Three-dimensional structure of the human transglutaminase 3 enzyme: binding of calcium ions changes structure for activation

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Transglutaminase (TGase) enzymes catalyze the formation of covalent cross-links between protein-bound glutamines and lysines in a calcium-dependent manner, but the role of Ca²⁺ ions remains unclear. The TGase 3 isoform is widely expressed and is important for epithelial barrier formation. It is a zymogen, requiring proteolysis for activity. We have solved the three-dimensional structures of the zymogen and the activated forms at 2.2 and 2.1 Å resolution, respectively, and examined the role of Ca²⁺ ions. The zymogen binds one ion tightly that cannot be exchanged. Upon proteolysis, the enzyme exothermally acquires two more Ca²⁺ ions that activate the enzyme, are exchangeable and are functionally replaceable by other lanthanide trivalent cations. Binding of a Ca²⁺ ion at one of these sites opens a channel which exposes the key Trp236 and Trp327 residues that control substrate access to the active site. Together, these biochemical and structural data reveal for the first time in a TGase enzyme that Ca²⁺ ions induce structural changes which at least in part dictate activity and, moreover, may confer substrate specificity.

Keywords: activation/calcium binding/transglutaminase/X-ray structure

Introduction

Transglutaminases (TGases; protein–glutamine:amine γ -glutamyl-transferases) are a family of calcium-dependent acyl-transfer enzymes that are ubiquitously expressed in mammalian cells. The common reaction performed by each of the nine TGase enzyme isoforms known in the human genome involves the activation of a target or donor protein-bound glutamine residue to form a thiol-acyl enzyme intermediate. This can be accepted by a nucleophilic group to accomplish the reaction. Of several potential nucleophiles, the most common is water, which results in the net deamidation of the target glutamine. However, if the nucleophile is the ϵ -amino group of a protein-bound lysine, an $N\epsilon$ -(γ -glutamyl)lysine intra- or interchain isopeptide cross-link is formed (Lorand and Conrad, 1984; Folk and Chung, 1985; Greenberg *et al.*, 1991; Melino *et al.*, 1998; Nemes and Steinert, 1999), leading to the formation of a stable, permanent and insoluble macromolecular complex.

TGases are expressed abundantly in stratified squamous epithelia where they are required for formation of the cell envelope barrier structure. The cell envelope of specialized epithelia such as the epidermis involves the accretion of several precursor structural proteins (Rice and Green, 1977; Kim *et al.*, 1990; Steinert and Marekov, 1995; Steinert *et al.*, 1998; Nemes *et al.*, 1999). To date, at least two different TGase enzymes are known to be required for effective barrier formation (Melino *et al.*, 1998; Nemes and Steinert, 1999) and include: the TGase 1 enzyme, which is usually membrane bound (Rice and Green, 1977; Steinert *et al.*, 1996); and the TGase 3 enzyme, which is cytosolic (Kim *et al.*, 1990, 1993). Both enzymes require activation by proteolysis at specific sites, which increases their specific activities by 2–3 orders of magnitude. Both of these TGases are also widely expressed in other tissues, including brain (Kim *et al.*, 1999) and muscle (Choi *et al.*, 2000).

While several TGase isoforms that have been well characterized to date preferentially use the sequence motif Gln–Gln–Val for the first step of the reaction (Lorand and Conrad, 1984; Folk and Chung, 1985), there nevertheless is a high degree of isoform substrate specificity. However, the features that specify which glutamine donor and lysine acceptor substrates may approach an enzyme isoform to engage in reaction remain unknown. For example, the human blood clotting factor XIIIa (hfXIIIa) enzyme uses fibrin almost exclusively for both reaction steps, resulting in its polymerization (Pisano *et al.*, 1968; Gladner and Nossal, 1983; Shen and Lorand, 1983). On the other hand, TGases 1 and 3 preferentially use different glutamines and lysines on the same substrates, such as loricrin and small proline-rich proteins 1, 2 and 3 (Candi *et al.*, 1995, 1999; Tarcsa *et al.*, 1998; Steinert *et al.*, 1999). Likewise, trichohyalin is recognized preferentially by TGase 3 (Tarcsa *et al.*, 1997). However, the bases of these substrate preferences remain unresolved.

The requirement for Ca²⁺ ions for TGase reactions in *in vitro* solution assays and *in situ* on tissue sections has been known for decades (Folk and Chung, 1985; Lorand *et al.*, 1987; Melino *et al.*, 1998). However, the numbers of ions, their location in the three-dimensional structures of TGases and precise role(s) in the activation and function are not yet understood. The three-dimensional structures of the zymogen and activated forms of the hfXIIIa enzyme isoform have been solved (Yee *et al.*, 1994, 1996; Weiss *et al.*, 1998). The activated enzyme possesses a single Ca²⁺ ion located in the vicinity of residues 473–490 in the catalytic core domain (Fox *et al.*, 1999). However, binding of a Ca²⁺ ion in this site does not significantly change the structure and does not result in activation *per se*, but may somehow contribute to the subsequent binding of substrates (Fox *et al.*, 1999). The human TGase 2 enzyme can bind as many as six Ca²⁺ ions with an apparent average

Fig. 1. The Ca^{2+} content of TGase 3. **(A)** Binding of Ca^{2+} to the zymogen and the activated form in the presence of Ca^{2+} ions was measured by equilibrium dialysis at 4°C . **(B)** Isothermal calorimetric titration of Ca^{2+} binding to TGase 3. To solutions of the zymogen and activated forms were added a series of injections of 5, 10 and 20 μl of CaCl_2 and the heat absorbed or liberated was recorded (upper panel). The lower panel records the net heat changes. The heat of dilution determined from injections of a solution without protein were subtracted to determine heat changes due solely to Ca^{2+} binding. The data shown are representative; the SD of replicates was $<5\%$.

affinity constant of $90 \mu\text{M}$ (Bergamini, 1988), but their properties and location in the structure remain unknown. In addition, the structure of a fish TGase enzyme (fTG, perhaps equivalent to mammalian TGase 2) has been presented (Noguchi *et al.*, 2001). The structure does not show a Ca^{2+} ion (Noguchi *et al.*, 2001), but *in vitro* activity assays with it have routinely incorporated Ca^{2+} (Yasueda

et al., 1994). Thus, the role of Ca^{2+} ions in TGase structure and function remains unresolved, but a role in activation and/or substrate specificity is plausible.

