Roles of Calcium Ions in the Activation and Activity of the Transglutaminase 3 Enzyme*

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The transglutaminase 3 enzyme is widely expressed in many tissues including epithelia. We have shown previously that it can bind three Ca\(^{2+}\) ions, which in site one is constitutively bound, while those in sites two and three are acquired during activation and are required for activity. In particular, binding at site three opens a channel through the enzyme and exposes two tryptophan residues near the active site that are thought to be important for enzyme reaction. In this study, we have solved the structures of three more forms of this enzyme by x-ray crystallography in the presence of Ca\(^{2+}\) and/or Mg\(^{2+}\), which provide new insights on the precise contribution of each Ca\(^{2+}\) ion to activation and activity. First, we found that Ca\(^{2+}\) ion in site one is exchanged with difficulty, and it has a binding affinity of \(K_d = 0.3\ \mu M\) (\(\Delta H = -6.70 \pm 0.52\ \text{cal/mol}\)), which suggests it is important for the stabilization of the enzyme. Site two can be occupied by some lanthanides but only Ca\(^{2+}\) of the Group 2 family of alkali earth metals, and its occupancy is required for activity. Site three can be occupied by some lanthanides, Ca\(^{2+}\), or Mg\(^{2+}\); however, when Mg\(^{2+}\) is present, the enzyme is inactive, and the channel is closed. Thus Ca\(^{2+}\) binding in both sites two and three cooperate in opening the channel. We speculate that manipulation of the channel opening could be controlled by intracellular cation levels. Together, these data have important implications for reaction mechanism of the enzyme: the opening of a channel perhaps controls access to and manipulation of substrates at the active site.

Transglutaminases (TGases)\(^1\) are ubiquitous enzymes that are used widely in biology for many different purposes. There are nine different genes for TGases in the human (1–5). Typically, TGases recognize and activate a protein-bound Gln residue by formation of a thiol-acyl intermediate form. The recognition of a Gln residue may be highly specific, such as apparently the case of the factor XIIIa, TGase 3, and TGase 4 enzymes, or with rather low specificity as for the TGase 2 enzyme. Next, this acyl intermediate is approached by a nucleophilic second substrate, which transfers onto the Gln residue. Commonly, the nucleophile is water, resulting in the net deamidation of a target Gln residue. If the nucleophile is the \(\epsilon-\text{NH}_2\) of a protein-bound Lys residue, an isopeptide \(\epsilon-(\gamma\text{-glutamyl})\text{lysine}\) cross-link is formed. As this cannot be cleaved in animal cells, controlled TGase activity thereby provides an efficient way for the formation of stable, insoluble macromolecular complexes. Other nucleophiles used include polyamines (to form mono- or bi-substituted/cross-linked adducts) or -OH groups to form ester linkages (as in the case of the membrane-bound TGase 1 enzyme to link epidermis-specific ceramides required for barrier function) (6). The TGase 2 enzyme can bind GTP nucleotides, and there is a reciprocal relationship between this binding and transamidation reactivity (7–9), presumably because the GTP binds in the vicinity of where cross-linking substrates must gain access to the enzyme for reaction (10). It remains to be demonstrated whether other TGase isoforms can also manipulate nucleotides.

In addition, TGase enzyme transamidation reactions require Ca\(^{2+}\), in both \textit{in vitro} assays and \textit{in vivo} (1–5). In the few cases measured, the Ca\(^{2+}\) concentration required to activate an enzyme isoform (>500 \(\mu M\)) is far higher than net intracellular Ca\(^{2+}\) ion concentrations (about 100 \(\text{nM}\)) (11). Also, Ca\(^{2+}\) is not required for GTP binding (7–9). Thus manipulation of intracellular Ca\(^{2+}\) concentrations could afford an effective way to control TGase functions, including cross-linking.

