Bioorganic Chemistry 57 (2014) 186–197

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Acyl transfer mechanisms of tissue transglutaminase

Jeffrey W. Keillor *, Christopher M. Clouthier, Kim Y.P. Apperley, Abdullah Akbar, Amina Mulani

Department of Chemistry, University of Ottawa, 10 Marie-Cure, Ottawa, Ontario K1N 6N5, Canada

ARTICLE INFO

Article history:
Available online 25 June 2014

Keywords:
Enzyme mechanism
Acyl transfer
Transglutaminase
Transamidation

ABSTRACT

Tissue transglutaminase (TG2) is a calcium-dependent enzyme that catalyses several acyl transfer reactions. The most biologically relevant of these involve protein-bound Gln residues as an acyl-donor substrate, and either water or a primary amine as an acyl-acceptor substrate. The former leads to deamidation of Gln to Glu, whereas the latter leads to transamidation, typically resulting in protein cross-linking when the amine substrate is a protein-bound Lys residue. In this review, we present an overview of over fifty years of mechanistic studies that have led to our current understanding of TG2-mediated hydrolysis and transamidation.

1. Introduction

Transglutaminases (TGases, EC 2.3.2.13) are a family of calcium-dependent enzymes that are involved in protein cross-linking [1,2]. Their first detected activity was their ability to incorporate low-molecular-weight primary amines into proteins [3]. More typically, TGases catalyse transamidation between the γ-carboxamide group of a protein-bound glutamine residue and the ε-amino group of a protein-bound lysine residue, forming an N^{ε}-(γ-glutamyl)lysine isopeptide bridge [1,2] (Scheme 1). This transamidation reaction represents the archetypical protein "spot-welding" or "cross-linking" activity shared by all members of the TGase family [1].

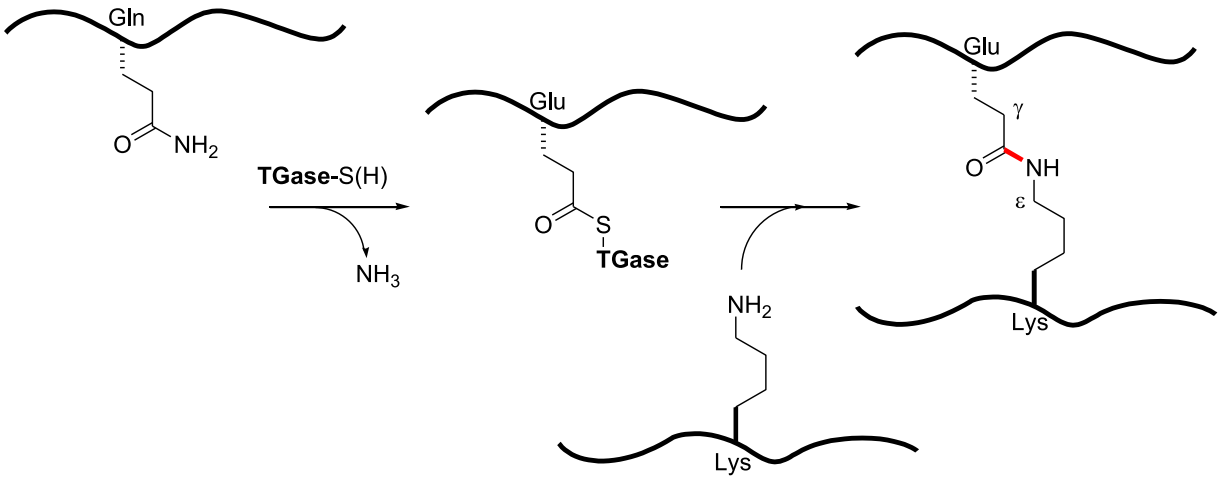
Several different human TGases have been characterised; in accordance with the rest of the family, all those listed below are regulated by calcium. Keratinocyte transglutaminase (TG1) is a membrane-bound and cytosolic protein, found in keratinocytes and in the brain. It is involved in cell-envelope formation, and has been associated with inherited lamellar ichthyosis [1]. Epidermal transglutaminase (TG3) is a protease-activated cytosolic protein. It is found in squamous epithelium and in the brain, and acts in cornified [4] cell-envelope formation [1]. Prostate transglutaminase (TG4), is involved in semen coagulation in rodents [1]. TG5, a recent addition to the TGase family, is expressed in stratified squamous epithelia such as the upper layers of the epidermis and in human hair follicle; it has also been proposed to catalyse formation of the cornified cell envelope, along with TG1 and TG3 [5]. TG6, another recent member of the family, has been found in human carcinoma lines with neuronal characteristics. Similarly to TG2, it has allosteric binding sites for both Ca^{2+} and GTP [6]. TG7 has equally been found in the skin epidermis, but little is currently known about its substrates [7] and physiological significance. Plasma transglutaminase (FXIII) [1,8] exists as a proenzyme tetramer of two pairs of non-covalently associated subunits, A and B; the dissociated FXIIIa is the active form. It is found in the cytosol, as well as extracellularly in blood plasma; it is activated by thrombin, which catalyses its dissociation to the active A subunit, and is involved in blood coagulation and the stabilization of blood clots against plasmin degradation.

Tissue transglutaminase (TG2), the enzyme of interest in this review, is ubiquitously expressed in tissues. It is predominantly found in the cytosol, but it is also located in the nucleus and membrane, as well as on the cell surface and even extracellularly. Given its widespread localisation and potential context-dependent activity, it has been proposed to serve many different functions. It is activated by Ca^{2+} and allosterically deactivated by GTP and/or by disulphide bond formation (see below) [1]. Furthermore, TG2 is a unique member of the TGase family due to its apparent ability to hydrolyse ATP and GTP; when bound to the latter, TG2 has been proposed to function as a classic G-protein [9]. However, this review will focus exclusively on its well-documented acyl transfer catalysis.

Among the acyl transfer reactions catalysed by TG2, transamidation and deamidation have the most obvious physiological relevance. Transamidation results in protein cross-linking when the amine substrate is a protein-bound lysine residue (Scheme 1), and leads to post-translation modification of glutamines when small molecule primary amines serve as acyl-acceptor substrates. Hydrolytic deamidation of glutamine residues to glutamate residues occurs when water acts as an acyl-acceptor 'substrate'. While additional acyl-transfer reactions have been proposed for other TGases, scant evidence exists in support of TG2-mediated intramolecular transamidation (i.e. cyclisation) [10] or transesterification [1,11,12]; this review will only discuss the mechanism of the extensively studied transamidation and hydrolysis reactions.

* Corresponding author.
E-mail address: jkeillor@uottawa.ca (J.W. Keillor).

http://dx.doi.org/10.1016/j.bioorg.2014.06.003
0045-2068/



Scheme 1. Archetypical TGase-mediated protein cross-linking.

Although TG2 is localised predominantly in the cytosol [13], its transamidation activity in this locale is generally inferred to be dormant, due to the low intracellular concentration of Ca^{2+} and the relatively high concentration of GTP [14]. However, extracellularly, where the concentration of Ca^{2+} is higher, its main function is transamidation [15]. It is important to note, however, that the extracellular activity of TG2 may be further regulated by disulphide bond formation and reduction (see below) [16,17]. TG2 is involved in various processes, ranging from cellular differentiation to apoptosis, and including cell adhesion and matrix assembly [18]. In particular, it has been associated with cell differentiation in neurons, astrocytes and fibroblasts [19], and has been shown to be necessary for neuronal differentiation in human neuroblastoma SH-SY5Y cells [20]. TG2 has also been shown to act as an adhesion receptor for fibronectin on the cell surface [21], and its role in programmed cell death relates to the prevention of macromolecule leakage as well as the inflammatory response [1].

Due to its role in extracellular matrix stabilisation, TG2 has been implicated in conditions such as fibrosis and atherosclerosis [9]; in the latter case, it may catalyse the incorporation of lipoprotein into atherosclerotic plaque [22]. TG2 has also been implicated in a variety of diseases. These include Alzheimer's and Huntington's, but the greatest body of evidence has been gathered for its role in celiac disease [1]. TG2 has been shown to be an autoimmune antigen of celiac disease, with gliadin being its preferred substrate [23]. It has been shown that TG2 itself participates in epitope selection by the hydrolytic deamidation of the glutamine residues of gliadin [24]. Other studies have shown [25] that TG2 activity is increased in Alzheimer's diseased brains. The enzyme is associated with the accumulation of fibrillar β-amyloid in extracellular plaque, as well as with the tau protein, which is involved with the tangles; both have been shown [1,26,27] to be substrates for TG2. Finally, huntingtin, a cytosolic protein involved in Huntington's, is also a substrate for TG2 [28]. The number of CAG repeats in the gene coding for huntingtin is related to the age of onset of the disease [29]; studies have shown that huntingtin containing a pathological length of polyglutamine residues (coded for by the CAG repeats) is a better substrate for TG2 transamidation than its shorter, non-diseased counterpart [28,29].