In this study, we have explored the functional and structural requirements of Ca^{2+} ions in the TGase 3 system. We demonstrate that binding of three Ca^{2+} ions is needed for activity. Furthermore, comparisons of the solved three-dimensional structures of the zymogen and activated forms demonstrate that on binding of Ca^{2+} ions to the latter, a channel opens to expose two key residues near the active site. Thus, we conclude that binding of Ca^{2+} ions in the TGase 3 system facilitates substrate binding and perhaps specificity.

Results and discussion

Human TGase 3 binds three Ca^{2+} ions

In initial studies, we explored the Ca^{2+} requirements for the zymogen and activated forms of human TGase 3. First, by exhaustive equilibrium dialysis into buffer containing increasing concentrations of $^{45}\text{Ca}^{2+}$, we found that the zymogen does not acquire label. However, direct quantitative biophysical measurements employing the inductively coupled plasma-mass spectrometry dynamic reaction cell (ICPMS-DRC) (West Coast Analytical Service, Santa Fe Springs, CA) demonstrates the existence of 1.0 mol of Ca^{2+} ion/mol and the absence of any other metals (Figure 1A). When we treated the zymogen with a solution of Chelex and EGTA, we were unable to remove significant amounts of it. However, denaturation in 8 M urea or 1% SDS released the Ca^{2+} ion, but the enzyme did not re-acquire a Ca^{2+} ion on removal of the denaturants. This is probably due to improper refolding as the protein subsequently could not be activated. To identify the source of this ion, we expressed a mini-culture of baculovirus (Kim *et al.*, 2001) in the presence of $^{45}\text{Ca}^{2+}$ and discovered that the zymogen sequesters a Ca^{2+} ion from the insect cells during expression. Next, we proteolyzed the TGase 3 zymogen with dispase and repeated the equilibrium dialyses. This TGase 3 enzyme is active and has acquired two additional Ca^{2+} ions (Figure 1A).

The thermodynamics of the acquisition of the two Ca^{2+} ions were determined by isothermal titration calorimetry (Figure 1B). When the proteolyzed TGase 3 was assayed in the presence of CaCl_2 , the binding data indicate 2.0 low affinity sites (average $K_d = 4 \mu\text{M}$) with $\Delta H = -4.639 \pm 0.15 \text{ kcal/mol}$. Such an exothermic reaction implies significant stabilization of the enzyme on binding. However, it has not been possible to determine the properties of the Ca^{2+} -binding site in the zymogen. Based on the avidity with which it acquires a Ca^{2+} ion during expression, we suspect that the K_d of this site should be in the nM range, typical of the $[\text{Ca}_i^{2+}]$ of eukaryotic cells.

The three Ca^{2+} ions are located in the 50 kDa portion of TGase 3

Activation of the zymogen requires specific cleavage at Ser469 (Kim *et al.*, 1990), located in a hinge region that separates the catalytic core and β -barrel 1 domains. Typically, the resultant 50 and 27 kDa fragments remain associated together in the active enzyme. This cleavage is replicated *in vitro* by treatment with dispase (Figure 2A).

Fig. 2. Location of Ca²⁺ ions in TGase 3. (A) SDS-PAGE. Lane 1, proteolytic activation of TGase 3, yielding 50 and 27 kDa fragments that normally remain associated together; lane 2, purified 50 kDa portion; lane 3, 77 kDa zymogen. (B and C) TGase 3 activities of the zymogen (lanes 1 and 2), the 77 kDa disperse-proteolyzed form (lanes 7 and 8) and the separated 50 (lanes 5 and 6) and 27 (lanes 3 and 4) kDa forms were measured by incorporation of [¹⁴C]putrescine into casein (Folk and Cole, 1966). The – and + indicate the absence or presence of 5 mM Ca²⁺ in the standard assay reaction. (B) The proteins were repurified through a monoQ column and were not pre-treated with 1 mM CaCl₂. (C) The proteins were pre-treated with 1 mM CaCl₂ prior to repurification. In the controls, 5 mM EDTA or EGTA was added to the standard assays. (D) TGase 3 is active in the presence of lanthanides. The Ca²⁺ + and – row indicate the presence or absence of calcium in the reaction. The M³⁺ row denotes the presence of 1 mM lanthanide: lanes 1–3, Er³⁺; lanes 4 and 5, Sm³⁺; lanes 6 and 7, Tb³⁺; and lanes 8 and 9, Lu³⁺. In one experiment, 5 mM ErCl₃ was used instead of CaCl₂.

In addition, we have found that some cleavage can occur by physical manipulation of protein solutions, perhaps by autocatalysis, or contaminating proteases in buffers, so that the purified zymogen retains some activity (H.-C.Kim and P.M.Steinert, unpublished observations).

The following sets of experiments were performed to clarify the precise roles of Ca²⁺ ions. The disperse-proteolyzed zymogen form was freed from disperse by one step through a monoQ column. The intact zymogen and the proteolyzed protein were also reacted with 1 mM CaCl₂ for 1 h at 23°C and then rechromatographed. Similarly, the separated 50 and 27 kDa pieces were either treated or not treated with 1 mM CaCl₂, and separated on the same column. Under these conditions, following ICPMS-DRC analyses, the proteolyzed 77 kDa form and the 50 kDa fragment each contained one Ca²⁺ ion when not exposed to CaCl₂, and three Ca²⁺ ions after exposure to CaCl₂. However, the intact zymogen retained only one Ca²⁺ ion even if it had been treated with CaCl₂. The 27 kDa fragment contained no Ca²⁺ ion (data not shown). Thus all three Ca²⁺ ions are located in the 50 kDa part of TGase 3, consisting of the N-terminal β-sandwich and the catalytic core domains (see below).