Despite the evident essential role of Ca\(^{2+}\) ions in TGase cross-linking reactions, very little is known structurally/functionally why Ca\(^{2+}\) ions are in fact required. Recently however, we solved the structure of the zymogen and the activated form of the TGase 3 enzyme system (12, 13). Whereas the zymogen constitutively acquires one ion during expression (in baculovirus), it is insufficient for activity. Upon proteolytic cleavage of a loop segment connecting the active site domain to the \(\beta\)-barrel 1 domain, the cleaved enzyme can acquire two additional Ca\(^{2+}\) ions, and becomes fully active. These events coincide with the opening of a channel, which passes through the enzyme. Moreover, this channel exposes two tryptophan residues, which are believed to be important in the enzyme reaction mechanism (13). The channel is formed upon the opening of an existing deep cavity by movement of a \(\beta\)-strand loop (residues Gly\(^{322}\)–Ser\(^{325}\) so that Asp\(^{324}\) can coordinate with the Ca\(^{2+}\) ion that occupies the site nominally termed site three. We also showed that certain trivalent lanthanide ions can occupy sites three and/or two with retention of activity and an open channel. However, key questions remain unanswered. What are the relative contributions of the occupancy of each Ca\(^{2+}\) ion in the
Calcium Ions and Transglutaminase 3 Activity

two sites, and why is there a site two? Does the proteolyzed form in the absence of metal ions in sites two and three possess a channel, or partially opened channel, or no channel? Nor of the dialysate were withdrawn for liquid scintillation counting. Assays were done at 37 °C in 0.2 ml of 20 mM Tris-HCl (pH 7.5), 0.5 mM CaCl₂, 1 mM MgCl₂, and 1 mM EDTA. The EGTA solution was removed by dialysis against 20 mM Tris-HCl (pH 8.0). The concentration of zincom treated with dispase at 37° for 30 min and dialyzed five times against 20 mM Tris-HCl (pH 8.0) and 5 mM EGTA. The EGTA solution was removed by dialysis against 20 mM Tris-HCl (pH 8.0). The concentration of zincom treated with dispase at 37° for 30 min and dialyzed five times against 20 mM Tris-HCl (pH 8.0) and 5 mM EGTA. The EGTA solution was removed by dialysis against 20 mM Tris-HCl (pH 8.0). The concentration of zincom treated with dispase at 37° for 30 min and dialyzed five times against 20 mM Tris-HCl (pH 8.0) and 5 mM EGTA.

**Biophysical Studies of TGase 3—** The thermodynamic properties of Ca²⁺ binding to the activated TGase 3 were measured by isothermal titration calorimetry using a MicroCal VP-ITC calorimeter as described previously (13). Data were fitted to appropriate binding models and thermodynamic parameters were determined from nonlinear least-squares fits, using ORIGIN™ software. Quantitative measurement of metal ion contents was performed by the use of inductively coupled plasma-mass spectrometry dynamic reaction cell (ICPMS-DRC) using a PerkinElmer ELAN 6100 instrument. Prior to measurements, the TGase 3 (10 mg/ml) was treated with dispase at 37° for 30 min and dialyzed five times against 20 mM Tris-HCl (pH 8.0) and 5 mM EGTA. The EGTA solution was removed by dialysis against 20 mM Tris-HCl (pH 8.0). The concentration of zincom treated with dispase at 37° for 30 min and dialyzed five times against 20 mM Tris-HCl (pH 8.0) and 5 mM EGTA. The EGTA solution was removed by dialysis against 20 mM Tris-HCl (pH 8.0). The concentration of zincom treated with dispase at 37° for 30 min and dialyzed five times against 20 mM Tris-HCl (pH 8.0) and 5 mM EGTA. The EGTA solution was removed by dialysis against 20 mM Tris-HCl (pH 8.0). The concentration of zincom treated with dispase at 37° for 30 min and dialyzed five times against 20 mM Tris-HCl (pH 8.0) and 5 mM EGTA. The EGTA solution was removed by dialysis against 20 mM Tris-HCl (pH 8.0). The concentration of zincom treated with dispase at 37° for 30 min and dialyzed five times against 20 mM Tris-HCl (pH 8.0) and 5 mM EGTA.

**TGase 3 Activity Assay—** TGase activity was assayed by incorporation of [14C]putrescine into casein (25). Assays were done at 37 °C in 0.2 ml of 20 mM Tris-HCl (pH 7.5), 0.5 mM CaCl₂, 1 mM MgCl₂, and 1 mM EDTA.