2. Overview

2.1. Structure

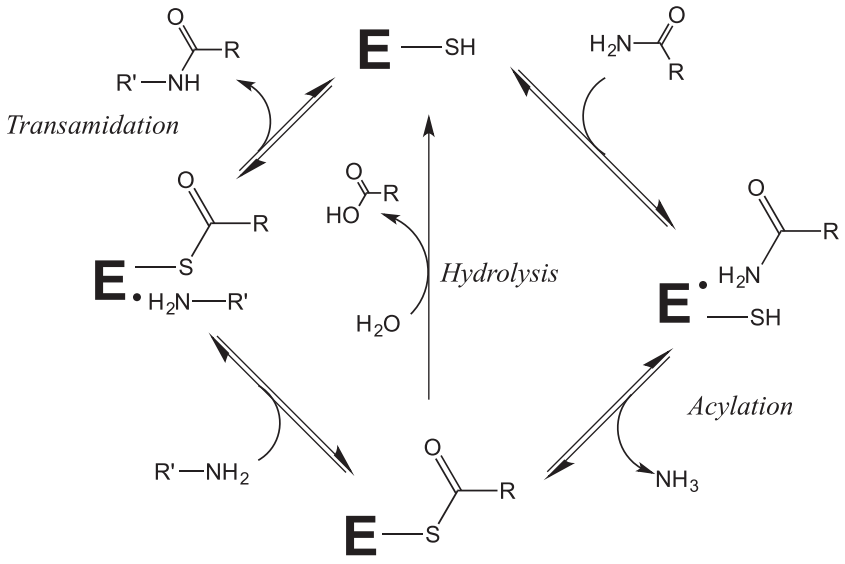
TG2 is a monomeric protein composed of 685–691 amino acids, having a molecular mass of 76–85 kDa. It contains four domains, including an *N*-terminal β-sandwich, a catalytic α/β-domain, and two *C*-terminal β-barrels [16,30]. All TGase enzymes are encoded by a family of closely related genes. Alignment of these genes reveals a high degree of sequence similarity and similar gene organisation as well as significant conservation of intron distribution and intron splice type [31]. The active site residues that form the catalytic triad (comprised of Cys277, His335, Asp358 in TG2) are conserved throughout the enzyme family, as are the residues that form the active site 'tunnel' that binds substrate residue sidechains (comprised of Trp241, Trp332 and Thr360) [16].

The essential Cys residue of the catalytic triad (of guinea pig liver TGase, gplTG2) was identified by Folk and co-workers, through labelling experiments using ^{14}C-labelled acyl donor substrate analogues [1,32–34]. They also demonstrated the calcium dependence of enzyme activity through the same set of seminal experiments [9,32–34]. Much more recently, site-directed mutagenesis studies have shown that TG2 binds up to six Ca^{2+} ions and that transglutaminase activity is decreased or lost when any of the Ca^{2+} binding sites were mutated, validating the essential nature of this metal for catalysis [35].

2.2. Catalytic cycle

In general, TG2-mediated acyl transfer is known to occur *via* a modified ping-pong mechanism [36]. First, the acyl donor substrate is bound by the enzyme and reacts with the active site thiolate, resulting in the release of one equivalent of ammonia and formation of an acyl-enzyme intermediate (Scheme 2). If an amine acyl acceptor substrate is present, it is bound by the acyl-enzyme, whose thiolester then reacts with the bound amine, resulting in regeneration of the free enzyme and release of the transamidation product. In the absence of free amine, water can regenerate the free enzyme, resulting in the hydrolytic deamidation of the initial acyl donor substrate.

Phenomenologically, the acyl transfer reactions catalysed by TGases and in particular the amide hydrolysis reaction, – resembles that of the cysteine proteases, whose active sites comprise a cysteine nucleophile and a basic residue that functions as a general acid/base in the catalytic mechanism. Since TGases share a core structural fold with the papain-like cysteine proteases, both families have been classified within the same superfamily in the Structural Classifications of Proteins database [37]. Bioinformatic analysis of archaeal genomes led to the identification of a superfamily of proteins homologous to eukaryotic TGases and the suggestion that eukaryotic TGases evolved from ancestral proteases [38].



Scheme 2. Modified ping-pong mechanism of TG2-mediated acyl transfer reactions.

2.3. Unified mechanism

A more detailed unified mechanism is shown in Scheme 3. By way of an overview, the active form of the enzyme is the imidazolium thiolate shown as form **I**. The side chain of a select Gln residue is bound in a tunnel leading down to active site (see Michaelis complex **II**). Nucleophilic attack by the active site thiolate on the amide substrate carbonyl leads to formation of tetrahedral intermediate **III**. The subsequent decomposition of **III** gives acyl-enzyme **IV** and results in expulsion of one equivalent of product ammonia, presumably with general acid assistance by imidazolium. Acyl-enzyme **IV** now has two fates – either aminolysis or hydrolysis, the selectivity of which is discussed below. If a suitable amine acyl-acceptor substrate (such as the side chain of an accessible Lys residue) is present, it is bound in a second binding tunnel to form Michaelis complex **V**. The active site imidazole group now functions as a general base to deprotonate the (neutral) amine during its attack on the thiolester carbonyl. This attack, forming tetrahedral intermediate **VI**, has been shown to be rate-limiting for a variety of acyl-donor and acyl-acceptor substrates. The decomposition of **VI** results in the release of transamidation product and the expulsion of thiolate, regenerating the free enzyme in its catalytically competent form. In the absence of suitable acyl-acceptor substrate, water can infiltrate the active site of acyl-enzyme **IV**. Subsequent activation by the general base imidazole, as observed in the cysteine proteases, leads to attack on thiolester to form tetrahedral intermediate **VII**. The decomposition of **VII** expels thiolate to regenerate the active site and releases the deamidation product glutamic acid.

3. TG2 acylation

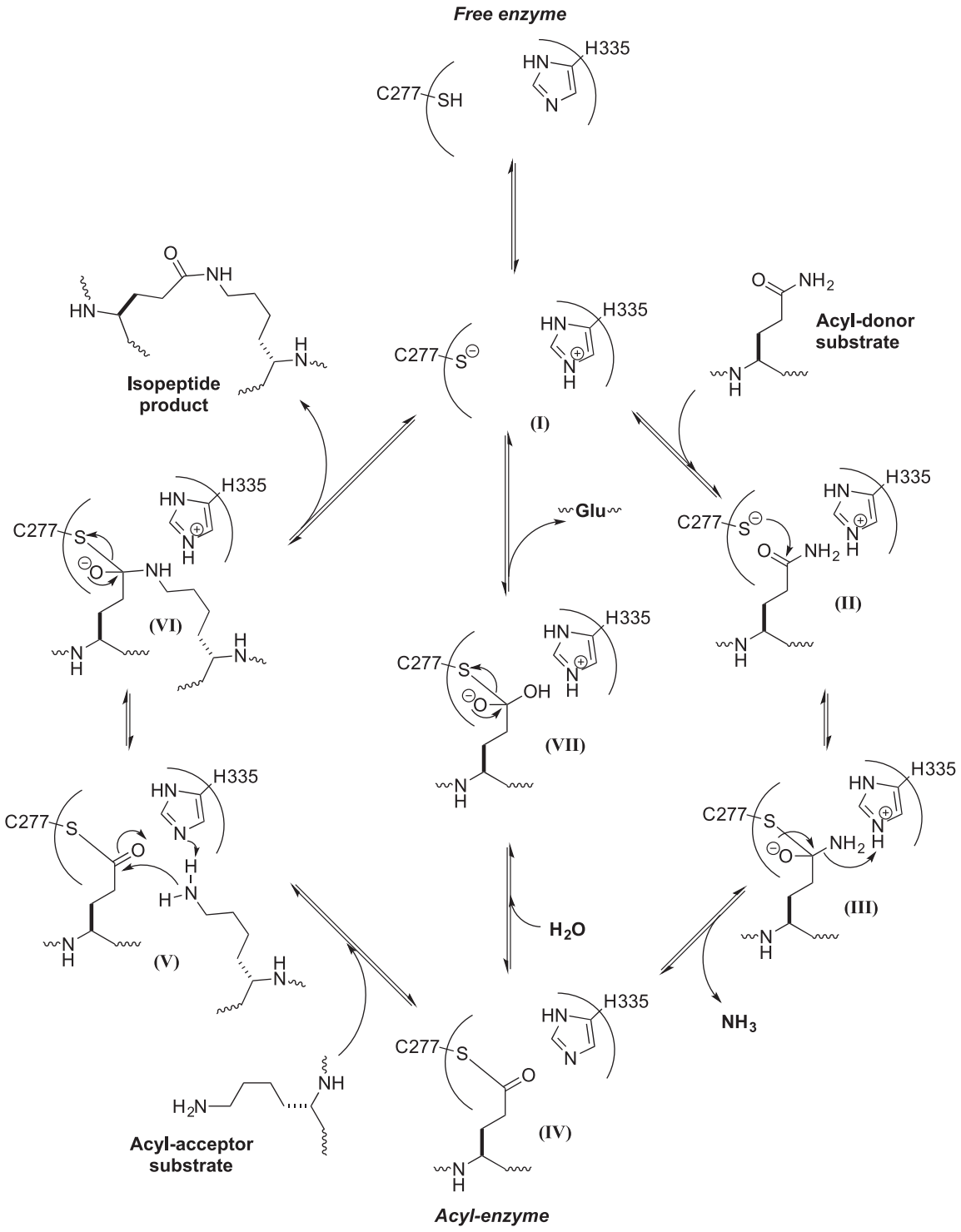
3.1. Acyl-donor substrate specificity

TG2 is relatively selective regarding which Gln residues are modified by acyl-transfer reactions, but much less selective with regard to lysine residues or primary amines. Early studies with guinea pig liver TGase (gplTG) by Folk and Cole reported that Gln alone does not serve as a substrate, nor do the peptides Gln-Gly and Gly-Gln-Gly; however, Cbz-Gln-Gly, Cbz-Gln-GlyOEt, and benzoyl-Gly-Gln-Gly are functional [39]. From this work it was noted that the *N*-terminal Cbz group plays an important role in conferring affinity for small peptidic substrates. Additional insight was provided by Chica et al. in 2004, in a kinetic study that featured a series of synthetic dipeptides bearing an *N*-terminal Cbz- or Boc-group [40]. All Cbz-peptides displayed similar K_M and k_{cat} values, whereas the Boc-dipeptides did not appear to act as donor substrates, showing no reactivity with gplTG. Owing to its simple structure, reasonable affinity and commercial availability, Cbz-Gln-Gly has been the most commonly used non-protein acyl donor substrate for many years.