The binding of two additional Ca²⁺ ions activates TGase 3

Each of these proteins that had not (Figure 2B) or had (Figure 2C) been pre-treated with 1 mM CaCl₂ was then assayed in the absence or presence of 5 mM CaCl₂ in the standard TGase assay. The 27 kDa form had no activity under any conditions (Figure 2B and C, columns 3 and 4). The intact zymogen and the 50 and 77 kDa proteolyzed proteins possessed no activity until exposed to Ca²⁺: without pre-treatment with 1 mM CaCl₂, they were inactive (Figure 2B, columns 1, 5 and 7, respectively) until 5 mM CaCl₂ was added to the assay (Figure 2B, columns 2, 6 and 8). The zymogen possessed 10% maximal activity, possibly due to random cleavage. However, the 50 and 77 kDa proteolyzed forms which had been pre-treated with 1 mM CaCl₂ possessed substantial activity (40–50% of maximum) in the absence of 5 mM CaCl₂ in the assay (Figure 2C, columns 5 and 7). This activity increased toward 100% with 5 mM CaCl₂. This increase may be due to an equilibrium exchange of either or both of the two Ca²⁺ ions: in the absence of CaCl₂ in solution, the bound ions may partly vacate, resulting in net partial loss of activity.

Table I. X-ray diffraction data collection and refinement statistics

	Zymogen	Activated
X-ray experimental data		
Space group	P_1	P_1
Cell dimensions	$a = 57.71 \text{ \AA}$, $b = 67.52 \text{ \AA}$, $c = 116.21 \text{ \AA}$ $\alpha = 97.24^\circ$, $\beta = 90.15^\circ$, $\gamma = 98.67^\circ$	$a = 57.49 \text{ \AA}$, $b = 116.54 \text{ \AA}$, $c = 60.77 \text{ \AA}$ $\alpha = 93.23^\circ$, $\beta = 92.99^\circ$, $\gamma = 89.51^\circ$
Resolution limit ($\text{\AA}$)	2.20	2.10
No. of observations/unique reflections	521 626/75 013	558 858/80 238
Overall $I/\sigma(I)^a$	11.3 (2.9)	14.9 (4.1)
Data completeness ^a	94.6 (93.0)	97.9 (91.8)
Refinement statistics		
No. of reflections used	72 799	78 516
Resolution limit ($\text{\AA}$)	20–2.20 (2.28–2.20)	20–2.10 (2.17–2.10)
$R_{\text{crystal}}^b/R_{\text{free}}^c$ (%) ^c	18.20/22.50	18.87/23.34
Protein atoms/dimer	10 580	10 500
No. of solvent molecules	904	1013
No. of ions in TGase 3		
(Calcium/chloride/bromide)	2/2/2	
(Calcium/chloride/ β -octyl glucoside)		6/2/2
Average temperature factors ($\text{\AA}^2$)		
Protein/solvent	25.39/27.95	25.76/31.22
Calcium/chloride/bromide ions	31.25/25.17/28.61	
Calcium/chloride/ β -octyl glucoside		29.29/37.98/50.87
R.m.s. deviation from ideal values		
Bond lengths ($\text{\AA}$)	0.006	0.005
Bond angle ($^\circ$)	1.24	1.28
Dihedral angles ($^\circ$)	25.54	25.44
Improper angles ($^\circ$)	0.74	0.73

^aValues in parentheses correspond to the highest shell.

^b $R_{\text{crystal}} = \Sigma |F_o(h) - F_c(h)| / \Sigma F_o(h)$, where $F_o(h)$ and $F_c(h)$ are the observed and calculated structure factor amplitudes for reflection h .

^c R_{free} was calculated against 10% of the complete data set excluded from refinement.

Together, the data indicate that the tightly bound Ca^{2+} ion in the zymogen is necessary but not sufficient for enzyme activity. However, the acquisition of two additional Ca^{2+} ions in a pre-treatment regimen, and in the absence of added CaCl_2 in the assay, is sufficient to activate the enzyme or at least allow the enzyme reaction to proceed in the presence of substrate. Historically, therefore, use of the standard TGase 3 assay conditions with 2–5 mM CaCl_2 (Kim *et al.*, 1990) allows the binding of these two additional activating Ca^{2+} ions.

Other lanthanide trivalent cations can substitute for Ca^{2+} to retain TGase 3 activity

Other lanthanide trivalent cations can bind to hfXIIIa in the general vicinity of its sole Ca^{2+} ion site (Fox *et al.*, 1999). Accordingly, the above experiments with the TGase 3 forms were repeated with four different lanthanide ions, Er^{3+} , Sm^{3+} , Tb^{3+} and Lu^{3+} . First, we found that neither could replace the tightly bound Ca^{2+} ion of the zymogen. Secondly, pre-treatment of the activated enzyme with 1 mM lanthanide, followed by purification through the monoQ column, and then assay for TGase activity in the absence of 5 mM CaCl_2 , revealed significant activity in each case, with somewhat higher activity with Sm^{3+} (Figure 2D). Thirdly, when we added standard 5 mM CaCl_2 to the TGase assay, activities increased, with somewhat higher activity with Sm^{3+} (Figure 2D). Direct measurement and the X-ray data (see below) of the Er^{3+} form of activated TGase 3 revealed the presence of a net of two Er^{3+} ions and one Ca^{2+} ion before addition of 5 mM

CaCl_2 , and only three Ca^{2+} ions afterward. Thus, Ca^{2+} ions are the preferred cation for the TGase 3 enzyme and will displace other ions. Further, we incubated the Er^{3+} form in the presence of 5 mM ErCl_3 , and found high activity comparable with that for CaCl_2 (Figure 2D), i.e. the one Ca^{2+} /two Er^{3+} ion form is about as active as the three Ca^{2+} ion form. In addition, we have repeated these experiments with Yb^{3+} , and found that TGase 3 acquires a net of one Yb^{3+} ion/mol, and the two Ca^{2+} /one Yb^{3+} ion form is comparably active (data not shown). In contrast, incubation of hfXIIIa with >40 μM lanthanide inhibited this enzyme (Achyuthan *et al.*, 1989), due to binding of a Yb^{3+} ion in a novel site (Fox *et al.*, 1999).

