TRANSGLUTAMINASES IN GLUTEN SENSITIVE DISEASES

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Jorunn Stamnæs

ABBREVIATIONS

APC	antigen presenting cell
CD	celiac disease
CE	capillary electrophoresis
csTG2	cell surface transglutaminase 2
DH	dermatitis herpetiformis
DTT	dithiothreitol
ECM	extracellular matrix
FITC	fluorescein isothiocyanate
GA	gluten ataxia
GSD	gluten sensitive disease
GSH	reduced glutathione
GSSG	oxidized glutathione
HLA	human leukocyte antigen
IAA	iodoacetic acid
IAM	iodoacetamide
IELs	intraepithelial lymphocytes
LIF	laser induced fluorescence
MHC	major histocompability complex
MS	mass spectrometry
PDI	protein disulfide isomerase
TGase	transglutaminase
TG2	transglutaminase 2

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INTRODUCTION

TRANSGLUTAMINASES

THE FAMILY OF TRANSGLUTAMINASES

Transglutaminases (TGases) (EC 2.3.2.13) are a family of evolutionary conserved enzymes found in almost all living organisms. Their primary function is the formation of covalent cross-links within and between peptides and proteins. This activity is Ca^{2+} dependent and specifically targets glutamine residues which are cross-linked to primary amines, creating very stable **iso-peptide bonds**.

Whereas most prokaryotes only have one TGase isoform, the human organism encodes nine isoforms of which eight are believed to exert cross-linking activity (Table 1) [1]. Six of the nine human TGase genes are located within two gene clusters; transglutaminase 2 (TG2), TG3 and TG6 are encoded on chromosome 20q11-12 while TG5, TG7 and 4.2 are encoded on chromosome 15q15 [2]. This suggests a close relationship and a recent evolution of several of the human TGase isoforms.

Transglutaminase cross-linking activity serves numerous of biological functions in the human body, from skin barrier formation and blood clotting to extracellular matrix assembly. Although some of the TGases remain to be fully characterized on the protein level, it appears that their biological functions both can be interrelated and strictly isoform specific. Some TGases are ubiquitously expressed, others are found only in certain tissues or cellular compartments, and some are zymogens requiring proteolytic activation (Table 1). Together with differences in substrate specificities, this should allow for a tight regulation and coordination of the collective transglutaminase activity in the human body.

Table 1. **Members of the human transglutaminase family**

Protein	Gene	Gene locus	Mw (kDa)	Tissue expression[1]	Localization	Function
Factor XIIIa[2]	*F13A1*	6p24-25	83	Platelets, chrondrocytes, placenta, plasma, synovial fluid, dermal dendritic cells	Cytosolic, extracellular	Blood coagulation [3], bone growth [4]
TG1	*TGM1*	14q11.2	90	Keratinocytes, brain	Membrane, cytosolic	Cell-envelope formation [5]
TG2	*TGM2*	20q11-12	80	Ubiquitous	Cytosolic, nuclear, membrane, cell surface, extracellular	Multiple [6]
TG3[3]	*TGM3*	20q11-12	77	Squamous epithelium, brain	Cytosolic	Cell-envelope formation [5]
TG4	*TGM4*	3q21-22	77	Prostate	Unknown	Semen coagulation in rodents [7]
TG5[3]	*TGM5*	15q15.2	81	Ubiquitous except for the CNS and lymphatic system	Unknown	Unknown
TG6	*TGM6*	20q11	78 [8]	Unknown	Unknown	Unknown
TG7	*TGM7*	15q15.2	?	Ubiquitous	Unknown	Unknown
Band 4.2[4]	*EPB42*	15q15.2	72	Red blood cells, bone marrow, fetal liver, spleen	Membrane	Membrane skeletal component [9]

Table 1 Members of the human transglutaminase family *[3-9] [1]Only tissues of high expression level are listed [2] Thrombin activated [3] Protease activated [4] No transglutaminase activity. Table 1 is modified from [1].*

TRANSGLUTAMINASE 2

Transglutaminase 2 (TG2) is the extraordinary member of the transglutaminase family. It is by far the most studied and best characterized isoform, and since its discovery in 1957 [10] a vast array of biological functions have been ascribed to this protein. Some of these functions are interrelated while others could well have been performed by completely unrelated proteins.

Biochemistry of TG2

Structure and conformation

TG2 is a monomeric protein with no reported posttranslational modifications and no disulfide bonds in its native state [11]. Similar to most of its family members, the enzyme consists of four domains; an N-terminal domain (harboring a fibronectin binding site) followed by a large, catalytic domain (harboring the active site C277) which is connected to two C-terminal β-barrel domains through a solvent exposed loop (Fig.1A). TG2 is an allosteric enzyme and both enzymatic activity and conformation is regulated by binding of the small ligands GTP (K_i ~90μM) and Ca^{2+} (K_a ~1mM) [12]. The GTP bound form of the enzyme assumes a compact conformation and is catalytically inactive [13]. In the absence of GTP and in the presence of

Ca^{2+}, the enzyme will assume a more open conformation where the active site is exposed and the enzyme is catalytically active. The open and closed conformation can be resolved by native polyacrylamide gel electrophoresis (nPAGE) [14, 15].