In 2000 Ohtsuka et al. studied the selectivity of gplTG towards various synthetic glutamine peptides [41]. They noted that Cbz-Gln-Phe and Cbz-Gln-Leu reacted more efficiently than Cbz-Gln-Gly, while no reactivity was detected for Cbz-D-Gln-Phe. Also, the length of the carboxamide side chain (i.e. $-(CH_2)_nCONH_2$) is critical; Cbz-Asn-Phe ($n = 1$), Cbz-Adp(NH_2)-Phe ($n = 3$) and Cbz-Asu(NH_2)-Phe ($n = 4$) were not recognised as acyl donor substrates.

Sugimura et al. further investigated [42] the preferred sequence of acyl donor peptide substrates by screening of a phage-displayed random peptide library using biotin cadaverine as an acyl acceptor substrate. Reactive peptide sequences that were biotinylated through TG2-mediated cross-linking were isolated and identified, then genetically fused to GST and re-evaluated as substrates. Of the 40 peptides identified as high-affinity substrates, 39 contained a Gln residue close to the *N*-terminus (separated by one or two residues), which is consistent with other findings [38,42–46]. Keresztessy et al. also screened a phage-displayed random peptide library and noted that about 70% of substrate sequences present a polar residue *N*-terminal to the reactive Gln residue [43]. Further specificity studies have shown a preference for Pro in the 'Q + 2' position (QxP), whereas QP and QxxP do not appear to serve as substrates [42,43,45]. Affinity for the QxP peptide motif has also been confirmed in parallel studies with gliadin peptide derivative substrates [24,47]. Further analysis revealed the preference for a hydrophobic residue (W, M, L, I or V) at position Q + 3 [42,43,45]. To summarise all of these studies, TG2 has a high affinity for Gln peptides of the sequence *z*Q*x*P*h* (where *z* is a polar amino acid residue, *x* is any residue and *h* is a hydrophobic residue).

Amine probes have since been used to identify TG2-reactive Gln residues on certain proteins in complex biological systems.



Scheme 3. Unified mechanism of TG2-mediated acyl transfer reactions.

Although too numerous to name here, Facchiano et al. have compiled an impressive list of protein glutamine donor substrates identified in this way [48].

3.2. Mechanistic studies

As mentioned above, and reviewed in 1983 [49], Folk and coworkers definitively demonstrated the role of Cys277 as the active site nucleophile present in the active site. More recently, Lee et al. mutated this residue to serine [50] and demonstrated that the C277S mutant has no transamidation activity, confirming the Cys277 residue is essential for acyl transfer activity. Similarly, Micanovic et al. found [51] that replacement of the catalytic His residue of the homologous Factor XIIIa with Asn abolished enzyme activity, suggesting by extension that His335 of TG2 is equally important. Likewise, Hettasch and Greenberg [52] found that replacement of the active site Asp residue of Factor XIIIa (D358 in TG2) reduced transamidation activity by more than 95%. Subsequently, Case and Stein [53] studied the solvent deuterium isotope effect on k_{cat}/K_M for gplTG2-catalysed hydrolysis of Cbz-Gln-Gly.

Similar types of isotope effects have been measured for the reactions of papain and other acyl transferases that possess active site Cys/His diads, so the authors compared their experimental value ($^{D}k_{cat}/K_M = 0.45$) to those measured previously for mechanistically related enzymes. This led them to propose that the Cys/His diad of TG2 exists predominately as the neutral thiol/imidazole species in the resting state, although the acylation step is rate-limited by attack of the active site thiolate (**I**, Scheme 3) on the γ-carboxamide group of the substrate Gln residue in the first Michaelis complex (**II**) [53].

The first X-ray structure of human TG2 was published in 2002 by Cerione and co-workers [30]. Their structure (PDB: 1KV3) was found to form a complex with GDP, a known deactivating ligand [54], suggesting that it may not represent the active form of the enzyme (Fig. 1). But in 2007 Khosla and co-workers published [16] a second structure of human TG2 (PDB: 2Q3Z), at 2-Å resolution and in a distinctly extended (or open) conformation (Fig. 2). This TG2 structure was obtained after reaction between the active enzyme and a pentapeptide irreversible inhibitor (Ac-P(DON)LPF-NH_2). From this structure the authors noted that the carbonyl of their substrate analogue inhibitor appeared to be hydrogen bonded to both the indole group of Trp241 and the backbone amide group of Cys277, (Fig. 2 inset) leading the authors to suggest these interactions may stabilise the oxyanion of the tetrahedral intermediate (**III** of Scheme 3) and the transition state leading to its formation (Fig. 3).

It is noteworthy that without the benefit of this structure, Iismaa et al. had noted [55] in 2003 that Trp241 is conserved in all enzymatically active forms of eukaryotic TGases and conspicuously absent from the catalytically inactive member of the TGase family, band 4.2 [56], strongly suggesting that this residue plays an important role during catalysis. Indeed, when the authors prepared a series of mutants they found the substitution of the Trp241 side chain had little effect on the Michaelis constant but significant lowering of the catalytic rate constant, suggesting the involvement of this residue in transition state (rather than ground state) stabilization. Of the potential interactions proposed by the authors, the one that would lead to transition state stabilization is the formation of a hydrogen bond with the developing oxyanion (Fig. 3).

Additional evidence that this transition state is rate-limiting was provided by the work of Gravel et al. [57]. They synthesised a variety of acyl-donor substrate analogues bearing a *p*-nitrophenol leaving group, with different heteroatoms substituting for the γ–methylene group on the glutamyl side chain; rates for pre-steady state acylation were then measured by stopped-flow techniques. The K_M values measured for glutamyl and carbonate esters (X = CH_2 and O, respectively, in Fig. 4) were found to be nearly identical at 1.5 mM and 1.6 mM respectively. However, the catalytic constant measured for the more electrophilic glutamyl ester was 85 s^{-1}, whereas the same rate constant was only 35 s^{-1} for the less electrophilic carbonate ester. This dependence on electrophilicity is more consistent with rate-limiting formation of tetrahedral intermediate **III** rather than its decomposition.

Molecular modelling has also been used to formulate hypotheses of how different residues of TG2 may participate in the acylation step. For example, in 2004 Chica et al. constructed a model of Michaelis complex **II** by docking Cbz-Gln-Gly into the active site tunnel [40]. However, without the insight provided by Khosla's 'open' structure [16] from 2007, Chica exposed the active site tunnel of the available 'closed' structure of Cerione [30] by rotating Trp241 out of position, rather than by displacing Tyr516 in an unforeseen and unusually large conformational change [40]. While this may diminish the relevance of Chica's conclusions, the approach remains valid. To that end, for this review we used the 'open' structure [16] of Pinkas et al. as a more appropriate starting point, removed the irreversible inhibitor and docked the simple substrate Cbz-Gln-Gly in its place. Specifically, the active site for the docking experiments was first defined to include all residues located within 8 Å of Cys277 (38 residues including Trp241, His335, and Trp332) and flexible ligand docking was conducted using the Molecular Operating Environment (MOE) suite of software. From the resulting lowest energy model, shown in Fig. 5, one can see that the distance between the thiol of active site

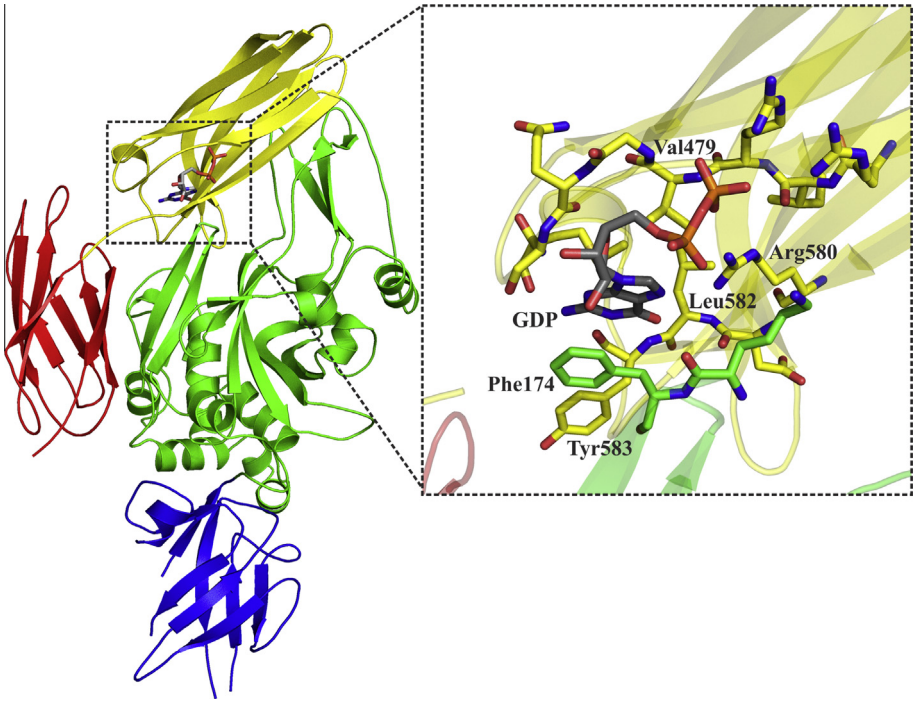


Fig. 1. Crystal structure [30] of TG2 in its 'closed' conformation (PDB: 1KV3). Inset: residues involved in binding of GDP.