**TGase 3 Ca²⁺ Ion Binding Assay—** Calcium binding was determined by equilibrium dialysis (26) allowing equilibration of the enzyme (0.5–0.8 mg/ml) against 100 volumes of buffer containing 20 mM Tris-HCl (pH 7.5) and 0.1 mM dithiothreitol supplemented with 46 mM β-glycerophosphate (500 cpm/μmol) up to 10 mM. After 48 h at 4 °C, aliquots of the enzyme solution and of the dialysate were withdrawn for liquid scintillation counting. The results were normalized to the protein concentration determined by absorbance at 280 nm.

**RESULTS AND DISCUSSION**

The purpose of this study is to better understand the differential structural/functional roles of cations in the three known positions of the TGase 3 enzyme system and to explore their
roles in the activation process. We have therefore solved the structures of three additional forms, and selected features are summarized in Table II. Briefly, the overall structure of TGase 3 consists of four folded domains that are common to the structures solved here and previously (13). The amino-terminal \( \beta \)-sandwich domain of TGase 3 (first 134 amino acids) consists of nine strands of \( \beta \)-sheets interspersed with three \( \alpha \)-helices adopting two four-stranded antiparallel sheets twisted about 50\(^\circ\) with respect to each other to form a distorted \( \beta \)-barrel. The catalytic core domain (residues 135–472) consists of 15 \( \beta \)-sheets interspersed with 15 \( \alpha \)-helical segments and is \( \alpha/\beta \)-type. It contains a central twisted six-stranded antiparallel \( \beta \)-sheet motif, which separates two clusters of \( \alpha \)-helices. The longest \( \alpha \)-helix of 16 residues is located in the center of the molecule and harbors the active site Cys\(^{272} \) residue, which is buried in this domain. Other members of the catalytic triad, His\(^{330} \) and Asp\(^{353} \), are located on adjacent strands of \( \beta \)-sheets. The barrel 1 and barrel 2 domains consist of largely of \( \beta \)-sheets arranged in barrel-like conformations, span from residues 473–592 and 593–692, respectively. Residues 462 to 471 form a highly flexible solvent-exposed loop that links the last \( \alpha \)-helical segment of the catalytic core domain to the first \( \beta \)-strand of the barrel 1 domain. This flexible hinge region harbors Ser\(^{469} \), the cleavage site used for proteolytic activation of the TGase 3 zymogen. All structures possess two non-proline cis peptide bonds: Arg\(^{388} \)–Tyr\(^{399} \) in the strand prior to the loop of \( \alpha \)-helix that contains the active site Cys\(^{272} \) residue; and Asn\(^{382} \)–Phe\(^{383} \) in a loop adjoining two \( \alpha \)-helices of the core domain. Furthermore, the Gly\(^{367} \)–Pro\(^{368} \) cis-peptide bond is conserved in location in all TGases.

**The Structure of Form I Is Essentially Identical to the Activated TGase 3**—First, we wanted to know whether the channel is opened by cleavage of the enzyme alone, that is, before insertion of metal ions at sites two and three. Proteolysis of the TGase 3 zymogen with dispase cleaves at Ser\(^{469} \) the flexible hinge of sequences that joins the active site domain to \( \beta \)-barrel 1 domains. This form I, measured in the presence of dispase but with no added Ca\(^{2+} \), is fully active (Table II). Its x-ray diffraction crystal structure is shown in Fig. 1a. The refinement crystallographic statistics are listed in Table I. The x-ray models consist of two crystallographically independent monomers of 692 residues. Both monomers have missing density for a flexible loop between residues 461–479. The r.m.s. difference between the 673 Ca carbon atoms of the two monomers is 0.41 Å, indicating that the Cα backbone structures are almost identical. Each monomer has three Ca\(^{2+} \) ions, and an open channel (Fig. 1b). However, its structure and coordinations of the Ca\(^{2+} \) ions (Fig. 2, form I and Table III) are essentially identical to those of the activated enzyme solved previously (Ref. 13 and fourth item of Table II). It turns out that the dispase used contains high levels of Ca\(^{2+} \) salts, so that during proteolysis, the cleaved enzyme acquires the two additional Ca\(^{2+} \) ions from the solution to occupy sites two and three. However, the crystal packing arrangement of the two monomers in the asymmetric unit of the structure is the P2\(_1\) space group, different from the P1 space group seen previously (13). Accessible surface area calculations for interface between the monomers in the asymmetric unit give a value of 2694 Å\(^2\) for the area buried upon formation of the two crystallographic monomers.