Overall topology and domain structure of zymogen and activated forms of TGase 3

The X-ray diffraction crystal structures of the zymogen and activated form (Kim *et al.*, 2001) were solved in order to understand better the structural and functional significance of the Ca^{2+} activation properties of TGase 3. The refinement details are shown in Table I.

While the zymogen and activated enzyme function in solution as monomers (Kim *et al.*, 1990), the X-ray models of the human TGase 3 zymogen and the activated forms consist of two crystallographically independent monomers of 692 residues per asymmetric unit, with overall resolution of 2.2 and 2.1 $\text{\AA}$, respectively (Figure 3). In the zymogen, one monomer has missing density for a flexible loop between residues 461 and 479, and the second monomer has missing density for residues

460–472. In the active enzyme, residues 460–480 are missing in both monomers. The r.m.s. differences between the C α carbon atoms of the two monomers in the zymogen and the activated form are 0.39 and 0.30 Å, indicating that the C α backbone structures are almost identical. The dimensions of each monomer are $\sim 110 \times 66 \times 51$ Å, and the protein molecule is reminiscent of a flattened triangle. The activated form has one β -octylglucoside molecule in the barrel 1 domain (Figure 3B), acquired during crystallization to prevent twinning (Kim *et al.*, 2001).

The zymogen and activated forms have well-organized structures consisting of four folded domains that are similar in organization and structure to the hfXIIIa (Yee *et al.*, 1994) and fTG enzymes (Noguchi *et al.*, 2001). The N-terminal β -sandwich domain of TGase 3 contains the first 134 amino acids and consists of nine strands of β -sheets interspersed by three α -helices. The catalytic core domain extends from residues Asn135 to Gly472 and consists of 15 β -sheets interspersed with 15 α -helical segments. The longest α -helix of 16 residues is located in the center of the molecule and harbors the active site Cys272 residue. Other members of the catalytic triad, His330 and Asp353, are located on adjacent strands of β -sheets. This catalytic triad is structurally similar to that of other TGases (Yee *et al.*, 1994; Noguchi *et al.*, 2001). The barrel 1 and barrel 2 domains span residues 473–592 and 593–692, respectively. The active site is shielded from contact with the solvent because it is buried within a narrow cleft with walls formed by two β -sheet strands of the catalytic core domain and the C-terminus of barrel 1. Residues 462–471 form a flexible solvent-exposed loop that links the last α -helical segment of the catalytic core domain to the first β -strand of the barrel 1 domain. This hinge region harbors Ser469, the cleavage site for proteolytic activation of the zymogen. This residue is flanked by polar residues, predicted to lie near the surface of the protein, which may be involved in recognition by an activating protease.

The arrangement of the two monomers in the asymmetric unit of the zymogen and the activated form crystal structures are different from those of hfXIIIa (Yee *et al.*, 1994; Weiss *et al.*, 1998) or fTG (Noguchi *et al.*, 2001). Notably, in TGase 3, three of the strands of the β -pleated sheet of the barrel 2 domain of one monomer form intermolecular contacts with four strands of the β -pleated sheet of the β -sandwich domain of the second monomer. Also, there are salt bridge interactions between residue Glu65 of one monomer and Arg653 of the second monomer, hydrogen bonding of Asn50 with Glu621, and other intermolecular contacts that are mediated through water molecules. Accessible surface area calculations for the interface between the monomers in the asymmetric unit give a value of 5375 Å² for the area buried upon formation of the two crystallographically independent monomers. As the protein is a monomer in solution (Kim *et al.*, 1990), this interface may not be functionally relevant and is imposed by crystal contacts within the unit cells. In contrast, there is only one molecule of the fTG enzyme in its asymmetric unit (Noguchi *et al.*, 2001).

There are two non-proline *cis* peptide bonds in the zymogen and active forms that have been conserved in the hfXIIIa (Weiss *et al.*, 1998) and fTG (Noguchi *et al.*, 2001) enzymes. One is Arg268–Tyr269 in the loop prior to

the strand of α -helix that contains the active site Cys272 residue, and the other is Asn382–Phe383 in a loop adjoining two α -helices of the core domain. The latter appears to stabilize a Gly367–Pro368 *cis* peptide bond.

Consistent with the above biochemical data, the crystal structure of the TGase 3 zymogen has one Ca²⁺ ion/monomer in the catalytic core domain (Figure 3A). This is retained in the activated form, which also has two additional ions in the catalytic core domain (Figure 3B). These sites are numbered 1–3 in Figure 3C. We found no evidence for other partially occupied cation-binding sites.

The binding of three Ca²⁺ ions induces changes in the structure of activated TGase 3

Comparisons of the C α atomic alignments of the solved TGase 3 zymogen and activated forms reveal high degrees of identity (Figure 3C), but there are four changes. One is located in a flexible surface loop in the N-terminal β -sandwich domain. Three other changes are observed in the catalytic core domain in the vicinity of each of the three Ca²⁺-binding sites (colored green in Figure 3C). There are also high degrees of similarity in the C α alignments of the solved hfXIIIa, fTG and activated TGase 3 enzymes (Figure 3D, colored red, green and yellow, respectively). A detailed analysis of these four solved structures is in progress.