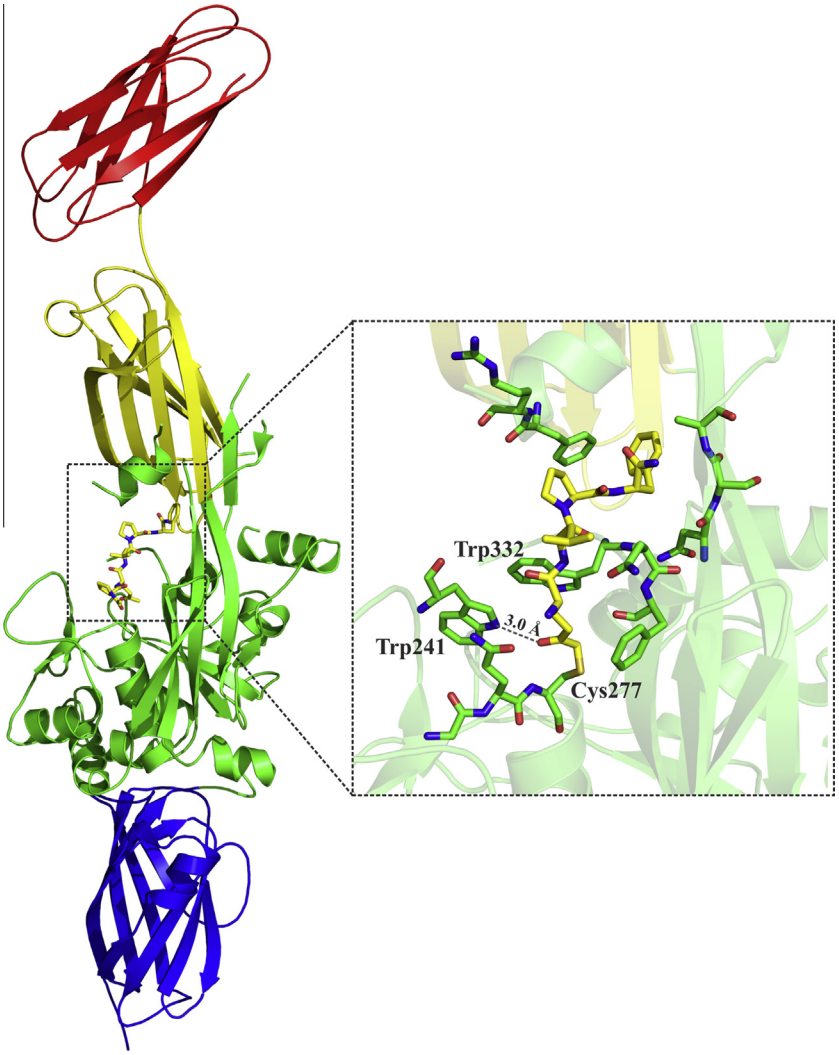


Fig. 2. Crystal structure [16] of TG2 in its 'open' conformation (PDB: 2Q3Z). Inset: residues interacting with bound irreversible inhibitor Ac-P(DON)LPF-NH_2.

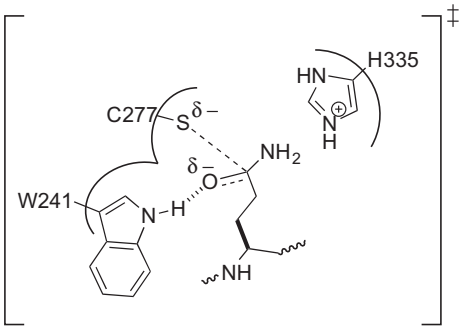


Fig. 3. Rate-limiting transition state of acylation step.

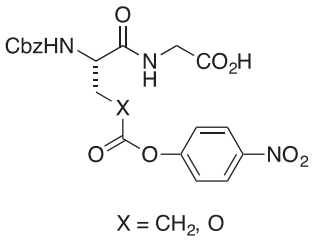


Fig. 4. Glutamyl derivatives studied [57] as acyl donor substrate analogues.

Cys277 and the imidazole of His355 is 3.2 Å, while the indole nitrogen of Trp241 is within 3.6 Å of the carboxamide carbonyl (see Fig. 3). Furthermore, the proximity between His335 and the carboxamide NH_2 group (4.9 Å) illustrates the feasibility of the imidazolium group acting as a general acid in the expulsion of ammonia (**III** to **IV**), playing the same role as the analogous residue of the cysteine proteases.

4. TG2 deacylation

4.1. Hydrolysis

In the absence of a primary amine acceptor, the acyl-enzyme intermediate can undergo hydrolysis to regenerate free enzyme, albeit much more slowly than aminolysis [58]. Fleckenstein et al. studied [24] these deacylation reactions in the presence of excess free amine by capillary zone electrophoresis and noted that transamidation was favoured over hydrolysis by a ratio of 6:1. However, upon lowering the pH from 7.5 to 6.0, the ratio of the initial rates of transamidation to hydrolysis dropped to 1:2. The authors suggested that at the lower pH, His335 of the catalytic triad would

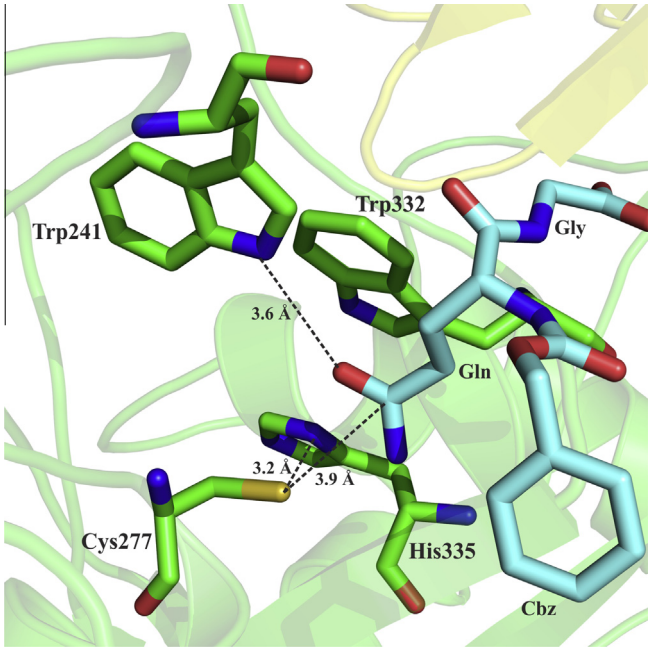


Fig. 5. Cbz-Gln-Gly docked (herein) into the active site of 'open' form TG2, using PDB structure 2Q3Z.

be increasingly protonated, and less capable of acting as a general base in the aminolysis reaction (see **V** to **VI** in Scheme 3). However, this explanation does not account for the increase in hydrolytic activity, for which the active site histidine is presumably also required as a general base, as it is in the cysteine protease mechanism. It seems more likely that a second basic residue that is required only for deprotonation of alkyl ammonium substrate (prior to further deprotonation by the active site general base) may be more affected by the drop in pH over this range (see below).

Case and Stein also studied the deacylation reaction with the goal of determining its rate-limiting step [53]. Specifically, they studied the solvent deuterium isotope effect on the rate constant for enzymatic hydrolysis. Similarly to their study of enzymatic acylation, they estimated the kinetic isotope effects (KIEs) for each microscopic rate constant, using fractionation factors, in order to compare the experimental value with those calculated for different rate-limiting scenarios. We propose that one can assume that the decomposition of the tetrahedral intermediate (**VII** in Scheme 3) to yield products would *not* involve general acid-assisted departure of thiolate, since in the microscopic reverse reaction, thiols do not require general base assistance in nucleophilic attacks, but rather react as their thiolates. With this assumption, we may re-visit the authors' calculations and suggest that the predicted KIE for breakdown of the tetrahedral intermediate would be close to 1. From that, we can show that the experimentally obtained KIE of 3.6 corresponds closely to a single mechanistic scenario, where decomposition of the tetrahedral intermediate to give the product is faster than its decomposition to return to the acyl enzyme, suggesting general-base assisted attack by water is the rate-limiting step for hydrolytic deacylation (Fig. 6).

4.2. Acyl-acceptor substrate specificity

Some of the first experiments to characterise the amine substrate specificity of TGases were carried out by Gross et al. on FXIIIa and guinea pig liver (tissue) TGase [11]. After testing series of alkyl amines as acyl-acceptor substrates, they concluded that the specificity for branched aliphatic amines depended on the length of the

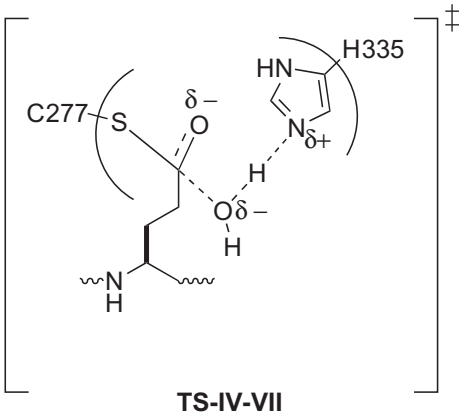


Fig. 6. Rate-limiting transition state for hydrolytic deacylation.

carbon chain, with a preference for those with a chain length identical to that found in lysine, the protein's natural substrate. This trend was still present, though less dramatic, for straight-chain amines. N^{α}-hippuryl-L-lysine amide was found to be favoured over the D-isomer, although this stereospecificity is not absolute, as opposite preferences have been observed for different substrate analogues with gplTG. The authors also found that peptides having a Lys residue flanked on both sides by Gly residues served as substrates of similar efficiency as straight-chain aliphatic amines. However, they noted that having a Leu residue *N*-terminal to the Lys residue improved substrate affinity, while having the Leu in the *C*-terminal position was detrimental.

Lorand and co-workers [59] studied αB-crystallin as an acyl-acceptor substrate, while Groenen et al. [60] studied βA3-crystallin as an acceptor substrate. Both groups showed that both proteins contain multiple reactive Lys residues, located in the *C*- and *N*-terminal regions, respectively. Sequence analysis did not reveal a consensus motif, but rather suggested that conformational flexibility and accessibility may be the most important factors for reactive Lys residues. However, Groenen et al. [60] also noted that not all Lys residues in the same protein domains act as amine substrates, suggesting that a certain degree of sequence specificity may exist.