**Form II: the Proteolyzed Enzyme Without Ca\(^{2+} \) Ions at Sites Two and Three**—We were able to quantitatively remove the two Ca\(^{2+} \) ions by chromatography through a mono Q column (confirmed by use of ICPMS-DRC), and this form II was crystallized. It has one molecule of 692 residues with missing density for residues 462–478 in its asymmetric unit of the P2\(_1\) space group (Fig. 1c and Table II). This enzyme is inactive, and
the channel is closed (Fig. 1d). Comparisons of its structures and the coordination of its single Ca$^{2+}$ ion at site one reveal near identity to the uncleaved zymogen (Fig. 2, form II and Table III, and Ref. 13). The only significant structural change is that the loop Ile223–Val231 containing Asp228 has shifted away so that Asn227 and Asn229 instead coordinate with the Ca$^{2+}$ ion in site one. Both are solvent-exposed yielding direct coordination with the Ca$^{2+}$ ion, thereby binding the ion even more tightly. In summary, these data show that metal ions must occupy sites two and/or three to open the channel necessary for activity.

The Ca$^{2+}$ Ion in Site One Contributes Significant Stabilization to TGase 3—Previously, we were unable to assess the energetic consequences of binding a Ca$^{2+}$ ion at site one. However, by use of more rigorous procedures, we were able to remove 85% of this ion, as assessed by titration with $^{45}$Ca$^{2+}$ and direct quantitative measurements employing ICPMS-DRC (Fig. 3a). By use of microcalorimetry, the binding data was obtained for the Ca$^{2+}$ ions (Fig. 3, b and c). At the lowest Ca$^{2+}$ ion concentrations, there is one high affinity binding site (average $K_d = 0.3 \mu M$) with $\Delta H = -6.70 \pm 0.52$ kcal/mol. Addition of further Ca$^{2+}$ ions indicated two other low affinity sites (average $K_d = 3.0 \mu M$) with $\Delta H = -3.73 \pm 0.16$ kcal/mol. The shape of the latter part of the curve and values are consistent with the previous reported data for Ca$^{2+}$ ion binding at sites two and three (13). Thus the first part of the curve corresponds to binding at site one. Such an exothermic reaction implies significant stabilization of the zymogen.

Form III: Structural Differences That Affect Activity and Function—Previously, we showed that lanthanides can occupy

| Form                                      | Site 1 | Site 2 | Site 3 | TGase activity | Channel | Data          |
|-------------------------------------------|--------|--------|--------|----------------|---------|---------------|
| Zymogen                                   | Ca$^{2+}$ |       |        | <5             | Closed  | Ref. 13       |
| I Zymogen proteolyzed with dispase        | Ca$^{2+}$ | Ca$^{2+}$ | Ca$^{2+}$ | 100 ± 1        | Open    | This study    |
| II I purified on MonoQ column             | Ca$^{2+}$ | Ca$^{2+}$ | Ca$^{2+}$ | <5             | Closed  | This study    |
| III II + CaCl$_2$                         | Ca$^{2+}$ | Ca$^{2+}$ | Mg$^{2+}$ | 6 ± 3          | Closed  | This study    |