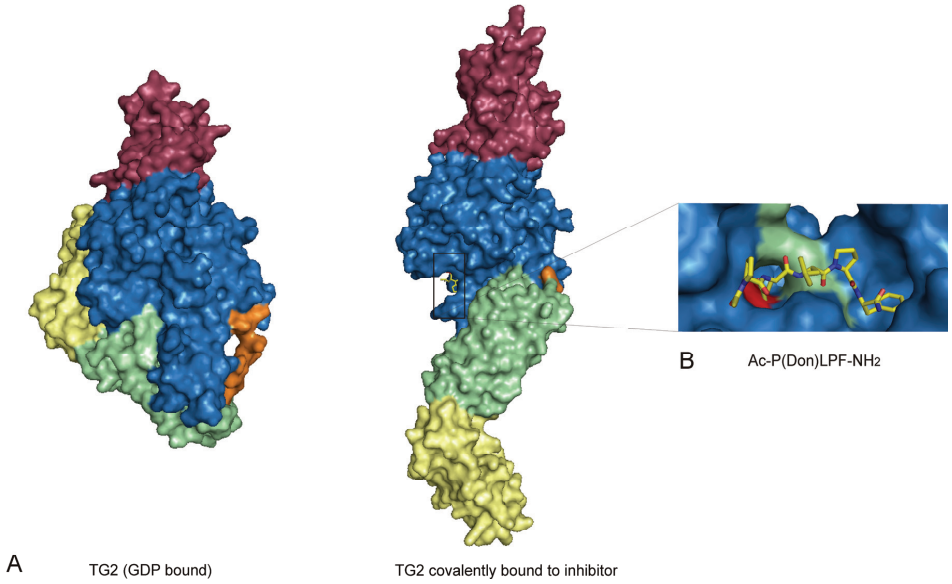

Figure 1 Structure and conformation of TG2 *A) TG2 consists of 4 domains; an N-terminal β-sandwich domain (dark red), the catalytic core domain (blue) and two C-terminal β-barrels (light green and light yellow) connected via a solvent exposed loop (orange). The dramatic change in conformation between the two crystal structures includes major peptide backbone changes within the catalytic domain of the enzyme.B) The peptide-like inhibitor Acetyl-P(Don)LPF-NH_2 is covalently linked to the active site C277 (red). Incoming acyl-acceptor substrates are believed to enter from the opposite side through a hydrophobic tunnel formed by the flexible indole groups of W241 and W332 (light green).The proline residue in +2 of the inhibitor war head group DON induces a remarkably good fit of the inhibitor to the active site. Figures are modified from[16, 17].*

Two crystal structures have been reported for TG2, displaying dramatically different conformations (Fig.1A). Liu *et.al* described the enzyme in the closed GDP bound conformation

[16], while Pinkas *et.al* recently reported an open, fully extended conformation [17]. In the closed conformation, the active site is filled by the 1st C-terminal β-barrel (Fig.1A, light green), and Y516 forms a hydrogen bond with C277 which stabilizes this conformation [15, 16]. In the open conformation, the active site is occupied by an irreversible, peptide-like inhibitor, suggesting that this conformation represents the thioester linked enzyme-substrate intermediate (Fig.1B, see next section). Interestingly, this conformation harbors a vicinal disulfide bond between C370 and C371. No function has yet been ascribed to this bond. Although not catalytically active, TG2 can still assume an open conformation in the absence of Ca^{2+} (as resolved by nPAGE). This suggests that small, but undoubtedly important, changes in conformation must be induced upon binding of Ca^{2+}. TG2 in a Ca^{2+} bound conformation remains to be crystallized and resolved.

Enzymatic activity

Most transglutaminases, including TG2, utilizes a papain-like cysteine-histidine-aspartate catalytic triad (C277, H335, A358 in TG2) to catalyze deamidation and transamidation [18]. In the presence of Ca^{2+}, the active site cysteine thiol-group (C277) will perform a nucleophilic attack on the γ-carboxamide group of a glutamine residue side chain (Fig.2). A thioester bond is formed between the glutamine residue side chain and the cysteine upon release of the first product, ammonia. The thioester linked TG2-substrate complex is then attacked by a nucleophilic primary amine (transamidation) or a water molecule (deamidation) displacing the enzyme and releasing a transamidated or deamidated product. Transamidation and deamidation is generally believed to be the rate-limiting step of the enzymatic reaction, although this remains disputed [19-22]. Transamidation has traditionally been regarded as the preferred reaction for TG2 where the presence of primary amines would efficiently inhibit deamidation. TG2 can also act as an iso-peptidase and further hydrolyze transamidated products introducing an indirect route of deamidation. This is however not a favored reaction and has so far only been demonstrated for transamidated products of small, primary amines [23, 24].