In further work, Groenen et al. also probed [61] the impact of residues *N*-terminal to the amine donor Lys; while the authors initially found that significant variability could be tolerated in this position, suggesting that TG2 did not require a strict consensus sequence, they did note that a Pro residue hampered the transamidation reaction. Additional experiments showed that both Pro and Gly in this position had the greatest adverse effect, which is unsurprising given their influence on secondary structure [62]. Further studies by Grootjans et al. showed [63] that in αA-crystallin mutants bearing a reactive Lys as the penultimate residue of the accessible *C*-terminus [60], the following relative reactivity was observed for the preceding position: Gly, Asp < Pro, His, Trp < Ser, Ala, Leu, Tyr, Asn < Val, Arg, Phe [63]. These results are in agreement with the early work of Gross et al. [11] as well as that of Groenen et al. [61,62].

Finally, Csósz et al. recently attempted [64] to statistically analyse the structural motifs of both Gln and Lys residues that are TG2-reactive, and showed that the reactive residues are often located in regions that are not well-resolved in crystal structures. Lys substrate residues were found to be located in both ordered and intrinsically disordered regions, less specificity than that for the glutamine acyl donor substrate, referring to the previous work of Grootjans et al. [63] Overall, the authors concluded that, instead of looking only at amino acid sequences, it is also necessary to consider other spatial features to explain the interactions observed between TG2 and its substrates [64]. This conclusion would appear

to summarise the results of most investigations within the admittedly broad specificity shown by TG2 for its amine substrates.

4.3. Transamidation mechanistic studies

As mentioned above, the first reported crystal structure of the 'open' form of the enzyme, notably with ligand bound in the accessible active site, was not published until 2007 by Pinkas et al. [16] From this structure (Fig. 2), it was shown [16] that the catalytic Cys277 is located in a hydrophobic tunnel bridged by residues Trp241 and Trp332, which reside on separate loops in the active site (Fig. 7). Trp241 was proposed to guide donor substrate binding, whereas Trp332 was postulated to regulate acyl acceptor substrate binding. Mutants W241A and W332A showed [16] no detectable activity, according to a coupled enzyme assay for ammonia detection [65], presumably due to perturbation of substrate binding sites and/or the active site pocket itself. Interestingly, Keillor et al. would later show [66] that the W332F mutant retained significant activity (also observed [55] by Iismaa et al.) but with distinctly different amine substrate *specificity*, confirming the importance of this residue to substrate binding. Pinkas et al. proposed [16] that these bridging Trp residues and their corresponding loops separate, analogous to a drawbridge, in order to release the enzyme-bound transamidated product after transamidation completion (Fig. 7). Additional mutagenesis experiments showed that Thr360 is also involved in transamidation; its replacement by alanine resulted in the ratio of transamidation to hydrolysis dropping from 3.6 to 0.11. Thr360 is conserved across all catalytically active members of the TGase family, and is located [16] on the solvent-exposed entrance of the putative acyl acceptor-binding tunnel (see Fig. 7), suggestive of a specific role in amine binding.

As discussed above for the acylation step, Pinkas et al. also observed [16] that the carbonyl of their enzyme-bound inhibitor formed hydrogen bonds with the indole of Trp241 and the backbone amide of Cys277, leading them to propose that these residues stabilize the oxyanion intermediates for both enzyme acylation and deacylation (both **III** and **VI** in Scheme 3) in a similar fashion.

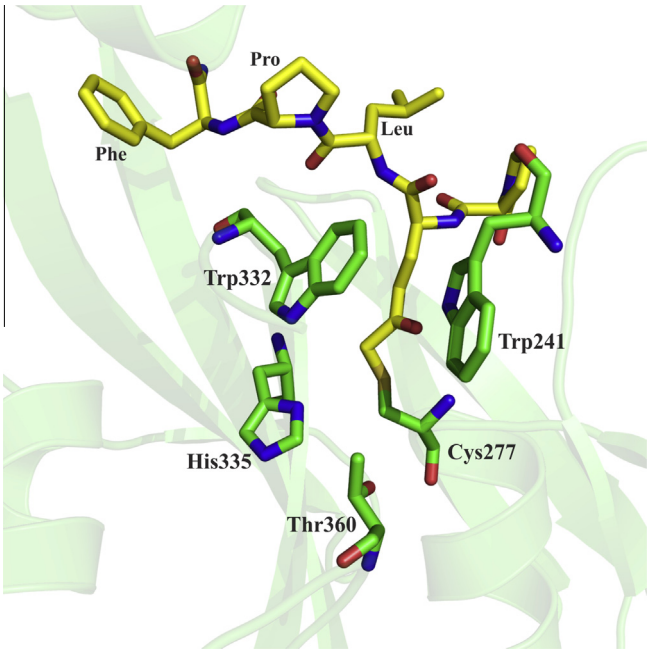
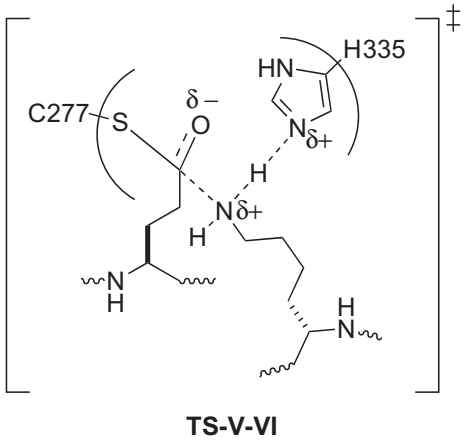


Fig. 7. Close-up view of putative acyl-acceptor substrate binding site of TG2 'open' form structure [16].

This proposal is consistent with the previous results of Iismaa et al., [55] showing importance of Trp241 catalytic efficiency (see above and Fig. 5).

In one of the first detailed structure–function studies of TG2-mediated transamidation, Leblanc et al. used a *p*-nitrophenyl ester substrate analogue as an acyl-donor substrate and as acyl-acceptor substrates, a series of six different primary amines, whose pK_{NH+} values ranged from 5.6 to 10.5 [58]. Under these conditions, acylation was rapid and deacylation was rate-limiting and dependent on amine basicity (nucleophilicity). Using a rate law including total amine concentration, a Brønsted plot was constructed for k_{cat}/K_M that gave a slope of $\beta_{nuc} = -0.37 \pm 0.08$; however, if only the basic form of the amine is considered, this slope changes to $\beta_{nuc} = 0.7 \pm 0.1$. Later, Case and Stein extended this investigation by a similar approach, but using different acyl-donor substrates (namely Cbz-Gln-Gly and *N*,*N*-dimethylcasein, NMC) [53]. Even under their conditions, using amide acyl-donor substrates, acylation was still faster than deacylation, which showed a similar dependence on acyl-acceptor amine basicity. Adding their data to those obtained by Leblanc et al., [58] they constructed a Brønsted plot whose slope was $\beta_{nuc} = 0.51 \pm 0.07$. The fact that the Cbz-Gln-Gly data points fell on the same line as that of the Leblanc study strongly suggests that acylation is rapid and deacylation is rate-limiting for both of the acyl donor substrates studied [53], while the linearity of the Brønsted plot indicates that no change in the rate-limiting step occurred over the series of amines studied, and deacylation remains the rate-limiting step [58]. The shallow positive slopes of the Brønsted plots suggest a moderate degree of positive charge buildup on the attacking amine nitrogen, consistent with a proposed transition state structure, in which the charge is delocalised between the attacking amine and the His335 imidazole that acts as a general base for catalysis (Fig. 8). Fleckenstein et al. later concurred with this proposed mechanism for transamidation, where His335 removes a proton from the amine substrate during the rate-limiting step of the deacylation reaction [24]. Case and Stein also noted [53] that while acylation from NMC was still faster than rate-limiting deacylation, rate differences were observed relative to the glutamyl dipeptide series. This serves as an important reminder that different acyl donor substrates may form different acyl-enzyme intermediates, having different conformations and dynamics, resulting in a difference in reaction rates with the second (amine donor) substrate.

Leblanc et al. also used solvent kinetic isotope effects (KIEs) to study the proton in flight at the transition state of the purported rate-limiting step (Fig. 8). Since the experiments were performed at a pH (or pD) of 7.0, within the broad plateau of the pH-rate



TS-V-VI

Fig. 8. Rate-limiting transition state for deacylation by transamidation.

profile [65,67,68], the effect on enzyme ionisation state was assumed to be negligible, assuring that the observed reaction rates were due to kinetic effects, and not equilibrium effects. For each acceptor substrate (aminoacetonitrile, glycine methyl ester and *N*-acetyl-L-lysine methyl ester) studied, a significant KIE was observed (5.9, 3.4, 1.2 respectively), suggesting that there was indeed a proton in flight at the rate-limiting transition state. Furthermore, the magnitude of the observed KIE was found to vary with amine acidity and therefore degree of proton transfer at the transition state [58].

Finally, a key feature of the aminolysis mechanism noted by many authors [24,53,58] is the requirement for initial deprotonation of acyl-acceptor substrate, which exists predominantly as its alkyl ammonium form in bulk solution, prior to a second deprotonation by the active site general base during nucleophilic attack on the acyl-enzyme thiolester (see Scheme 4). High-resolution structural data recently obtained [69] for FXIII, a closely related TGase, may provide insight regarding the double deprotonation. Specifically, the authors proposed [69] that in addition to the well-established catalytic triad, two other residues, His342 and Glu401, facilitate nucleophilic attack by the amine substrate. This pair of residues attains a productive spatial arrangement upon calcium binding in their structure, and is highly conserved across all human TGases. In Khosla's 'open form' structure [16], the corresponding putative base is His305, although it is not immediately adjacent to the acyl-donor binding site. It is important to note that as long ago as 1994, Yee [70] noted the catalytic function of corresponding residue His342 of FXIII, while Hettasch and Greenberg [52] showed that the H342A mutant showed strikingly reduced activity. This suggests by extension that His305 of TG2 may be important to its mechanism, possibly in the role proposed in Scheme 4. Given the symmetry of the acylation and deacylation steps in the transamidation reaction, in this role His305 would probably also participate, in its imidazolium form, in the protonation of ammonia during its departure, prior to its release into bulk solvent as ammonium.