Fig. 1. Conformations of the forms I (a and b), II (c and d), and III (e and g) solved in this study. The upper row shows the solved structures of the three forms. This is nominally the front side of the enzyme. The amino-terminal $\beta$-sandwich (red), catalytic core (blue), $\beta$-barrel 1 (magenta), and $\beta$-barrel 2 (orange) domains are shown. The Ca$^{2+}$ ions are shown in yellow, the sole Mg$^{2+}$ ion in cyan. Below are shown the electrostatic surface potential images. The acidic and basic residues are colored red and blue, respectively. The electrostatic potentials, including Ca$^{2+}$ and Mg$^{2+}$ ions, have been mapped onto the surface plan from $-15 kT$ (deep red) to $+15 kT$ (deep blue). The open channel is clearly evident in b. In g, the back side of the enzyme has a deep cavity; the front side (f) remains closed.
sites two and/or three with retention of full activity (13). But the major multivalent metal cation in cells is Mg^{2+}. Accordingly, we explored conditions to occupy sites two or three with Ca^{2+} ions. First, we measured TGase 3 activity in the presence of MgCl\textsubscript{2} and/or CaCl\textsubscript{2} (Fig. 4a). With MgCl\textsubscript{2} alone, there were only traces (<10%) of activity. With CaCl\textsubscript{2}, maximal activity was acquired by about 0.6 mM in the absence and presence of MgCl\textsubscript{2}. Addition of higher concentrations of MgCl\textsubscript{2} inhibited activity by 10–20%. Second, we titrated form II with different molar ratios of these ions and found by employing ICPMS-DRC that in the range of 0–2.5 mM CaCl\textsubscript{2} or MgCl\textsubscript{2}, a at low ratios (0.1) of Ca^{2+}/Mg^{2+} the TGase 3 contains one of each ion only (Fig. 4b). Form II rapidly acquired a second Ca^{2+} ion by ratios ~0.2, then gradually began to displace the sole Mg^{2+} ion by ratios ≥0.3, and finally Mg^{2+} ion was lost by ratios ≤0.7 (Fig. 4b). The one Ca\textsuperscript{2+}/one Mg\textsuperscript{2+} enzyme had no activity; the two Ca\textsuperscript{2+}/one Mg\textsuperscript{2+} enzyme had very low activity, and the three Ca\textsuperscript{2+} enzyme had full activity (Fig. 4b).

Attempts were therefore made to crystallize form II decorated with one Ca\textsuperscript{2+}/one Mg\textsuperscript{2+} and one Mg\textsuperscript{2+}/two Ca\textsuperscript{2+}. By use of MgCl\textsubscript{2} and an ATP salt, employed because they allowed introduction of Mg\textsuperscript{2+}, and the ATP does not bind to TGases (27), we were able to recover only the second of these, termed form III. We reconfirmed by ICPMS-DRC in crystals the presence of one Mg\textsuperscript{2+} and two Ca\textsuperscript{2+} ions and no other metals.

The x-ray diffraction crystal structure of form III model has two crystallographically independent monomers of 692 residues per asymmetric unit at 2.4 Å in the P1 space group (Table I and Fig. 1e). Both monomers have missing density for a flexible loop between residues 462 and 478 and the r.m.s. difference between the C\textsubscript{α} backbone structures is 0.30 Å, indicating that the C\textsubscript{α} backbone structures are almost identical. Accessible surface area calculations for interface between the monomers in the asymmetric unit give a value of 2688 Å\textsuperscript{2} for the area buried upon formation from each crystallographically independent monomers. As the protein is a monomer in solution (28), this interface may not be functionally relevant and is imposed by crystal contacts within the unit cells. Notably, the channel is closed (Fig. 1, f and g). There are major differences in the coordinations of the metal ions (Fig. 2, form III and Table III): while sites one and two are heptacoordinated as in form I, site three is coordinated with only six oxygens in