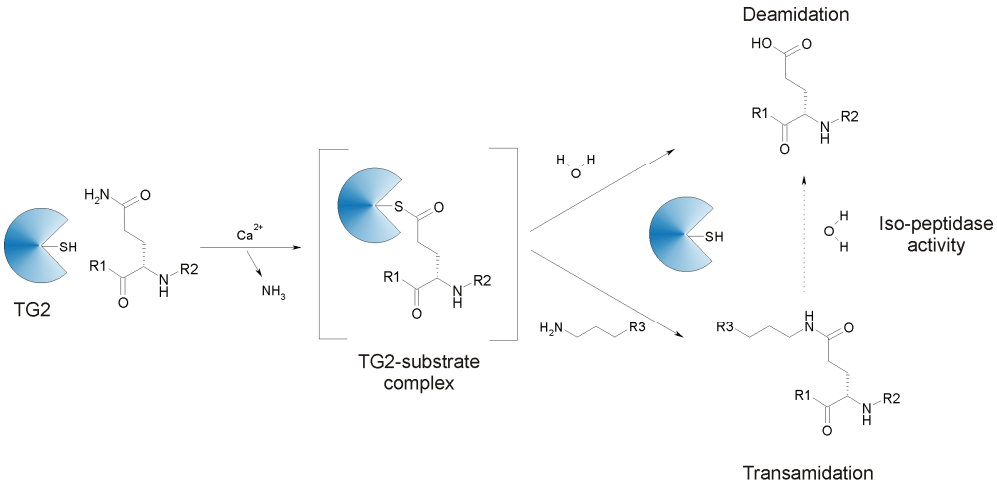

Figure 2 Enzymatic activity of TG2 *The catalysis follows a modified Ping-Pong mechanism [22] where the active site C277 attacks the glutamine residue side chain of an acyl-donor substrate and forms a covalent thioester enzyme-substrate intermediate upon release of the first reaction product, ammonia (acylation). The thioester complex is then attacked by an incoming primary amine (acyl-acceptor) or a water molecule which displaces the enzyme upon release of a transamidated or deamidated product (deacylation). The enzyme can further hydrolyze the transamidated product to a deamidated product (iso-peptidase activity). (R1, R2; polypeptide or protein, R3; NH_2, CH_3, peptide or protein) Figure is modified from [25].*

Substrate specificity

Glutamine residues are the only amino acids which can act as **acyl-donor substrates** for TG2. Targeting of glutamine residues in peptides is strongly governed by the primary sequence flanking the glutamine residue [22]. Preferred sequence motifs for peptide substrates have been identified both through peptide library approaches and phage-display library assays which all have reported a remarkably strong influence of proline residue positioning. This was first described by Vader *et.al* [26], who addressed deamidation of gluten peptides by guinea pig TG2, and reported that a proline residue in position +2 of a glutamine residue was extremely beneficial while proline in +1 and +3 completely protected against targeting. A similar observation was made almost simultaneously by Fleckenstein *et.al* [27], who addressed guinea pig TG2 mediated

transamidation of scanning peptide libraries. This “proline effect” has later been reproduced for human TG2 using phage display peptide libraries [28, 29]. Taken together, these studies indicate that the sequence -QXP(hydrophobic)- appears to be a highly preferred motif for peptide substrate targeting by TG2. Glutamine targeting in intact protein substrates will in addition be influenced by the secondary and tertiary structure of the protein. While TG2 is highly specific in its acyl-donor substrate targeting, a wide range of primary amines can act as **acyl-acceptor substrates** for transamidation. However, the primary amine group should preferably be followed by an un-branched hydrocarbon chain of at least 3 carbon atoms [22, 30]. This requirement ensures that only the ε-amine group of lysine residues can be cross-linked while peptide and protein N-termini will not be targeted by TG2. Transamidation to lysine residues creates very stable iso-peptide bonds within and between peptides and proteins. Cross-linking of proteins can also occur using both ends of bivalent small primary amines like spermidine. A long list of small biogenic amines have been identified as acyl-acceptor substrates for TG2 and other TGases (spermidine, putrescine, histamine, and serotonin and more) [24, 31-34], although the role of these amines in relation to TG2 cross-linking activity remains to be elucidated.

Biology of TG2

Expression and localization

TG2 is constitutively expressed in several tissues. It is most abundant in the liver and small intestine but can be up-regulated in **most cell types and tissues** in response to tissue injury and stress signals [35, 36]. Increased TG2 expression is often observed upon cell differentiation and maturation of for example macrophages and dendritic cells [37-39]. A major fraction of the enzyme is localized in the cytosol (~80%) while smaller amounts can be found in the nucleus and mitochondria [1, 40, 41]. TG2 is also excreted, despite lacking both stabilizing disulfide bonds and a leader sequence commonly required for excreted proteins. Once externalized, TG2 can either stay associated with the cell surface or become deposited in the extracellular matrix protein network where it binds tightly to fibronectin [42-44]. The localization of the enzyme appears by large to determine its biological function [1, 45].

A multifunctional protein

TG2 in the **extracellular matrix** (ECM) is primarily believed to act as a **cross-linking enzyme** where numerous of proteins have been identified as substrates [43]. This activity is of pivotal importance during tissue injury and wound healing due to the formation of mechanically and proteolytically stable iso-peptide bonds [46]. The Ca^{2+} concentration in the ECM fluctuates in the millimolar (0.5-3mM) range which is sufficient for constitutive activation of TG2 enzymatic activity *in vitro*. However, a recent report showed that extracellular TG2 is inactive under normal conditions but transiently activated upon tissue damage [47]. It is not clear whether this burst of activity derives from newly excreted or released enzyme, or reactivation of the enzyme pool already localized in the ECM.