The Ca²⁺ ion-binding site 1 of the zymogen

This site encompasses residues Asn224–Asn229. The Ca²⁺ ion is positioned 13.7 Å above the C α carbon atom of Cys272 in the active site (Figure 4A). The Ca²⁺ ion adopts an octahedral coordination by forming direct contacts with the main chain carbonyl oxygen atoms of Ala221, Asn224, Asn226 and Asn229, the carbonyl side chain oxygen of Asn224, and a water molecule. A summary of oxygen atoms that are potentially involved in the coordination of this (and the other two Ca²⁺) ions is presented in Table II. Interestingly, the location of this Ca²⁺ ion site is retained in the activated TGase 3 (Figure 4B), but there are changes. The Ca²⁺ ion is heptacoordinated in an environment that can be best described as a distorted pentagonal bipyramid by forming direct contacts with the main chain carbonyl oxygen atoms of Ala221, Asn224 and Asn226, the carbonyl side chain oxygen of Asn224 and Asp228, and a water molecule. The loop Ile223–Val231 containing Asn229 has shifted away and Asp228 instead coordinates with the Ca²⁺ ion. In this way, the Ca²⁺ ion in the activated TGase 3 is shielded by the carbonyl side chain oxygen of Asp228, which is now located in a tight turn between β -strands and α -helix and is an outlier in the Ramachandran plot. The Asp228 side chain in the zymogen form ($\phi = 47.6$, $\psi = 40.6$) is exposed, while in the activated form it is buried ($\phi = -144.9$, $\psi = 19.7$). In addition, the $\langle B \rangle$ value is decreased from 34.52 to 24.36 Å². Indeed, the $\langle B \rangle$ value of this Ca²⁺-binding site has been reduced from 31.30 to 19.11 Å² (Table II). Together, these data show that the Ca²⁺ ion has been buried by the movement of the Asp228 side chain.

There is a high probability of calcium being the metal present in the crystal structure of the zymogen in this position. First, the zymogen acquires a Ca²⁺ ion during expression, and the ICPMS-DRC analyses indicate 1.0 Ca²⁺ ion/mol and the absence of any other metals.

Secondly, the mean peak size in the $|F_o| - |F_c|$ difference electron density map was at the 10σ level (large positive difference peak) for each Ca^{2+} ion in each independent monomer in the zymogen and the activated forms. Thirdly, this Ca^{2+} ion site was investigated further by close inspection of anomalous electron density maps. The clarity of the $2|F_o| - |F_c|$ electron density maps, the observed bond distances and the coordination geometry strongly support the assignment of this Ca^{2+} -binding site. In refinement modeling analyses, when one water molecule replaced the Ca^{2+} ion at this point, the $\langle B \rangle$ value refined to a value of $\sim 3 \text{ \AA}^2$, implying that a water molecule in this location does not have enough electrons to match the X-ray diffraction data adequately. Finally, the com-

pared $\langle B \rangle$ values as well as bond–valence calculations using parameters from Brese and O’Keeffe (1991) result in a valence of 2.3 for a Ca^{2+} ion in the zymogen and the activated form, supporting this assignment as a calcium site.

Site 1 of TGase 3 is generally similar to a novel Yb^{3+} site found at the dimer 2-fold axis near residues Asp270 and Glu272 of hfXIIIa (Figure 3D), where binding results in inhibition of this enzyme. Asp270 is conserved as Asp227 in TGase 3, but Glu272 is replaced by Asn229. The residue Asn229 coordinates with the Ca^{2+} ion in the zymogen, but, in activated TGase 3, the loop bearing this residue moves away so that a stronger coordination forms with Asp228 instead. As the equivalent loop bearing

Glu272 does not change in hfXIIIa on activation (Fox *et al.*, 1999), this may explain why a lanthanide ion in this site inhibits hfXIIIa but does not affect activated TGase 3.

This Ca²⁺ ion may be required for maintenance of the correct three-dimensional structure of the active site region of TGase 3. Further, its enhanced coordination

after enzyme activation suggests that it is essential for enzyme activity.

The Ca²⁺ ion-binding site 2 in activated TGase 3 is homologous to that in hfXIIIa

One of the two new Ca²⁺-binding sites acquired in the activated TGase 3 exists near the end of the catalytic core

Fig. 3. Stereo images of the solved structures. Solved structures of dimers of (A) human TGase 3 zymogen. (B) The activated form in the presence of Ca²⁺. (C) Superposition of C_α traces. Each is a pair of stereo diagrams showing the overall secondary structures. The four domains are the β-sandwich (red), the catalytic core (green), barrel 1 (magenta) and barrel 2 (orange). The Ca²⁺ ions are shown in yellow. In (A), the Cl⁻ ion (cyan) between the β-sandwich and core, and the Br⁻ ion (blue) in barrel 1 domains are shown. In (B), a single molecule of β-octylglucoside is visible. In (C), the gray color represents the zymogen and the magenta color is the activated form. The three Ca²⁺ ion-binding sites are numbered 1–3. The three changed loop regions of the activated form, Ile223–Val231, Leu319–Ser325 and Tyr439–Asp446, are shown in green. The Ca²⁺ ion in site 1 superimposes in both structures. (D) Stereo overlay images of the C_α traces of the solved structures of fXIIIa (red), fTG (green) and activated TGase 3 (yellow) enzymes. Calcium ions are also included. The cleavage site is marked by an arrow in (A).

Fig. 4. Stereo views of the key residues involved in the Ca^{2+} ion-binding sites in the zymogen and activated forms of TGase 3. (A and B) Site 1. The Ca^{2+} ion in the zymogen (A) forms direct contacts with the main chain carbonyl oxygen atoms of Ala221, Asn224, Asn226 and Asn229, the carbonyl side chain oxygen of Asn224, and a water molecule, but, in the activated form (B), the Ca^{2+} ion forms direct contacts with the main chain carbonyl oxygen atoms of Ala221, Asn224 and Asn226, the carbonyl side chains oxygen of Asn224 and Asp228 and a water molecule. In this case, the Ca^{2+} ion is shielded further by the loop Ile223–Val231 bearing Asp228. Asp228 in the activated form is buried, while in the zymogen it is exposed. (C) Sites 2 and 3. Site 2 in the activated TGase 3 exists near the end of the catalytic core domain by the α -helical segment leading to the loop which connects to the barrel 1 domain, encompassing residues Asn430–Glu448. The Ca^{2+} ion forms contacts with the carbonyl side chain oxygen groups of Asn393, Glu443 and Glu448, the main chain carbonyl oxygen atoms of Ser415 and two water molecules. The third Ca^{2+} -binding site in the activated TGase 3 occurs in the vicinity of the loop segment leading to the catalytic His330 active site. This Ca^{2+} ion forms contacts with the carbonyl side chain oxygens of Asp301, Asp303 and Asp324, the side chain atoms of Asn305, the main chain carbonyl oxygen atoms of Ser307 and a water molecule. The loop bearing residues Leu319–Ser325 has moved from its position in the zymogen, thereby creating a channel through the molecule. In all images, gray represents the water oxygen atom.