| Table III | The distance of all residues within 3 Å of the specified calcium ion site |
|-----------|---------------------------------------------------------------|
| Site one  | Residue | Atom | Distance (Å) | Residue | Atom | Distance (Å) | Residue | Atom | Distance (Å) |
|           | Ala\textsuperscript{221} | O | 2.25 | Ala\textsuperscript{221} | O | 2.68 |
|           | Asn\textsuperscript{224} | O | 2.49 | Asn\textsuperscript{224} | O | 2.96 |
|           | Asn\textsuperscript{224} | OD1 | 2.31 | Asn\textsuperscript{224} | OD1 | 2.91 |
|           | Asn\textsuperscript{226} | O | 2.12 | Asn\textsuperscript{226} | O | 2.29 |
|           | Asp\textsuperscript{226} | OD1 | 2.54 | Asp\textsuperscript{226} | OD1 | 3.13 |
| Site two  | Asn\textsuperscript{393} | OD1 | 2.28 |
|           | Ser\textsuperscript{415} | O | 2.45 |
|           | Glu\textsuperscript{443} | OE1 | 2.37 |
|           | Glu\textsuperscript{443} | OE2 | 2.77 |
|           | Glu\textsuperscript{448} | OE1 | 2.70 |
|           | Water\textsuperscript{393} | O | 2.18 |
| Site three| Asp\textsuperscript{301} | OD2 | 2.13 |
|           | Asp\textsuperscript{303} | OD1 | 2.82 |
|           | Asn\textsuperscript{305} | OD1 | 2.69 |
|           | Asn\textsuperscript{305} | ND2 | 2.93 |
|           | Ser\textsuperscript{307} | O | 2.29 |
|           | Asp\textsuperscript{324} | OD2 | 2.63 |
|           | Water\textsuperscript{36} | O | 2.34 |
an octahedral geometry. Furthermore its coordinations were shorter and did not involve the conserved residue Asp 324.

To identify which of sites two or three possessed the Mg$^{2+}$ ion, we initially modeled both for Ca$^{2+}$. After several rounds of building and refinement, a decrease in the B factor value for Ca$^{2+}$ relative to the surrounding side chain atoms and relatively short distances to nearby oxygen atoms (2.0–2.2 Å), indicated that site three most likely contained the Mg$^{2+}$ ion.

Second, we calculated the mean peak size in the Fo–Fc difference electron density maps at above 4σ level (large positive difference peak) for each metal ion in sites two and three in each independent monomer and found that site two is most likely Ca$^{2+}$ and site three Mg$^{2+}$. The clarity of the simulated annealing Fo–Fc omit electron density map, the observed bond distances, and the coordination geometry strongly support the assignment of Mg$^{2+}$ at site three. Finally, compared (B) values as well as bond valence calculations using parameters from Brese and O’Keeffe (29) provided support for this assignment as Mg$^{2+}$ ion. We note that the bond distances to the metal-oxygen ligand atom typically range from 2.0–2.2 Å for Mg$^{2+}$ and 2.2–2.9 Å for Ca$^{2+}$. In addition, the smaller size of Mg$^{2+}$ ions determines a preference for six coordinations, rather than an energetically more favorable seven for Ca$^{2+}$ (30). Furthermore, only the side chain carbonyl oxygen atom of Asn305 is coordinated in a monodentate manner to Mg$^{2+}$, whereas Ca$^{2+}$ coordinates in a bidentate manner with both the side chain atoms of Asn305 in form I that contains a Ca$^{2+}$ ion at site three. Thus the Asn 305 residue plays an especially important role in discriminating between Ca$^{2+}$ and Mg$^{2+}$ ions. Nevertheless, superpositions of the Ca$^{2+}$ and Mg$^{2+}$ of forms I and III show that the locations of the metal ions are similar. Notably however, comparisons of their crystal structures reveals that the Mg$^{2+}$ ion at site three results in a contraction of the coordination sphere, and a less energetically favored conformation because of a weaker interaction with Asn305. Moreover, the Mg$^{2+}$ ion in site three is unable to coordinate with residue Asp 324 from the Gly 322–Ser325 loop segment motif. Thus this loop occupies the same position as in the zymogen and no channel forms. On the other hand, some lanthanide metal ion (Er$^{3+}$ or Tb$^{3+}$) forms were as active as the Ca$^{2+}$ form (13). The likely reason is that for example Ca$^{2+}$ and Tb$^{3+}$ both possess spherical filled outer electronic subshells and are of similar size; their effective ionic radii for 7-fold coordination are 0.98 and 1.06 Å, respectively.

In the sequence Asp 320-Lys-Gly-Ser-Asp-Ser325 involved in metal ion binding at site 3, we suggest that Gly 322 plays a
pivotal role. Because the Gly residue lacks a side chain, it provides a greater range of backbone torsion angles than any other amino acid. Having a Gly residue modulate the range of motion or pliability of this sequence motif by 9 Å thereby allows specific side chain interactions of Asp with a Ca$^{2+}$ ion. Such flexibility could thus influence the affinity and kinetics of metal ion binding or release and simply control the ability to discriminate between metal ions of different size, as seen here for Ca$^{2+}$ and Mg$^{2+}$ ions. Indeed, there are numerous established precedents for this concept (31–33). In particular, mutagenesis experiments on EF-hand calcium-binding proteins has revealed that Gly residues can greatly affect the affinity, specificity, and/or stability of metal binding (31).