TG2 in the **cell cytosol** has been ascribed a multitude of functions. The Ca^{2+}-deficient and GTP-rich environment will mainly keep the transglutaminase activity of TG2 quiescent. TG2 not only binds but also **hydrolyzes GTP** and can act as a small G protein, activating phospholipase C by the α-$_{1B}$ adrenergic receptor [48]. Studies with TG2 knock out mice have revealed a role in macrophage phagocytosis and clearance of apoptotic cells [49, 50] and TG2 is reported to have both pro-apoptotic and anti-apoptotic functions depending on its conformation and cellular

localization [45, 51]. A recent report indicated that TG2 turnover in the cytosol can be regulated by SUMOylation, which prevents ubiquitylation and proteosomal degradation [52]. Although originally characterized *in vitro*, TG2 also exerts a **protein disulfide isomerase (PDI) activity** recently described to play a role in regulation of mitochondrial respiratory chain function [40, 53]. The PDI activity is independent of the active site C277, indicating that TG2 must harbor additional reactive cysteine residues. Although usually quiescent, intracellular TG2 can also exert **cross-linking activity.** A rise in intracellular Ca^{2+} during apoptosis activates TG2 and induces massive cross-linking of cellular proteins to reduce leakage and tissue inflammation [54-56]. Further, TG2 can cross-link or deamidate proteins like α,β-crystalline, vimetine and various heat shock proteins [57-60], and can also transamidate small GTPases of the Rho family (RhoA, RhoB, Rcb) rendering them constitutively active [61, 62]. Rho activity plays an important role in mitosis, cell adhesion and migration [63, 64], thereby relating the transglutaminase activity of cytosolic TG2 to these events.

RhoA activation can also be mediated through **cell surface associated TG2** (csTG2) which can specifically associate with β_1 and β_3-integrins and thereby act as a co-receptor for fibronectin [65]. This induces integrin clustering and formation of focal adhesion points which in turn activates RhoA and promotes cell adhesion and migration [66]. Integrin-associated csTG2 can be found in podosomes of monocytes adhering to and migrating on fibronectin [67] suggesting that csTG2 mainly is found in specialized structures in adherent or migrating cells. CsTG2 can also associate with heparane sulphate chains of proteoglycans like syndecan-4, introducing a second, integrin independent function in cell adhesion and migration [68]. Notably, exogenous TG2 can also associate with cell surfaces as addition of soluble fibronectin-TG2 complexes could improve adhesion of fibroblasts [68, 69]. Although not yet demonstrated, this suggests that TG2 already deposited in the ECM can associate with and perhaps also be taken up by migrating cells.

The mechanism behind TG2 cell surface deposition is not clear although TG2 can associate with underglycosylated β-integrins which indicates association already within the cell [65]. Turnover is, at least in part, regulated by constitutive endocytosis together with low-density lipoprotein-like receptor 1 (LPR1) and integrins where the integrins are recycled while csTG2 will undergo lysosomal degradation [70]. Although not required for cell surface localization, enzymatic activity seems to be necessary for deposition of TG2 into the ECM [71] and a recent report

indicated that TG2 activity, and cell surface versus ECM deposition, might in fact be regulated by TG2 nitrosylation [72]. Whether csTG2 generally is active is not clear but this is likely to depend both on cell type and binding partners. Further, we have limited knowledge on the true abundance of csTG2 as few antibodies are considered to efficiently recognize TG2 in its cell surface associated context. Reports using a novel monoclonal antibody specific for csTG2 indicated abundant expression on most cell-types, in contrast to observations made with other TG2 specific antibodies which typically give no or only weak staining of cell surfaces [73].

TG2 in disease

TG2 has been implicated in multiple disorders affecting most organs from the central nervous system to liver, eyes and bone tissue [74-77]. The role of the enzyme is however poorly understood in most of the cases. The use of transgenic mouse models and TG2 knock out mice are emerging as valuable tools to further decipher TG2 biology which is a prerequisite to understand its role in disease [78].

GLUTEN SENSITIVE DISEASES

Gluten sensitive diseases (GSDs) are a collective term embracing diseases induced by the ingestion of dietary gluten, where symptoms are alleviated upon removal of gluten from the diet [79]. Of these, celiac disease is the most frequent and best characterized.

CELIAC DISEASE

Clinical aspects

Celiac disease (CD) is a chronic inflammatory disorder of the small intestine caused by an inappropriate immune response towards the environmental antigen gluten from wheat and related proteins from rye and barley [80]. The disease is characterized by infiltration of intraepithelial lymphocytes (IELs), villous atrophy, flattening of the mucosa and crypt hyperplasia giving symptoms like diarrhea, malnutrition and fatigue [81-83]. Disease onset spans from 1-2 years of age (upon introduction of gluten in the diet) till late adult life. The disease is prevalent among Caucasians (1:100) [84, 85] although this number is likely to be underestimated as adults in particular might present with only weak or diffuse symptoms rendering them undiagnosed [86].

Genetics

The **genetic factor** of CD became evident from the strong heritability of the disease and concordance in twins [87, 88]. The strongest association comes from the HLA haplotypes *DQA1**05, *DQB1**02 and *DQA1**03, *DQB1**0302 encoding the MHC class II molecules DQ2.5 and DQ8 [89]; more than 90% of CD patients are DQ2 or DQ8 positive. However, of all DQ2/DQ8 positive individuals in the population (30%), only a fraction develop CD [90]. Thus, DQ2/DQ8 seems necessary but not sufficient and additional factors must be crucial to precipitate the disease. In fact, the HLA-association is believed to account for only 50% of the genetic risk [91] and several non-HLA candidate genes have been identified through genome wide association scans [92]. Follow up studies have identified additional genes, and several of these have now been replicated on the population level [93, 94]. The individual risk contributions from these genes are minute compared to HLA, making them difficult to identify in small population studies. Importantly, most of them play a functional role in the immune system [93] suggesting

that a sum of genetic factors is required. Identification of the causal variants and functional analysis of these gene polymorphisms are expected to shed new light on the pathogenesis of CD.