Table II. The distance and *B*-values of all the residues within 3.5 Å of the specified calcium ion site

	Residue	Atom	Zymogen		Residue	Atom	Activated	
			Distance (Å)	 (Å ²)			Distance (Å)	 (Å ²)
Site 1	Ala221	O	2.50	31.30	Ala221	O	2.31	19.11
	Asn224	O	2.50		Asn224	O	2.26	
	Asn224	OD1	2.35		Asn224	OD1	2.36	
	Asn226	O	2.55		Asn226	O	2.32	
	Asn229	O	2.78		Asn228	OD1	2.39	
	Wat516	O	2.40		Asn228	OD2	2.17	
Site 2					Wat508	O	2.46	29.56
					Asn393	OD1	2.19	
					Ser415	O	2.30	
					Glu443	OE1	2.49	
					Glu443	OE2	2.60	
					Glu448	OE1	2.86	
					Wat586	O	2.18	
					Wat54	O	2.32	
Site 3								39.22
					Asp301	OD2	2.36	
					Asp303	OD1	3.19	
					Asn305	OD1	2.23	
					Asn305	ND2	2.95	
					Ser307	O	2.78	
					Asp324	OD2	2.93	
					Wat959	O	2.51	

domain near the α -helical segment leading to the loop which connects to the barrel 1 domain, encompassing residues Asn430–Glu448. This Ca²⁺ ion is 23 Å below the C $_{\alpha}$ carbon atom of the active site Cys272 residue. The site resembles that of the sole known Ca²⁺-binding site of hfXIIIa and is thought to adopt an EF-hand-like conformation (Fox *et al.*, 1999). The Ca²⁺ ion adopts a heptacoordinated conformation forming direct contacts with the carbonyl side chain oxygen groups of Asn393, Glu443 and Glu448, the main chain carbonyl oxygen atoms of Ser415, and direct ligation to two water molecules (Figure 4C; Table II). The residues involved in the coordination with Ca²⁺ ion binding are different from those of hfXIIIa. In hfXIIIa, the Ca²⁺ ion lies in an acidic pocket formed by Asp438, Glu485 and Glu490, but the Ca²⁺ ion coordinates only with the main chain carbonyl oxygen of Ala457 and four water molecules. However, in both enzymes, binding of a Ca²⁺ ion promotes no (hfXIIIa) or only minor (TGase 3) changes in shape that do not affect in any obvious way the conformations of residues near the active site.

We have also crystallized the activated TGase 3 with Er³⁺ and Yb³⁺ complexes in the absence of Ca²⁺ ions. The refinement analyses revealed that the activated form of TGase 3 contained a net of two Er³⁺ ions or one Yb³⁺ ion. The mean peak sizes in the $|F_o| - |F_c|$ difference electron density maps were found to be at the 15 σ level (large positive difference peak) for both metals. The anomalous electron density maps indicate that the Yb³⁺ ion is located in site 2, and the two Er³⁺ ions are located in sites 2 and 3 of TGase 3. In each case, they engage the same oxygen atoms as the Ca²⁺ ions, as described above (data not shown: refinement analyses in progress). These data imply

that Ca²⁺ ions in site 2 can exchange with other cations more easily than site 3.

The binding of Ca²⁺ ion in the third binding site in activated TGase 3 opens a channel toward the active site

The third Ca²⁺ ion-binding site acquired in the activated TGase 3 enzyme is novel. It occurs in the vicinity of a loop segment consisting of residues Asp320–Ser325 leading to the catalytic residue His330 in the active site. This Ca²⁺ ion is 22 Å from the C $_{\alpha}$ carbon atom of the active site Cys272 residue and 17 Å from the C $_{\alpha}$ carbon atom of His330. The precise location of this loop is different in the zymogen when compared with the activated TGase 3. In the latter, the loop has moved and becomes directly bonded to the Ca²⁺ ion via the carbonyl side chain oxygen of Asp324, and thereby opens a channel through the protein. The Ca²⁺ ion adopts a heptacoordinated stack with direct contacts with the carbonyl side chain oxygens of Asp301, Asp303 and Asp324, the side chain atoms of Asn305, the main chain carbonyl oxygen atoms of Ser307 and a water molecule (Figure 4C, Table II).

The channel is clearly visible on the electrostatic surface potential image (Figure 5). Figure 6A shows an enlargement of this zone revealing that on one side the channel is conical in shape, extending from the surface of the protein toward the catalytic triad. In addition, the guanidinium side chain of Arg396 changes its orientation to form a hydrogen bond with Glu586 in the activated form, which allows the hole to pass through to the reverse side (Figure 6B). The movement of the loop and opening of the channel expose Trp236 and Trp327 on the upper and outer surface of the channel (Figure 6A).

Fig. 5. Structural comparison of the zymogen and activated form. The upper and lower rows represent images rotated 180° with respect to each other, to show the channel in the active form. The electrostatic surface potential maps are shown in the center. The acidic and basic regions are colored red and blue, respectively. The arrow denotes a channel that opens on binding of a Ca^{2+} ion in site 3. On the left and right are secondary structure images of the zymogen and the activated TGase 3 in the same orientations. The electrostatic potential, including calcium ions, has been mapped onto the surface plan from -15.0 kT (deep red) to $+15.0$ kT (deep blue).