5. Conformational regulation

5.1. Structural considerations

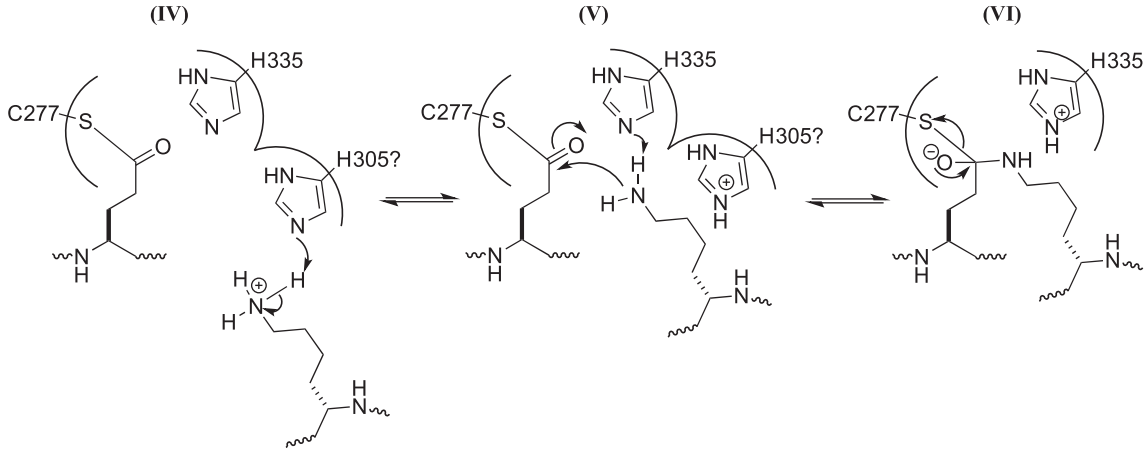
As mentioned above, early studies revealed that TG2 is activated by Ca^{2+} binding [32] and deactivated by GDP/GTP binding [54]. In terms of TG2 conformation, early SAXS results suggested the existence of a compact form of TG2 in the presence of GDP [71]. These results, in combination with fluorescence and dynamic CD studies, also suggested that TG2 may show conformational flexibility and the ability to adopt an alternative extended conformation upon exposure to Ca^{2+} [71,72]. More recently, X-ray crystallography has shown [16,30] that human TG2 can adopt at least two dramatically different tertiary structures that may be representative of large conformational changes related to ligand binding and the regulation of activity.

As mentioned above, Cerione's structure of human TG2 in complex with GDP [30] provided a picture of how guanine nucleotide binding diminishes TG2 activity. This structure comprises four structural domains: an *N*-terminal β-sandwich, a catalytic core, and two *C*-terminal β-barrel domains that are folded back over the core domain, giving a compact or 'closed' conformational form (Fig. 1). The GDP binding site is a hydrophobic pocket located in a groove between the catalytic core (Ala147 to Asn460) and the first β-barrel (Gly472 to Tyr583) domains [30], made up of residues Phe174, Val479, Met483, Leu582, and Tyr583 (see inset, Fig. 1).

In this conformation, flexible loops located within the first β-barrel effectively block substrate access to the active site. Furthermore, these loop rearrangements appear to be 'latched' in place by the formation of a dipole interaction between the phenol side chain of re-positioned Tyr516 and the nucleophilic thiol group of active site Cys277 (Fig. 9) [30]. The authors also proposed that residues Ile416-Ser419 may form a cavity in which the binding of Ca^{2+} could weaken GDP binding and allow for "activation" of TG2 [30].

Fig. 2 shows Khosla's structure of human TG2, irreversibly inhibited by pentapeptide Ac-P(DON)LPF-NH_2, in a dramatically different 'open' conformation [16]. To ensure that this structure was not an artefact of crystallography, the authors measured the hydrodynamic radii (R_h) of TG2 in solution, in the presence of GDP and after reaction with their inhibitor. These values were found [16] to be in excellent agreement with the initial 'closed form' structure of the enzyme [30] and the authors' 'open form' structure, respectively. The latter values are also in good agreement with previous small angle neutron scattering measurements obtained for TG2 activated by calcium in solution [73].

Structural overlay and comparison of the open and closed forms of TG2 shows that although the *C*-terminal β-sandwich domains are displaced by approximately 120 Å, the *N*-terminal and catalytic domains remain largely unchanged, suggesting the conformational regulation of activity may be due to occlusion of substrate binding sites rather than perturbation of the catalytic machinery [30]. However, as mentioned above, it has also been noted that the 'open' crystal structure of TG2 also differs from the 'closed' structure in that it contains an additional disulphide bond, namely



Scheme 4. Proposed role for His305 as a second base in the reaction of alkyl ammonium acyl-acceptor substrates.

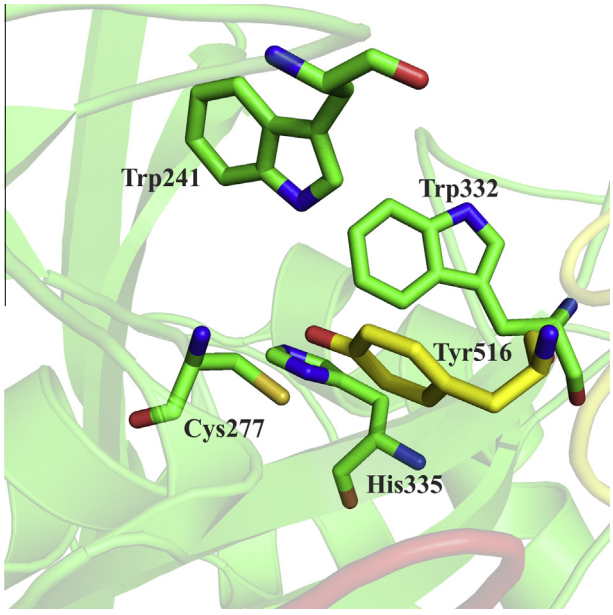


Fig. 9. Tyr516 (from β-barrel 1) inserts into the active site of the 'closed' (inactive) conformation of TG2 [30].

between vicinal cysteine residues Cys370 and Cys371 [16,17]. This disulphide bond has been suggested to act as a redox activated conformational switch, in that it must be reduced in order to allow the enzyme to adopt an open and catalytically active conformation. In this way, this disulphide bond may serve as an additional mode for regulation of activity *in vivo* [16,17].

5.2. Gel electrophoresis studies

In order to directly monitor how ligand binding may affect TG2 conformation under native conditions, several groups have conducted native PAGE (nPAGE) studies. In a detailed study by Begg et al. [74] nPAGE gels were run for wild-type and mutant variants of TG2 in the absence and presence of GTP (5, 25 and 500 μM). In the absence of GTP, TG2 was found to migrate predominantly in a single band, whereas the addition of 500 μM GTP resulted in the appearance of a faster migrating (presumably 'closed') form of TG2. Subsequently, the groups of Keillor and Khosla independently performed additional nPAGE studies to determine the influence of GDP, $CaCl_2$ and synthetic reversible and irreversible inhibitors on TG2 conformation [16,75]. Both research groups found that in the absence of ligand, TG2 presented two bands by nPAGE, corresponding to a population of (slowly interconverting) open and closed forms. Further, running gels in the presence of GDP/GTP resulted in a shift in TG2 conformation to the faster migrating species (closed form) while incubation of TG2 with $CaCl_2$ resulted in a shift to the slower migrating (open) form of TG2. When Khosla et al. incubated TG2 with the same irreversible inhibitor they used in their crystallographic studies, they observed that TG2 showed a single slower migrating band on nPAGE, presumably corresponding to the open conformation X-ray structure [16].

Recently a method based on kinetic capillary electrophoresis (KCE) was adapted to allow for the real-time monitoring of effector-induced conformational dynamics of TG2 [76]. When purified TG2 was analysed by CE, two peaks were observed, where the faster migrating peak corresponds to a family of extended (open) conformations and the slower eluting peak corresponds to a family of more compact (closed) conformations (Fig. 10). This observation is in excellent agreement with the separation achieved previously by native PAGE [16,74,75]. Supplementation of the KCE running buffer with effector ligands known to influence TG2 conformation (either Ca^{2+} or GDP) resulted in a shift in the relative populations of the migrating species. Measuring this shift in the conformational equilibrium allowed ligand titration experiments to be performed, resulting in the determination of a K_d value for calcium (39 μM) in good agreement with previous work. This method also allowed, for the first time, the determination of rate constants for the conformational opening and closing of TG2 (k_{open} = 0.138 min^{-1} and k_{close} = 0.050 min^{-1} respectively) [76]. Importantly, since these rate constants are slower than k_{cat} values observed for TG2-mediated hydrolysis or transamidation, these conformational changes cannot take place during the catalytic cycle. Rather, it seems more likely that the conformational change occurs as part of the tight functional regulation of TG2.

5.3. FRET-based conformational assays

FRET-based assays have also been developed in order to study TG2 conformational changes. Shakhparonov and co-workers [77] modified the gene encoding for TG2 in order to fuse yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) to its *N*- and *C*-termini, respectively. In this design, the closed form of TG2 should allow FRET to take place between the two fluorescent proteins (due to the close proximity of the *N*- and *C*-termini of TG2 in this conformation) while the extended form would separate the fluorescent proteins and prevent FRET between the pendant fluorescent proteins (Scheme 5). This fusion protein was then expressed inside mammalian cells. Treatment of these cells with calcium ionophore A23187 resulted in rapid decrease in FRET, presumably due to the conformational shift from closed to open form TG2.

