regions w. coverage

Similar in all states

iTG2 + Ca²⁺
Open conformation

More dynamic when oxidized
A

Glu8  Lys30  Inhibitor
Asp94  Arg19

Regions w/o coverage
Regions w. coverage
Stabilized by epitope-1 mAbs
Stabilized by epitope-2 mAbs

B

Epitope 1

Relative binding (%)

Epitope 2

Epitope 3

Epitope 4 / Other

Relative binding (%)
Supporting Information

Activity-regulating structural changes and autoantibody epitopes in transglutaminase 2 assessed by hydrogen/deuterium exchange

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SI METHODS

**Mass spectrometry.** Samples were run using a Waters HDX-Manager with a desalting flow provided by an Agilent 1260 Infinity quaternary pump and a gradient flow provided by a nanoAcquity UPLC Binary Solvent Manager (Waters). For measurements on intact proteins (global-exchange analysis), a 50 µL/min gradient flow (5% B to 50% B over 3 min) was used, along with a MassPREP Micro Desalting Column (Waters) for desalting. For measurements on peptides (local-exchange analysis), a 40 µL/min gradient flow (5% B to 50% B over 12 min) was used, along with ACQUITY UPLC BEH C18 1.7 µm 2.1 x 5 mm Vanguard Pre-Columns and 1.0 x 100 mm analytical columns for desalting and peptide separation, respectively. A column containing immobilized pepsin was placed between the loop and the desalting trap to digest the samples. Samples were subjected to ESI-MS using a Waters Synapt G1 mass spectrometer with detector MCP voltage of 1700 V for intact proteins or 1950 V for peptides. Rigorous washing steps were performed between each injection.

**Data analysis.** Mass spectra of intact TG2 were deconvoluted using MaxEnt 1 in MassLynx v4.1. Peptides from peptic digests were identified from DDA MS/MS runs using ProteinLynx Global Server v2.4 (Waters) and MassAI v1.05 (MassAI Bioinformatics, http://www.massai.dk). Deuterium incorporation for intact proteins and peptides was quantified using DynamX v1.0 (Waters). Visualizations (PDB codes 2Q3Z and 1KV3) were created using VMD v1.9.1 (1).
SUPPORTING REFERENCES

SUPPORTING FIGURE LEGENDS

**Fig. S1.** Deconvoluted global-exchange spectra for effector-free WT TG2, effector-free iTG2 and iTG2 labeled in the presence of 5 mM CaCl₂. In the absence of Ca²⁺, iTG2 and the open conformation, State B, in WT TG2 were found to exhibit similar behavior during isotopic exchange, indicating that the inhibitor had a minimal impact on the dynamics of the open conformation. The inhibitor did, however, appear to prevent the formation of closed conformation, State A. Adding Ca²⁺ to the solution prior to labeling had a pronounced stabilizing effect on iTG2.

**Fig. S2.** Deuterium uptake plots for peptides located in the N-terminal, core and C-terminal domains of TG2. Error bars represent SD based on labeling triplicates.

**Fig. S3.** Saturation binding curves showing reactivity of mAbs targeting different epitopes to TG2 subjected to various treatments in ELISA. The human mAbs have previously been assigned to epitope 1-4 or “other” (2). The mouse mAb CUB7402, which targets a linear epitope, was included as a control that should give the same signal irrespective of TG2 conformation. (A) Comparison of mAb reactivity to Ca²⁺-bound iTG2 and GTP-bound TG2. (B) Comparison of mAb reactivity to non-oxidized, Ca²⁺-bound iTG2 and iTG2 that was oxidized prior to Ca²⁺ treatment.

**Fig. S4.** Deuterium uptake plots for TG2 peptides which exhibit a change in deuterium uptake upon antibody binding. Ca²⁺-bound iTG2 was incubated in the presence or absence of TG2-specific mAbs prior to labeling. Exchange time, in seconds, is shown on the x-axis and deuterium uptake is shown on the y-axis. For clarity, uptake plots for TG2-mAb complexes,
which did not result in altered deuterium uptake, are not shown. Dotted horizontal lines represent the observed deuterium uptake in a full-deuteration control. Error bars represent SD based on analytical duplicates of a single labeling. For each peptide, all shown TG2-mAb complexes were found to exhibit a different deuterium uptake than unbound TG2 in at least 2 of 3 data points, based on unpaired two-tailed t-tests (p < 0.05).