The immune response towards gluten

In contrast to most immune mediated diseases, the major antigen in CD is known. The disease is precipitated by the ingestion of **gluten**, and removal of gluten from the diet usually leads to complete recovery [95]. This is currently the only treatment for the disease. Gluten is the collective term for wheat storage proteins which can be further divided into gliadins and glutenins based on their solubility. Both fractions can elicit an immune response in celiac disease patients. Gluten proteins are extremely rich in glutamine (30%) and proline (15%) residues, the latter making them highly resistant towards intestinal digestive enzymes and brush border proteases [96-98]. Incomplete digestion leaves large polypeptides which can enter the lamina propria and be taken up by antigen presenting cells (APCs) [98]. It is still not clear how these peptides traverse from the intestinal lumen to the lamina propria, although several mechanisms have been suggested [99-101]. Importantly, some gluten peptides can induce **innate** immune responses. Several studies have focused on the α-gliadin derived peptide p31-43, which is not recognized by $CD4^+$ T cells but has a multitude of other functions, from increasing the number of IELs, induction of MIC and IL-15 to activation of MAP kinases [102-105]. However, the underlying mechanism remains to be elucidated and no receptor for p31-43 has so far been described. A very recent study reported toll-like receptor-4 stimulatory capacity of different parts of gluten, suggesting that several gluten derived peptides can trigger innate immune responses by more than one mechanism (Junker *et.al*, 13th International Celiac Disease Symposium, Amsterdam, April 2009).

Due to their proline-induced secondary structure, gliadin peptides are remarkably well accommodated by the disease associated DQ2 and DQ8 allowing efficient presentation of peptides to gluten reactive $CD4^+$ T cells [106]. Importantly, gliadin peptide binding affinity to DQ2 or DQ8 is strongly enhanced by the presence of negative charges in certain positions. These are introduced by TG2, which catalyzes highly specific deamidation of selected glutamine residues. As peptide-MHC affinity and peptide off-rate is of pivotal importance during T-cell activation, deamidation is likely to be crucial to mount an efficient T-cell response towards gluten

[107, 108]. The gluten specific **T-cell response** in CD is dominated by interferon-γ producing $CD4^+$ cells which dictate the inflammation in the celiac lesion [109]. Isolation and *in vitro* culturing of T-cell lines from small intestinal biopsies from celiac disease patients has facilitated the identification of several dominant gluten T-cell epitopes which drive this response [110-115]. CD patients also develop a humoral immune response, i.e. a **B-cell response,** towards gluten. Although antibodies towards native gluten peptides also can be found in healthy individuals, IgA towards deamidated gluten is emerging as a disease specific marker [116, 117]. A well established hallmark of CD is however the gluten-dependent production of IgA or IgG **anti-TG2 autoantibodies**, introducing TG2 as the major auto-antigen of the disease [118]. These antibodies are highly disease specific and are used as a diagnostic tool [119-121].

DERMATITIS HERPETIFORMIS

The prevalence of extra-intestinal disease manifestations in response to gluten is increasingly appreciated [122]. The most frequent manifestation is dermatitis herpetiformis (DH), a skin disorder characterized by sub-epidermal blistering predominantly affecting skin around the major joints [123]. These symptoms usually disappear upon removal of gluten from the diet. DH has a strong HLA association similar to CD and has been referred to as “celiac disease of the skin” [124]. Although approximately 30% of DH patients never experience intestinal symptoms, they frequently have increased number of IELs in the small intestine, which is reminiscent of latent or silent celiac disease [125]. Compared to CD, DH has a rather late onset with a mean age of 38 years [126].

Most DH patients have circulating anti-TG2 autoantibodies, but in addition they frequently have autoantibodies specific for another TGase isoform, TG3 [79, 127].The presence of IgA deposits in the dermal papillae of the skin is pathognomonic for DH and is used in diagnosis of the disease [128].

GLUTEN ATAXIA

Gluten can also induce neurological symptoms like ataxia in some individuals, giving rise to the term gluten ataxia (GA) [129, 130]. GA has been reported in 12-15% of CD patients and 12-41% of patients with ataxia of unknown origin [131, 132]. GA also has a late onset with a median age of 54 years [129]. The ataxia is believed to be caused by antibody mediated irreversible damage to Purkinje cells in the cerebellum; hence the symptoms in GA are not necessarily alleviated upon removal of gluten from the diet [129, 133-136]. Further, as brain biopsies can only be obtained from dead patients, the diagnosis of GA is both difficult and controversial [137].

As with DH, patients with GA may or may not have small intestinal symptoms but most patients have circulating anti-TG2 antibodies. Notably, GA patient were recently been found to have autoantibodies reactive towards a newly identified transglutaminase isoform, TG6 [8]. Thus, it appears that TG2 is not the only transglutaminase implicated in GSDs.