Independently, Truant and co-workers [78] prepared a similar fluorescence-based TG2 conformational sensor, also by fusing CFP and YFP to the *N*- and *C*-termini, respectively. They then used FRET-FLIM (fluorescent lifetime imaging microscopy) to determine TG2 conformation inside living cells. When these cells were treated with A23187 to increase intracellular Ca^{2+} levels, a decrease in FRET was observed, indicative of a conformational shift to open form TG2. Analysis of mutant variants of TG2 revealed that the R580A mutation (known to have compromised GDP/GTP binding) showed significantly less FRET compared to wild-type enzyme, suggesting this mutation shifts the TG2 conformational equilibrium towards the open form (in agreement with earlier nPAGE studies [74]). By way of contrast, the mutant W241A, known have compromised TG2 transamidation activity, did not show different conformational behaviour (presumably because the W241A mutant is still able to bind GDP).

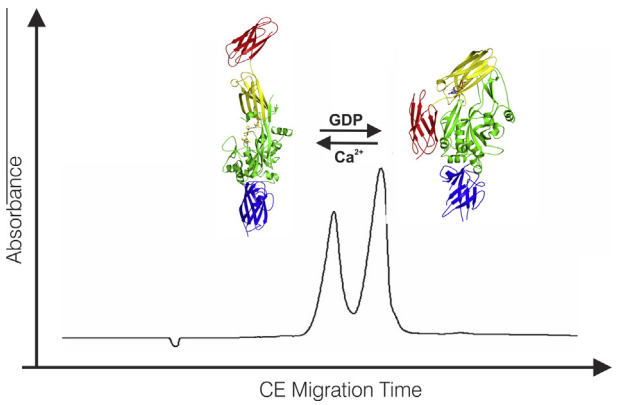
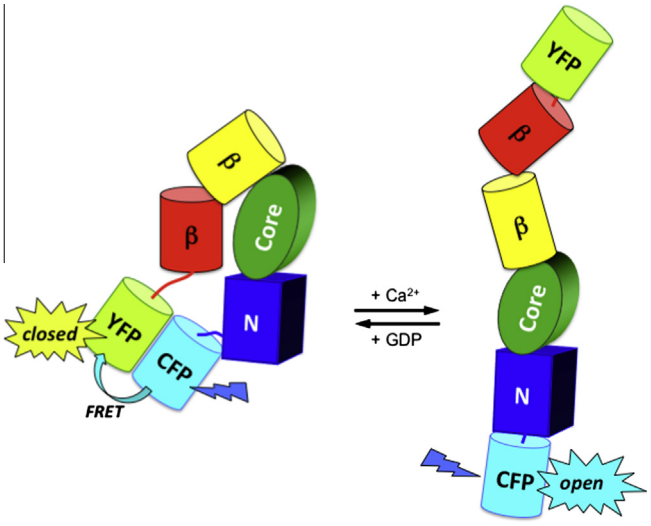


Fig. 10. Separation of families of 'closed' and 'open' conformations of TG2 by capillary electrophoresis.



Scheme 5. FRET-based conformational assay [77,78]. Excitation of CFP leads to FRET with YFP and yellow emission in closed form, but only cyan emission in open form.

This biosensor system was then applied to study the conformational effects of known TG2 inhibitors. When cells expressing the CFP-TG2-YFP fusion protein were treated with NC9, an irreversible inhibitor developed in the Keillor group [66,79], a significant decrease in FRET was observed, relative to untreated cells, indicating the irreversibly inhibited TG2 adopts a more extended conformation. Treatment of these cells with CP4d, a reversible and TG2-selective inhibitor [80] led to an enhanced FRET signal relative to untreated cells. This provides evidence for the intracellular efficacy of this inhibitor ($EC_{50} \sim 1\ \mu M$), which may affect TG2 activity by stabilizing a closed (inactive) conformation. Currently the mechanism of this conformational effect is not known – for example, inhibitor binding may result in a large-scale conformational change directly, or alternatively it may favour GDP binding. However, it raises the possibility that conformational stabilization may play a role in the mode of action of a small molecule TG2 inhibitor.

6. Perspectives

Judging from the topics that are presented above, and those that are noticeably absent, it is obvious that there remain several unanswered mechanistic questions. For example, what are the structural determinants for the selectivity of transamidation over hydrolysis? Where, and precisely how, is the amine substrate bound? To what extent is the transamidation reaction reversible? Furthermore, if one considers the biological roles of TG2, how is its mechanistic behaviour structurally controlled by its conformational dynamics and regulation?

However, in addition to these questions, there is another emerging physiological function that bears consideration. Namely, mounting evidence suggests that TG2 may play alternative *non-catalytic* roles within cells, 'moonlighting' as a conformation-dependent scaffold protein [81,82]. For example, in a recent study published by Ahn and co-workers [83], nuclear TG2 was found to modulate MMP-9 expression levels in cardiomyoblast cells *via* non-covalent interactions. Immunoprecipitation showed that TG2 in its closed form may affect the formation of a protein dimer complex that serves as a transcription modulator [83], potentially through protein–protein interactions. The ability of TG2 to form non-covalent (protein–protein) interactions within cells was also suggested in work published by Johnson and co-workers [84] who used yeast 2-hybrid screening to detect the association of TG2 with a subunit of the hypoxia inducible factor 1 (HIF1), preventing the formation of a heterodimer and resulting in reduced neuronal death under ischemic conditions. Finally, in further studies [85] probing the role of TG2 in ischemic cell death, Johnson and coworkers independently manipulated the conformation and transamidation activity of various TG2 mutants, allowing them to speculate that the primary functional role of TG2 in cell survival/death processes may be as a non-catalytic scaffold protein, although the exact nature of the binding partners remains unknown.

In this light, one may hope that future bioorganic mechanistic investigations may not only answer the remaining questions regarding TG2's catalytic behaviour, but also provide tools for studying the non-catalytic, conformation-dependent biological function of this complex and fascinating enzyme.

References

[1] L. Lorand, R.M. Graham, Nat. Rev. Mol. Cell Biol. 4 (2003) 140.
[2] C. Tabolacci, A. Lentini, B. Provenzano, S. Beninati, Amino Acids 42 (2012) 975.
[3] N.K. Sarkar, D.D. Clarke, H. Waelsch, Biochim. Biophys. Acta 25 (1957) 451.
[4] A. Kalinin, L.N. Marekov, P.M. Steinert, J. Cell Sci. 114 (2001) 3069.
[5] V. Pietroni, S. Di Giorgi, A. Paradisi, B. Ahvazi, E. Candi, G. Melino, J. Invest. Dermatol. 128 (2008) 2760.
[6] H. Thomas, K. Beck, M. Adamczyk, P. Aeschlimann, M. Langley, R. Oita, L. Thiebach, M. Hils, D. Aeschlimann, Amino Acids 44 (2013) 161.
[7] K. Kuramoto, R. Yamasaki, Y. Shimizu, H. Tatsukawa, K. Hitomi, Arch. Biochem. Biophys. 537 (2013) 138.
[8] D. Aeschlimann, M. Paulsson, Thromb. Haemost. 71 (1994) 402.
[9] J.S. Chen, K. Mehta, Int. J. Biochem. Cell Biol. 31 (1999) 817.
[10] T. Cabras, R. Inzitari, C. Fanali, E. Scarano, M. Patamia, M.T. Sanna, E. Pisano, B. Giardina, M. Castagnola, I. Messana, J. Sep. Sci. 29 (2006) 2600.
[11] M. Gross, N.K. Whetzel, J.E. Folk, J. Biol. Chem. 252 (1977) 3752.
[12] K.N. Parameswaran, L. Lorand, Biochemistry 20 (1981) 3703.
[13] M. Griffin, R. Casadio, C.M. Bergamini, Biochem. J. 368 (2002) 377.
[14] J.L. Piper, G.M. Gray, C. Khosla, Biochemistry 41 (2002) 386.
[15] L. Fésüs, M. Piacentini, Trends Biochem. Sci. 27 (2002) 534.
[16] D.M. Pinkas, P. Strop, A.T. Brunger, C. Khosla, PLoS Biol. 5 (2007) e327.
[17] J. Stamnaes, D.M. Pinkas, B. Fleckenstein, C. Khosla, L.M. Sollid, J. Biol. Chem. 285 (2010) 25402.
[18] G. Melino, M. Annicchiarico-Petruzzelli, L. Piredda, E. Candi, V. Gentile, P.J. Davies, M. Piacentini, Mol. Cell Biol. 14 (1994) 6584.
[19] Z. Balajthy, K. Csomós, G. Vámosi, A. Szántó, M. Lanotte, L. Fésüs, Blood 108 (2006) 2045.
[20] J. Tucholski, M. Lesort, G.V. Johnson, Neuroscience 102 (2001) 481.
[21] C.A. Gaudry, E. Verderio, D. Aeschlimann, A. Cox, C. Smith, M. Griffin, J. Biol. Chem. 274 (1999) 30707.
[22] J.M. Bowness, A.H. Tarr, R.I. Wiebe, Thromb. Res. 54 (1989) 357.
[23] W. Dieterich, T. Ehnis, M. Bauer, P. Donner, U. Volta, E.O. Riecken, D. Schuppan, Nat. Med. 3 (1997) 797.
[24] B. Fleckenstein, O. Molberg, S.-W. Qiao, D.G. Schmid, F. von der Mulbe, K. Elgstoen, G. Jung, L.M. Sollid, J. Biol. Chem. 277 (2002) 34109.
[25] G.V.W. Johnson, T.M. Cox, J.P. Lockhart, M.D. Zinnerman, M.L. Miller, R.E. Powers, Brain Res. 751 (1997) 323.
[26] L.K. Rasmussen, E.S. Sørensen, T.E. Petersen, J. Gliemann, P.H. Jensen, FEBS Lett. 338 (1994) 161.
[27] S.M. Dudek, G.V.W. Johnson, J. Neurochem. 61 (1993) 1159.
[28] M. DiFiglia, E. Sapp, K.O. Chase, S.W. Davies, G.P. Bates, J.P. Vonsattel, N. Aronin, Science 277 (1997) 1990.
[29] M.E. MacDonald, C.M. Ambrose, M.P. Duyao, R.H. Myers, C. Lin, L. Srinidhi, G. Barnes, S.A. Taylor, M. James, N. Groot, H. Macfarlane, B. Jenkins, M.A. Anderson, N.S. Wexler, J.F. Gusella, Cell 72 (1993) 971.
[30] S. Liu, R.A. Cerione, J. Clardy, Proc. Natl. Acad. Sci. USA 99 (2002) 2743.
[31] P. Grenard, M.K. Bates, D. Aeschlimann, J. Biol. Chem. 276 (2001) 33066.
[32] J.E. Folk, P.W. Cole, J. Biol. Chem. 241 (1966) 3238.
[33] J.E. Folk, P.W. Cole, J.P. Mullooly, J. Biol. Chem. 242 (1967) 4329.
[34] M. Gross, N.K. Whetzel, J.E. Folk, J. Biol. Chem. 250 (1975) 7693.
[35] R. Király, É. Csósz, T. Kurtán, S. Antus, K. Szigeti, Z. Simon-Vecsei, I.R. Korponay-Szabó, Z. Keresztessy, L. Fésüs, FEBS J. 276 (2009) 7083.
[36] J.E. Folk, J. Biol. Chem. 244 (1969) 3707.
[37] T.J.P. Hubbard, B. Ailey, S.E. Brenner, A.G. Murzin, C. Chothia, Nucl. Acids Res. 27 (1999) 254.
[38] K.S. Makarova, L. Aravind, E.V. Koonin, Protein Sci. 8 (1999) 1714.
[39] J.E. Folk, P.W. Cole, J. Biol. Chem. 240 (1965) 2951.
[40] R.A. Chica, P. Gagnon, J.W. Keillor, J.N. Pelletier, Protein Sci. 13 (2004) 979.
[41] T. Ohtsuka, M. Ota, N. Nio, M. Motoki, Biosci. Biotechnol. Biochem. 64 (2000) 2608.
[42] Y. Sugimura, M. Hosono, F. Wada, T. Yoshimura, M. Maki, K. Hitomi, J. Biol. Chem. 281 (2006) 17699.
[43] Z. Keresztessy, É. Csósz, J. Hársfalvi, K. Csomós, J. Gray, R.N. Lightowlers, J.H. Lakey, Z. Balajthy, L. Fésüs, Protein Sci. 15 (2006) 2466.