**Fig. S5.** Comparison of the deuterium uptake by TG2 produced in different expression systems. Recombinant human TG2 was either expressed in Sf9 insect cells or in *E. coli*. The two proteins contain slightly different purification tags (2), causing *E.coli*-produced TG2 to have a higher mass than the Sf9-produced enzyme. Deconvoluted mass spectra for GTP-bound TG2 and Ca\(^{2+}\)-bound iTG2, prior to deuteration and after 10, 100 and 1000 s exchange, are shown. The corresponding values for deuterium uptake are shown in tables and displayed in a deuterium uptake plot. The two TG2 preparations exhibit very similar deuterium uptake, indicating that there are no major differences in their folding. Note that the absolute deuterium uptake values shown here are greater than those displayed in Table S1. This is due to these data being recorded at a later date, using an optimized analytical workflow.

**Fig. S6.** Saturation binding curves showing the effect of introduced mutations on mAb binding to TG2 in ELISA. mAbs targeting different epitopes were tested for binding to WT and mutant TG2. The TG2-reactive mouse mAb CUB7402 was included as a control for equal coating of the TG2 variants. *(A)* Comparison of mAb reactivity to WT TG2, the R19S E153S M659S (REM) triple mutant and R19S TG2. *(B)* Comparison of mAb reactivity to WT TG2 and the triple mutants E8Q R9S D11N (ERD) and R28S E29Q K30E (REK). The mutants were constructed based on the sequence of the homologous protein TG3, which is not
recognized by the mAbs (2). (C) Comparison of mAb reactivity to WT, D94A and D97A TG2.

**Fig. S7.** Effect of TG2 mutations on protein conformation and binding to mAbs in the presence of Ca$^{2+}$. (A) Non-denaturing PAGE analysis of WT and mutant TG2 that had either been left untreated or incubated with GTP or a combination of Ca$^{2+}$ and the active-site inhibitor Ac-P(DON)LPF-NH$_2$. WT and mutants display the same distributions between open and closed conformations. (B) Reactivity of 53 TG2-specific mAbs to mutant iTG2 in the presence of Ca$^{2+}$ as determined by ELISA. Signals obtained with the indicated mutants are given relative to the signals obtained with WT iTG2. Red bars indicate medians. Compared to the results reported in Figure 4, the mutations have lower impact on the binding of several mAbs, when Ca$^{2+}$ is present.
Table S1. Deuterium uptake in different TG2 states

<table>
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<tr>
<th>Exchange time</th>
<th>State A</th>
<th>State B</th>
<th>TG2 + GTP</th>
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<td>10 s</td>
<td>118.6 (+/- 2.6)</td>
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<td>161.6 (+/- 3.0)</td>
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<td>1000 s</td>
<td>204.9 (+/- 5.9)</td>
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<td>197.1 (+/- 0.9)</td>
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Values represent the mass increase in Da ± SD based on analytical duplicates of a single labeling.
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Color coding matches the one used in Figure 2 on the open and closed structures of TG2. Deuterium uptake plots, which the identifications are based on, are shown in Figure S2.

† The relevant deuterium uptake plots are shown in Figure S4. Deuterium uptake was found to be significantly different upon mAb-binding, based on unpaired two-tailed t-tests (p < 0.05).

‡ Peptides could not be observed in the oxidized samples. Cysteine residues present in these peptides or at the pepsin cleavage site have presumably formed disulfide bonds.
State A

Effector-free wt TG2

State B

Effector-free iTG2

iTG2 + Ca^{2+}

Relative intensity

Da

Relative intensity

Da

Relative intensity

Da

Undeuterated

10 s exchange-in

1000 s exchange-in
N-terminal domain

Core domain
C-terminal domains