TRANSGLUTAMINASES IN GLUTEN SENSITIVE DISEASES

TRANSGLUTAMINASE 2 IN CELIAC DISEASE

TG2-mediated post translational modification of gluten peptides

TG2-catalyzed deamidation is likely to be crucial for the T-cell response towards gluten peptides in celiac disease and inhibition of this activity is expected to have therapeutic benefits [112, 138, 139]. Gluten peptides are in fact remarkably good peptide substrates for TG2 [12]. TG2 exerts highly specific targeting of only certain glutamine residues within peptides harboring gluten T-cell epitopes and the order of preference seems to mirror the frequency of T-cell responses towards various epitopes in celiac disease patients [12, 26, 27, 140]. The glutamine residue targeting is primarily governed by proline residues which are very frequent in gluten peptides. Proline in position +2 is particularly favorable as it induces a perfect fit of the peptide substrate with the active site of the enzyme [17]. Peptide deamidation *in vivo* should however be disfavored by the ample amounts of primary amines present on sites of TG2 expression. The relative amount of deamidation was however shown to increase with decreasing pH, suggesting that TG2 activity in low pH compartments like the small intestinal brush border or early endosomes could circumvent this problem although no published data has so far indicated this [27]. Thus, the importance of TG2 activity in the immune response towards gluten is clearly established, but we still lack knowledge on how and where this activity occurs.

TG2 autoantibodies

The gluten-dependent production of IgA (and IgG) anti-TG2 autoantibodies is a hallmark of CD. The autoantibodies are produced locally in the small intestine and can be detected in the circulation and in intestinal deposits where they bind TG2 in the ECM and the endothelium of small blood vessels [141-143]. Despite a continuous increase in the number of reported functions of these autoantibodies (from angiogenesis to cell cycle regulation and differentiation) no clear consensus has yet emerged [144-146]. An increasing body of evidence suggests that most of the observed effects could be mediated via RhoA although further studies are required to delineate this. Notably, several groups have addressed whether the autoantibodies can influence the catalytic activity of TG2. Esposito *et.al* [147] reported a dose dependent inhibition of TG2

transamidation using affinity purified IgA and IgG from CD patient sera, whereas no inhibition was observed using purified IgG from control subjects. Dieterich *et.al* [148] reported no difference in the inhibitory capacity of total IgA purified from CD patients and control subjects. Affinity purified anti-TG2 autoantibodies from CD patients exerted a dose dependent inhibition of transamidation (up to 80% inhibition). This was however deemed insufficient to block biologically active deamidating activity. In contrast, more recent work by Király *et.al* [149] indicated that CD anti-TG2 autoantibodies rather enhance the catalytic activity of TG2 by acting as chaperones which can stabilize the enzyme in advantageous conformations or by preventing otherwise rapid inactivation of the enzyme.

The mechanism behind the production of anti-TG2 autoantibodies remains to be experimentally demonstrated although several clues point in the same direction; i) the autoantibodies are only found in DQ2-positive individuals, ii) their presence in the circulation depends on the intake of gluten, and iii) no TG2-specific T cells have been isolated. Thus, the standing hypothesis is based on a hapten-carrier model where TG2-gliadin complexes are taken up by TG2-specific B cells. The B cells can then present gliadin peptides to $CD4^+$ T cells, which in return provides the necessary help to the B cells [150]. Two types of TG2-gliadin complexes can be formed. Transient thioester bound enzyme-substrate complexes are formed during catalysis which for gluten peptides have been found to be unusually stable [12]. TG2 can also create very stable iso-peptide linked complexes by cross-link gliadin peptides to six of its own lysine residues [151].

ROLE OF OTHER TRANSGLUTAMINASES IN GLUTEN SENSITIVE DISEASES

DH patients typically have circulating antibodies which can be subdivided into populations which recognize TG2, are cross-reactive with TG2 and TG3 or primarily recognize TG3 [79]. IgA deposits in the dermal papillae co-localizes with TG3 but not TG2 [79]. Indeed, TG3 is primarily expressed in the skin but not at the site of the antibody deposits, indicating that the complexes derive from the circulation. The gluten dependent antibody production and isotype suggest an intestinal origin. Although it is not clear whether TG3 is expressed in the small intestine, TG3 is now considered the main autoantigen of DH.

The diagnosis of GA is less straightforward than DH and CD and little is therefore known about the lesion and pathology of GA. Most GA patients have anti-TG2 autoantibodies. In addition they frequently have antibodies with high avidity for the recently identified TGase isoform TG6 [8]. Hadjivassiliou *et.al* also described the co-localization of IgA and TG6 within cerebellar structures in brain biopsies from deceased GA patients. TG6 was suggested to predominantly be expressed in neuronal cells. However, TG6 expression in control tissue did not overlap with immune complex deposits in GA, suggesting that also these immune complexes derive from circulation [8]. This paper was however the first to describe TG6 on the protein level and very little is therefore known about the expression, activity and role of TG6 in health and disease.

AIM OF THESIS

The overall aim of this thesis is to address various aspects of TG2 biochemistry to further improve our understanding of its role in celiac disease. In addition, related transglutaminase isoforms, now emerging as putative players in other gluten sensitive diseases, are briefly touched upon. More specifically, we aimed to focus on the following topics;

- TG2 mediated deamidation of gluten peptides is pivotal in celiac disease. Yet, acyl-acceptors are expected to be abundant in an *in vivo* setting. To shed light on this, we wished to address the still incompletely understood process of peptide substrate deamidation in the presence of acyl-acceptor substrates under various reaction conditions.

- Regulation of TG2 enzymatic activity is poorly understood. In relation to this, we looked at the reversible oxidative inactivation of TG2 which is likely to be relevant for regulation of TG2 activity in the ECM.