Notably, in the hfXIIIa and fTG enzymes, the equivalents of these tryptophan residues are predicted to play multiple important roles in the enzyme reactions. First, they are buried and must be exposed. Secondly, movements of their side chains would allow direct access of substrates to the buried active site. However, how these two residues become exposed so the conformations of their side chains can change to allow enzyme reaction has not been shown. Thirdly, these two tryptophan residues are thought to participate in oxanion intermediate formation with first the acyl glutamine donor and then the acceptor lysine substrates (Pedersen *et al.*, 1994). Because of near identities of sequences, we suggest that these predicted events should also occur in the TGase 3 system. Thus, based on our new data, we can predict specifically that the Ca^{2+} ion-induced formation of the channel and exposure of the two key tryptophan residues will facilitate the movements of their side chains as appropriate substrates approach. A complementary possibility is that the opening of this channel in the TGase 3 system confers specificity on which substrates can approach the tryptophan residues to engage the active site.

Conclusions

We have successfully solved the structures of two forms of a second mammalian TGase enzyme, the inactive TGase 3 zymogen and its activated form. Our X-ray data reveal the presence of three Ca^{2+} ions that are required for enzyme activity. The presence and function of the three ions have been confirmed by biochemical and biophysical assays. Our new data provide the first insights on the structural role that Ca^{2+} ions play in any of the TGase isoforms. The Ca^{2+} ion-binding site 1 in the zymogen is tightly bound and cannot be removed without protein denaturation. Because its coordination with residue side chains changes following activation, the data suggest that its presence is essential for activity. However, it is not sufficient for activity. For activation, two additional Ca^{2+} ions are required that change the structure of the enzyme. Further, their exothermic binding properties imply significant net enzyme stabilization. Our data imply that the intact loop of residues 462–471 in the zymogen (not visualized in Figure 3A) occludes binding of Ca^{2+} ions in sites 2 and 3, which is removed on cleavage at Ser469. Of these, site 2 is similar to the sole site of hfXIIIa, and binding of a Ca^{2+} ion

Fig. 6. Stereo view images of the novel channel opened by Ca²⁺ ion binding in site 3. Protein domains are colored as in Figure 3. (A) On one side, the electrostatic surface potential (black transparent) shows that the active site triad residues Cys272, His330 and Asp353 are buried and inaccessible. The movement of the loop bearing residues Asp320–Ser325 opens the channel in the activated TGase 3, and exposes the side chains of the Trp236 and Trp327 residues. (B) On the opposite side, the guanidinium group of Arg396 has moved to form a salt bridge with Glu586, allowing the hole to extend through the protein.

causes only a minor change in structure that has no discernible effect on access to the active site. However, binding of a Ca²⁺ ion in site 3 creates a channel which exposes the two key Trp236 and Trp327 residues that control access of substrates to the active site of TGase enzymes. We therefore suggest that the binding of the three Ca²⁺ ions, and especially at site 3, facilitates approach of substrates and/or directly activates the enzyme. Indeed, it is also possible that these changes reflect or regulate the unique substrate specificity properties of TGase 3. Finally, detailed comparisons of the high resolution structures of these two TGase 3 enzyme isoforms with those of the solved hfXIIIa and fTG

enzymes are now in progress (see also preliminary comparisons in Figure 3C and D). Coupled with the new insights into the critical role of Ca²⁺ ions in the activation process of the TGase 3 system, such analyses may reveal other changes that reflect TGase isoform substrate specificity and the mechanism of action.

Materials and methods

Protein expression, crystallization and data collection

Recombinant human TGase 3 zymogen was expressed in the baculovirus system and purified as described previously (Kim *et al.*, 2001). To prepare the activated form, a 12 ml solution of zymogen (17 mg/ml in a buffer of

20 mM Tris–HCl pH 8.0 containing 1 mM EDTA and 125 mM NaCl) was treated with 3 mg of dispase (Roche) at 37°C for 30 min. The active TGase 3 form was then recovered by passage through a monoQ column with a linear gradient of 0–500 mM NaCl, and eluted at 100 mM NaCl (Kim *et al.*, 2001). Whenever the zymogen is digested with dispase, most stays as the 77 kDa (activated) form, but a minority of the protein falls apart as the 27 and 50 kDa fragments. The fragments were eluted from the column at 125 and 350 mM NaCl, respectively.

Crystallization trials of the zymogen were performed using the hanging drop vapor diffusion method by adding the non-ionic detergent β -octylglucoside (0.1 mM) to 100 μ l of protein in 20 mM Tris–HCl pH 8.0, 1 mM EDTA and 125 mM NaCl at a concentration of 17 mg/ml. To a fresh 2 μ l of protein, 2 μ l of precipitant solution was added and equilibrated over a well solution containing 4% (w/v) PEG 20K and 100 mM Tris–HCl pH 8.5 at 15°C. The condition used for the activated TGase 3 in the presence of Ca^{2+} ions is somewhat different from that previously reported for the activated TGase 3 in the absence of calcium (Kim *et al.*, 2001). The crystallization of TGase 3 (15 mg/ml) was done using 0.1 mM β -octylglucoside in 100 μ l of protein in 20 mM Tris–HCl pH 8.0, 1 mM EDTA, 125 mM NaCl and 3.0 mM CaCl_2 . To a fresh 4 μ l of protein, 2 μ l of reservoir solution was added and equilibrated over a well solution containing 4–12% (w/v) PEG 6K, 100 mM bicine pH 9.0 and 1% dioxane at 21°C.

Thin, plate-like crystals were harvested into mother liquors containing 20% (v/v) 2-methyl-2,4-pentanediol as the cryoprotectant, and were then flash-cooled into liquid nitrogen with a rayon mounting loop (Oxford Cryosystems, Oxford, UK) prior to data collection. All data were collected at the National Synchrotron Light Source, Brookhaven National Laboratories, using beamline X9B, the ADSC Quantum 4 detector with a wavelength of 0.92 Å, crystal to detector distance of 205 mm and oscillation range of 1.0°, and exposure for 60 s. The Matthew's coefficient is 2.88 Å³/Da for the zymogen and 2.68 Å³/Da for the activated TGase 3, suggesting two molecules per asymmetric unit, with solvent content of 58 and 56%, respectively (Matthews, 1968). The diffraction data from sets of crystals were indexed, processed, scaled and merged using the HKL2000 suite of programs (Otwinowski and Minor, 1997). An attempt was made to collect multiwavelength anomalous dispersion data in which halide ions are introduced as anomalous scatterers in the cryoprotectant during a short soak (Dauter *et al.*, 2000). The crystal was soaked in 1 M NaBr for 45 s, but no anomalous scatterers could be obtained. However, in the zymogen structure, one bromide ion was detected.