Accordingly, these data establish that the metal binding properties of site three determine the precise coordination with neighboring atoms, which in turn directly affects the folding of the peptide chain.

**Cooperative Role of the Ca$^{2+}$ Ion in Site Two**—Fig. 4 showed that in the presence of high relative concentrations of Mg$^{2+}$ ions, the proteolyzed enzyme possesses the one Ca$^{2+}$ ion at site one and a sole Mg$^{2+}$ ion, and the enzyme is inactive. The data for form II presented above indicate that the Mg$^{2+}$ ion should be bound at site 3 only, leaving site two vacant. As relative Ca$^{2+}$ ion concentrations rise, the enzyme rapidly acquires a second ion (Fig. 4b), but the two Ca$^{2+}$/one Mg$^{2+}$ ion form III is still inactive. Our data indicate that this second Ca$^{2+}$ ion occupies site two. However, as relative Ca$^{2+}$ ion concentrations rise still further, the Mg$^{2+}$ ion at site three is displaced by Ca$^{2+}$, and only when a net of ≥1.5 Ca$^{2+}$ ions have been acquired, does the enzyme begin to display activity (Fig. 4b). We assume this corresponds to opening of the front side of the enzyme. The shape of the activity curve of Fig. 4b suggests that site two should be filled with the Ca$^{2+}$ ion first, and that activity is recovered as soon as site three is at least half-filled. These data therefore strongly imply that sites two and three cooperate with each other.

We explored the reason why site two is not occupied by a Mg$^{2+}$ ion in a high Mg$^{2+}$ ion environment. Examination of $2 F_o - F_c$ and simulated annealing $F_o - F_c$ omit electron density maps suggests that in order to comply with the obligatory 2.0–2.2 Å bonding range, a Mg$^{2+}$ ion could only coordinate with one neighboring amino acid atom at a time. The smaller size of Mg$^{2+}$ ion determines its preference for a coordination number of six with an octahedral geometry of its complexes and is less comfortable than Ca$^{2+}$ in accepting larger multidentate and anionic ligand groups. An alternative possibility for a Mg$^{2+}$ ion to coordinate with several amino acid atoms would impose severe local distortion of the peptide backbone. Both situations are energetically unfavorable. Thus, site two provides a highly constrained binding cavity for metal ions, so that only a Ca$^{2+}$ (or lanthanide) ion with an appropriate ionic radius and multidentate property can comfortably occupy site two.

The FXIIIa enzyme has one Ca$^{2+}$ ion at a location generally similar to site two of TGase 3 and lies in an acidic pocket formed by Asp, Glu, and Glu but the Ca$^{2+}$ ion coordinates only with the main chain carbonyl oxygen of Ala and four water molecules. However this ion does not confer any significant change in FXIIIa structure, and the enzyme is inactive. It remains to be determined whether other Ca$^{2+}$ ions are utilized by an active FXIIIa enzyme form and whether they too cooperate with each other to allow reaction.

**Structure of the Channel**—We note from the structure of thezymogen (13) and form II (Fig. 1c) the presence of a deep cavity on one side (nominally termed the back side) of the TGase 3 enzyme. We have seen in form I that the binding of the Ca$^{2+}$ ion in site three laterally displaces a loop following the strand bearing residues to Ser so that coordinates with the ion (Fig. 5a). This loop on the front side of the enzyme moves so as to open the deep cavity and thus a channel through the enzyme. However, when the Ca$^{2+}$ ion (weaker Lewis acid) is replaced by a Mg$^{2+}$ ion at this site three, the channel is closed (Fig. 1f and g). The side chain of Ser in the loop