- We further wished to study the conformation of cell surface TG2 to better understand the biological function and role of TG2 at this location.

- Finally, we aimed to address whether the closely related isoforms TG3 and TG6 share the ability of TG2 to utilize gluten peptides as substrates and whether their activity and expression indicates an active role of these isoforms in the gluten sensitive diseases DH and GA.

METHODOLOGICAL CONSIDERATIONS

Recombinant human transglutaminases

All the recombinant human transglutaminases used in this thesis were expressed in *E.coli*. His_6-tagged recombinant human TG2 was expressed in BL21 competent *E.coli* and purified according to *Piper et.al* [12]. The plasmid was a kind gift from Chaitan Khosla, Stanford University. TG2 produced in this system is typically highly active with reproducible batch-to-batch yield and level of activity. Plasmids encoding TG2 mutant constructs (C230A, C370A and C371A) were a kind gift from Daniel Pinkas, Stanford University. The mutants were expressed and purified similar to wild type TG2 except for the over night induction step which was performed at 22°C. TG3 and TG6 were provided by Daniel Aeschlimann, Cardiff University and were produced as described by Hadjivassiliou *et.al* [8]. Production of recombinant proteins in *E.coli* can potentially introduce aberrant modifications and disulfide bridges due to altered folding environment compared to eukaryotic cells, although this has not been reported for recombinant TG2.

Determination of TG2 enzymatic activity by quantification of reaction products

TG2 enzymatic activity and kinetic parameters are typically determined by quantification of one of its three reaction products; ammonia, deamidated or transamidated product. **Ammonia** is typically measured indirectly in a coupled assay developed by Keillor *et.al* [152] where glutamate dehydrogenase couples ammonium (produced by TG2) to ketoglutarate, forming glutamate upon consumption of NADH. The decrease in NADH is monitored spectrophotometricaly and serves as the readout. Although this method requires a large sample volume and therefore large amounts of enzyme and substrates per sample, it is typically performed in a high throughput 96well plate set-up making it suitable for determination of kinetic parameters. **Transamidation** is typically quantified by the incorporation of radioactively or fluorescently labeled acyl-acceptor substrates (^{3}H-putresine or monodansylcadaverin) into acyl-donor substrates like dimethylcaseine. This assay does not take into account any concomitant deamidation which might occur. **Deamidation** of peptide substrates can be quantified by mass spectrometry or capillary electrophoresis (CE). Quantification by mass spectrometry (MS) is rapid and requires small sample amounts, but is not suitable for quantification of very low percentages of deamidation (<5%) which might cause problems for accurate determination of V_{max} values.

Alternatively, **simultaneous monitoring of deamidation and transamidation** can be performed by CE-LIF (Laser induced fluorescence) detection. This allows the simultaneous monitoring of both reaction pathways and is therefore more correct than quantification of only transamidated product. This method was established by Fleckenstein *et.al* [27] and was used in paper I. Detection of only fluorescently labeled compound ensures a very high specificity and sensitivity. This method is however not sufficiently high-throughput for facile determination of kinetic parameters for multiple substrates compared to for example the ammonium release assay. Further, fluorescence labeling of the acyl-donor substrates is required. Introduction of large hydrophobic fluorescence groups will affect peptide solubility and can even change the substrate properties of some peptides; N-terminal labeling of the gluten peptide DQ2-α-I with FITC (FITC-QLQPFPQPQLPYP) actually introduces a second deamidation site (most likely at the N-terminal Q) in addition to the normally targeted -QLP- (J. Stamnæs, unpublished observation). Similar substrate behavior was however observed for the FITC-labeled peptides used in paper I and their unlabeled analogs. These peptides harbored only one glutamine residue.

Immunoprecipitation and protein identification by mass spectrometry

Mass spectrometry has over the last decades emerged as the gold standard for protein identification. Mohan *et.al* initially isolated the antigen of mAb 6B9 by immunoprecipitation from cell surface biotinylated cells followed by SDS-PAGE, western blotting and streptavidin detection which revealed one band of approximately 80kDa. Identification of this band was performed by affinity purification followed by SDS-PAGE, in-gel digestion with trypsin and LC-MS (ESI Q-ToF). The MS and MSMS spectra were searched against the NCBI protein databank using the search engine MASCOT which gave positive identification of a single peptide deriving from TG2. In paper II we also performed immunoprecipitation using 6B9 and were able to isolate an 80kDa antigen which after in-gel trypsin digestion and mass spectrometry (MALDI-ToF/ToF) repeatedly resulted in 4 prominent tryptic peptides which were not present in control samples. These peptides were found to derive from CD44, while no TG2 derived peptides were identified.

Identification of only one or four tryptic peptides from an in-gel protein digestion is very little. The low number of peptides can be due to several factors, from poor enzyme digestion due low amounts of protein, use of silver staining, loss of peptides in the work up process, poorly

calibrated mass spectrometers or the use of different search parameters when performing databank searches [153]. The discrepancy in protein identified is however more difficult to understand and can only be explained if one protein co-precipitates with the other. This is not an unlikely explanation, as TG2 associates with heparane sulfate chains of syndecan-4 and might therefore also associate with similar side chains of CD44. Nevertheless, it is puzzling that no CD44 derived peptides were identified from the original samples. However, the original identification of TG2 as the antigen of mAb 6B9 was not confirmed by use of other methods, in comparison with our identification of CD44 as the antigen of mAb 6B9.