[44] C. Esposito, I. Caputo, FEBS J. 272 (2005) 615.
[45] M.T. Pastor, A. Diez, E. Pérez-Payá, C. Abad, FEBS Lett. 451 (1999) 231.
[46] D. Aeschlimann, M. Paulsson, K. Mann, J. Biol. Chem. 267 (1992) 11316.
[47] L.W. Vader, A. de Ru, Y. van der Wal, Y.M.C. Kooy, W. Benckhuijsen, M.L. Mearin, J.W. Drijfhout, P. van Veelen, F. Koning, J. Exp. Med. 195 (2002) 643.
[48] A. Facchiano, F. Facchiano, Amino Acids 36 (2009) 599.
[49] J.E. Folk, Adv. Enzymol. Relat. Areas Mol. Biol. 54 (1983) 1.
[50] K.N. Lee, S.A. Arnold, P.J. Birckbichler, M.K. Patterson, B.M. Fraij, Y. Takeuchi, H.A. Carter, Biochim. Biophys. Acta 1202 (1993) 1.
[51] R. Micanovic, R. Procyk, W. Lin, G.R. Matsueda, J. Biol. Chem. 269 (1994) 9190.
[52] J.M. Hettasch, C.S. Greenberg, J. Biol. Chem. 269 (1994) 28309.
[53] A. Case, R.L. Stein, Biochemistry 42 (2003) 9466.
[54] K.E. Achyuthan, C.S. Greenberg, J. Biol. Chem. 262 (1987) 1901.
[55] S.E. Iismaa, S. Holman, M.A. Wouters, L. Lorand, R.M. Graham, A. Husain, Proc. Natl. Acad. Sci. USA 100 (2003) 12636.
[56] S.E. Iismaa, B.M. Mearns, L. Lorand, R.M. Graham, Physiol. Rev. 89 (2009) 991.
[57] C. Gravel, D. Lapierre, J. Labelle, J.W. Keillor, Can. J. Chem. 85 (2007) 164.
[58] A. Leblanc, C. Gravel, J. Labelle, J.W. Keillor, Biochemistry 40 (2001) 8335.
[59] L. Lorand, P.T. Velasco, S.N.P. Murthy, J. Wilson, K.N. Parameswaran, Proc. Natl. Acad. Sci. USA 89 (1992) 11161.
[60] P.J.T.A. Groenen, J.J. Grootjans, N.H. Lubsen, H. Bloemendal, W.W. Dejong, J. Biol. Chem. 269 (1994) 831.
[61] P.J.T.A. Groenen, H. Bloemendal, W.W. Jong, Eur. J. Biochem. 205 (1992) 671.
[62] P.J.T.A. Groenen, R.H.P.H. Smulders, R.F.R. Peters, J.J. Grootjans, P.R.L.A. Vandenijssel, H. Bloemendal, W.W. Dejong, Eur. J. Biochem. 220 (1994) 795.
[63] J.J. Grootjans, P.J. Groenen, W.W. de Jong, J. Biol. Chem. 270 (1995) 22855.
[64] É. Csósz, P. Bagossi, Z. Nagy, Z. Dosztanyi, I. Simon, L. Fésüs, J. Mol. Biol. 383 (2008) 390.
[65] N. Day, J.W. Keillor, Anal. Biochem. 274 (1999) 141.
[66] J.W. Keillor, R.A. Chica, N. Chabot, V. Vinci, C. Pardin, E. Fortin, S.M.F.G. Gillet, Y. Nakano, M.T. Kaartinen, J.N. Pelletier, W.D. Lubell, Can. J. Chem. 86 (2008) 271.
[67] D.D. Clarke, M.J. Mycek, A. Neidle, H. Waelsch, Arch. Biochem. Biophys. 79 (1959) 338.
[68] N. Day, Études mécanistiques de l'étape d'acylation de la transglutaminase tissulaire de foie de cobaye, Université de Montréal, 1999.
[69] M. Stieler, J. Weber, M. Hils, P. Kolb, A. Heine, C. Büchold, R. Pasternack, G. Klebe, Angew. Chem. Int. Ed. Engl. (2013).
[70] V.C. Yee, L.C. Pedersen, I. Letrong, P.D. Bishop, R.E. Stenkamp, D.C. Teller, Proc. Natl. Acad. Sci. USA 91 (1994) 7296.
[71] P. Mariani, F. Carsughi, F. Spinozzi, S. Romanzetti, G. Meier, R. Casadio, C.M. Bergamini, Biophys. J. 78 (2000) 3240.
[72] A. Di Venere, F. De Matteis, N. Rosato, A. Rossi, A.F. Agrò, G. Mei, J. Biol. Chem. 275 (2000) 3915.
[73] R. Casadio, E. Polverini, P. Mariani, F. Spinozzi, F. Carsughi, A. Fontana, P. Polverino de Laureto, G. Matteucci, C.M. Bergamini, Eur. J. Biochem. 262 (1999) 672.
[74] G.E. Begg, L. Carrington, P.H. Stokes, J.M. Matthews, M.A. Wouters, A. Husain, L. Lorand, S.E. Iismaa, R.M. Graham, Proc. Natl. Acad. Sci. USA 103 (2006) 19683.
[75] C. Pardin, I. Roy, R.A. Chica, E. Bonneil, P. Thibault, W.D. Lubell, J.N. Pelletier, J.W. Keillor, Biochemistry 48 (2009) 3346.
[76] C.M. Clouthier, G.G. Mironov, V. Okhonin, M.V. Berezovski, J.W. Keillor, Angew. Chem. Int. Ed. Engl. 51 (2012) 12464.
[77] M.S. Pavlyukov, N.V. Antipova, M.V. Balashova, M.I. Shakhparonov, Biochem. Biophys. Res. Commun. 421 (2012) 773.
[78] N.S. Caron, L.N. Munsie, J.W. Keillor, R. Truant, PLoS ONE 7 (2012) e44159.
[79] H.F. Al-Jallad, V.D. Myneni, S.A. Piercy-Kotb, N. Chabot, A. Mulani, J.W. Keillor, M.T. Kaartinen, PLoS ONE 6 (2011) e15893.
[80] C. Pardin, I. Roy, W.D. Lubell, J.W. Keillor, Chem. Biol. Drug Des. 72 (2008) 189.
[81] Z. Wang, M. Griffin, Amino Acids 42 (2011) 939.
[82] D. Park, S.S. Choi, K.S. Ha, Amino Acids 39 (2010) 619.
[83] J.-S. Ahn, M.-K. Kim, J.-H. Hahn, J.-H. Park, K.-H. Park, B.-R. Cho, S.-B. Park, D.-J. Kim, Biochem. Biophys. Res. Commun. 376 (2008) 743.
[84] A.J. Filiano, C.D.C. Bailey, J. Tucholski, S. Gundemir, G.V.W. Johnson, FASEB J. 22 (2008) 2662.
[85] G. Colak, J.W. Keillor, G.V.W. Johnson, PLoS ONE 6 (2011) e16665.