Structure solution and refinement

The crystal structure of the zymogen was solved by molecular replacement using AmoRe (Navaza and Saludjian, 1997) and a resolution range of 10–3.5 Å for the rotation search and between 10 and 3.0 Å for the translation search. There were two solutions to both the rotation and translation searches. These solutions gave rise to two molecules within each asymmetric unit. The polyalanine model of the hfXIIIa enzyme (PDB 1GGT; Yee *et al.*, 1994) was used as the initial search model. The initial *R*-factor after correctly orienting and positioning the two molecules was 44.60% for all the data from 10 to 3.0 Å resolution. The rigid-body refinement using CNS software (Brünger *et al.*, 1998), followed by simulated annealing applying strict 2-fold non-crystallographic symmetry (NCS) constraints (Kleywegt, 1996) to 3.0 Å resolution, improved the solutions, yielding a final *R*-factor of 39.8%. Subsequently, the polyalanine model was converted to the TGase 3 sequence using the program O (Jones *et al.*, 1991). Initial phases were improved using solvent flattening and histogram matching (CCP4, 1994) with 2-fold NCS molecular averaging (Kleywegt and Read, 1997). For final refinement, the NCS restraints were released. After many cycles of manual model rebuilding into SIGMAA-weighted $3|F_o| - 2|F_c|$ and $|F_o| - |F_c|$ maps, the refinement converged at an *R*-factor of 18.20% and an *R*_{free} of 22.50%. This included all low-resolution data from 25 Å resolution as bulk solvent correction. In order to avoid overfitting of the model, each step of the rebuilding procedure was monitored using the free *R*-factor and a residue real space correlation coefficient as a guide. For the activated TGase 3 data set with the additional Ca^{2+} ions, starting with one of the monomers of the preliminary zymogen model as the search probe, molecular replacement in AmoRe (Navaza and Saludjian, 1997) was used to search for the location of monomer in this crystal form in *P*₁ space group. A translation search yielded the correct solution (CC = 38.2%). The model was refined in CNS (Brünger *et al.*, 1998) and with the simulated annealing, positional and *B*-factor refinement protocols using a maximum-likelihood target (Brünger *et al.*, 1990). Anisotropic scaling and a bulk solvent correction were used, and the individual *B*-factors were refined isotropically. Except for the *R*_{free} set (a random sampling

consisting of 8.6% of the data set), all data between 20 and 2.1 Å (with no σ cut-off) were included in the refinement which was converged at an *R*-factor of 18.87% and *R*_{free} of 23.34% (Table I). The stereochemical quality of the structure was analyzed with the programs PROCHECK (Laskowski *et al.*, 1992) and CNS (Brünger *et al.*, 1998). Figures were generated with Molscript (Kraulis, 1991) and Raster3D (Merritt and Bacon, 1997).

Microcalorimetric titration studies of TGase 3

The thermodynamic properties of Ca^{2+} binding to the activated TGase 3 were measured by isothermal titration calorimetry using a MicroCal VP-ITC calorimeter. Prior to measurements, the TGase 3 (1 μ M) was dialyzed three times against 20 mM Tris–HCl pH 8.0 and 2 mM EGTA. The EGTA solution was removed by extensive dialysis against 20 mM Tris–HCl pH 8.0 and treated with Chelex 100. This TGase 3 was placed in a 1.38 ml sample cell. A 250 μ l syringe loaded with 0.5 mM standard CaCl_2 solution (Fisher Scientific) was used for a series of automatic injections of 10 μ l each into the protein solution. After each injection, a 5 min pause was allowed for reaching the baseline. Heat produced due to dilution was measured by injecting the Ca^{2+} solution into the sample cells from which TGase 3 protein was omitted. For each titration step, the heat of dilution was subtracted. Data were fitted to appropriate binding models, and thermodynamic parameters were determined from non-linear least-squares fits, using ORIGIN software.

TGase 3 activity assay

TGase activity was measured by incorporation of [¹⁴C]putrescine into casein (Folk and Cole, 1966). Assays were done at 37°C in 0.5 ml portions of 0.1 M Tris–HCl pH 7.5, containing 1% *N,N*-dimethylated casein (Sigma), 0.5 μ Ci putrescine (118 mCi/mmol), 1 mM dithiothreitol, 5 mM CaCl_2 and 1 mM EDTA. In some experiments, the assays contained 1 mM lanthanide, ErCl_3 , SmCl_3 , TbCl_3 or LuCl_3 , instead of CaCl_2 .

TGase 3 calcium-binding assay

Calcium binding was determined by equilibrium dialysis (Bergamini, 1988) allowing equilibration of the enzyme (0.5–0.8 mg/ml) against 100 vols of buffer containing 20 mM Tris–HCl pH 8.0 supplemented with increasing amounts of ⁴⁵ CaCl_2 (300 c.p.m./nmol) 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.

TGase 3 quantitative metal analysis

All plastic and glassware was incubated in 5 M HCl for 36 h and rinsed with double-distilled water that had been passed over a Chelex 100 column. For buffer and transition metal solutions, Chelex 100-treated water was used throughout. Prior to adjustment of the pH, Chelex 100 resin was added (0.5 g/100 ml) and the solution was stirred for 45 min; after adjustment of the pH, the solution was passed through a 0.2 μ m filter and stored in metal-free plastic vials at –20°C. Prior to measurements, the TGase 3 (10 mg/ml) was 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 bound metal ions were detected by use of a Perkin-Elmer ELAN 6100 ICPMS-DRC apparatus. The concentration of zymogen was 1.5 mg/ml.

Accession numbers

The structure factors and the coordinates of zymogen and the activated TGase 3 have been deposited in the Protein Data Bank with the accession number 1L9M for zymogen and 1L9N for the activated TGase 3.

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