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**Fig. 5.** Front (a) and back (b) views of the cavity/channel of the activated TGase 3 enzyme. The Ca$^{2+}$ ions in sites one, two, and three are colored lime green. In a, the transparent electrostatic surface potential shows that the active site triad residues Cys-His-Asp are buried and inaccessible. The movement of the loop bearing residues Asp-Ser in the activated enzyme opens the cavity to form a channel through the enzyme and exposes the side chains Trp and Trp residues, thought to be important in manipulation of substrates. In b, the guanidinium group of Arg participates in a salt bridge interaction with Glu at the entrance of the cavity. Also shown are the side chains of Arg and Trp residues, thought to be important in manipulation of substrates. In
sequence Asp$^{320}$-Lys-Gly-Ser-Asp$^{325}$ motif instead forms a direct bond via a water molecule to the main chain nitrogen of Leu$^{298}$ and the carbonyl oxygen of Asp$^{295}$ causing the front of the cavity/channel to be closed.

At the back side of the enzyme, the cavity is always open whether or not any metal ion occupies site three. The guanidinium side chain of Arg$^{396}$ near the opening of the cavity intrudes into the front side and Arg$^{570}$ at the opening of the cavity is lined by the side chains of Arg 570, Leu529 and the carbonyl oxygen of Asp 320 causing the front of the cavity/channel to be closed.

The cavity itself is lined by the side chains of Arg 570, Asn168 and is filled with a water molecules connected to each other through hydrogen bonds. The cavity is lined predominantly by hydrophilic side chains of Arg$^{220}$, Arg$^{570}$, Thr$^{167}$, and Asn$^{168}$ and is filled with a water molecules connected to each other through hydrogen bonds. The cavity is lined by the side chains of Asp$^{320}$, Met$^{318}$, Val$^{326}$ and Asn$^{328}$ (left interior). When viewing the back of the cavity, the guanidinium groups of three arginines (Arg$^{420}$, upper portion; Arg$^{396}$ in the interior) intrude into the cavity. Altogether, these data impose severe constraints on how this cavity/channel could be used by substrates: it is too deep for a Gln or Lys residue to penetrate the active site Cys 272 and 13.5 Å from the Cα atom of Thr 417, with a total volume of 90,414 Å$^3$. The entrance of the cavity is controlled by the side chain

CONCLUSIONS

One of the most intriguing aspects of catalysis by the TGase 3 enzyme is its dependence on two divalent metal ions. They occupy distinct binding sites and differ greatly in their affinities for various divalent metal cations. The physiological concentration of free Mg$^{2+}$ ions in resting eukaryotic cells is 1–2 mM, whereas that for free Ca$^{2+}$ ions is ~100 nM, so that there is about a 10$^4$ molar ratio. This consideration allows the following hypothesis for the control of function of the TGase 3 enzyme. First, any TGase 3 protein present in a cell will likely be in the inactive zymogen form and have a sole Ca$^{2+}$ ion in site one. But the flexible loop of residues 462–471 of TGase 3 is easily physically broken leading to activation (13, 28). Should any enzyme become proteolyzed or broken, we can conclude from the data of Fig. 4 that site three will readily acquire a Mg$^{2+}$ ion but the enzyme form will remain inactive. This therefore provides a simple but elegant mechanism to protect the cell from aberrant transamidation reactions by some proteolyzed TGase 3 enzyme. Only when global intracellular or microenvironmental Ca$^{2+}$ concentrations rises into the micromolar range will site two acquire a Ca$^{2+}$ ion, and the Mg$^{2+}$ ion from site three be displaced by a Ca$^{2+}$ ion (Fig. 4). The Ca$^{2+}$ ion occupancy at both sites cooperatively allow energetically favorable coordination with residues Asn 305 and Asp 324, resulting in a change in the conformation of the loop Asp$^{320}$-Ser$^{325}$, the channel opens, and the enzyme becomes active. How can Ca$^{2+}$ concentrations rise to the required high level in living cells? We speculate that local microenvironmental Ca$^{2+}$ ion levels could be greatly changed in living cells by association of the TGase 3 enzyme with other cellular constituents, as indeed has been demonstrated previously for the TGase 1 enzyme (35). Further studies are now necessary to understand how this channel is employed by substrates to effect reaction.

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