Flow cytometry staining of CD44 transfectants and bone marrow derived mononuclear cells

Verification of CD44 as the antigen of mAb 6B9 was performed by flow cytometry staining by comparing the staining pattern of mAb 6B9 with that of a well characterized anti-CD44 mAb MEM-263. Staining of Jurkat cells stably transfected with the most common splice variant of human CD44 conjugated to EGFP (CD44s-eGFP) was compared to control transfectants [154]. We also stained bone marrow derived mononucleated cells where expression of CD44 can be visualized as a very distinct and unique staining pattern [155]. As both experiments were performed with human cells, there is still a remote possibility that 6B9 recognizes an epitope of csTG2 which is dependent on CD44 co-expression. This can only be ruled out using murine cells transfected with human CD44, as 6B9 was reported not to stain murine cells.

Oxidation of TG2

In vitro oxidation of TG2 was performed by prolonged exposure to air or by incubation with various ratios of reduced and oxidized glutathione (GSH and GSSG). Treatment with GSH/GSSG can result in not only disulfide formation within proteins but also formation of GSH adducts. The presence of disulfide linked peptides and GSH adducts was assessed by MALDI ToF mass spectrometry. As a similar oxidation pattern was observed for TG2 oxidized by air and by treatment with GSH and GSSG, it is reasonable to assume that oxidation of TG2 in the presence of GSH and GSSG primarily results in intramolecular disulfide formation and not formation of stable GSH-adducts. No further oxidation (sulfenic or sulfonic acid) was observed.

SUMMARY OF PAPERS

In **paper I** we investigated the simultaneous deamidation and transamidation of TG2 peptide substrates in the presence of a primary amine. We observed that both events will occur in the presence of a primary amine and that good peptide substrates are less prone to direct deamidation due to rapid and facile transamidation. Poor peptide substrates have a higher ratio of deamidation to transamidation due to less transamidation. Further, the ratio of deamidation to transamidation was dependent on the reaction conditions and concentration of active enzyme. At higher concentrations of enzyme, good substrates were prone also to indirect deamidation through hydrolysis of transamidated product. These data demonstrate that deamidation of peptide substrates can occur under most reaction conditions and that detailed knowledge on TG2 expression and activity regulation *in vivo* is essential to predict the outcome of the enzymatic reaction.

In **paper II**, we report that the monoclonal antibody 6B9 described to be specific for cell surface transglutaminase 2 instead recognizes the cell surface proteoglycan CD44. We present results from a series of experiments which show that 6B9 does not recognize recombinant TG2 in any context, and that TG2 cannot be isolated through immunoprecipitation. Rather, we repeatedly identified peptides deriving from the glycoprotein CD44. Finally, we show that 6B9 only stains CD44 positive cells and not CD44 negative cells and that staining of bone marrow derived mononuclear cells is identical with 6B9 and with a well characterized CD44 specific mAb.

DH and GA patients have circulating IgA antibodies specific for TG3 and TG6 respectively. In **paper III** we addressed whether TG3 and TG6 can actively contribute to the gluten dependent production of these antibodies. We show that that both TG3 and TG6 can accommodate gluten peptides as substrates and specifically deamidate gluten T-cell epitopes. Further, both TG3 and TG6 were able to form covalent complexes with gluten, although to less extent than TG2. Our findings indicate that TG3 and TG6 might contribute to their own autoantibody production in DH and GA as suggested for TG2 in CD. This supports the notion that the anti-TG3 response in DH and anti-TG6 response in GA is the result of immune responses primarily directed against these enzymes and not against TG2.

UNPUBLISHED RESULTS

Oxidative inactivation of TG2

How TG2 activity is regulated *in vivo* is not known. We do however known that TG2 can be inactivated through reversible oxidation where enzymatic activity can be recovered upon treatment with reducing agents like DTT [156]. Thus, disulfide bridge formation must play a crucial role. TG2 has no reported disulfide bridges in its native state [11]. Several decades ago, extensive work by Folk and co-workers indicated that oxidative inactivation of TG2 does not involve the active site C277 [157-159]. It is difficult to extract a clear mechanism from this work but the findings are in line with more recent reports describing TG2 as a PDI where also this activity was independent of the active site cysteine [53]. This implies that TG2 also harbors other reactive cysteine residues. Intriguingly, a vicinal disulfide bond (between C370 and C371) was reported in the crystal structure of TG2 believed to represent the open and active conformation of the enzyme.

A recent report showed that extracellular TG2 *in vivo* is inactive during homeostasis [47]. Considering the oxidative environment in the extracellular space, oxidation of TG2 presents a plausible mechanism for inactivation. Intrigued by this possibility and the conflicting data in the literature, we aimed to identify cysteine residues involved in oxidative inactivation of TG2 and to determine the role of the vicinal disulfide bond reported in the crystal structure. Initially, we observed that TG2 subjected to prolonged gel filtration in the absence of reducing agents became inactive. This inactive TG2 migrated in nPAGE with an open conformation and could not assume a closed conformation upon incubation with GTP (Fig.3A). Treatment with DTT could partially recover both enzymatic activity and the ability to assume a closed conformation (Fig.3A, B).

To identify cysteine residues labile for oxidation, TG2 was incubated with various ratios of reduced and oxidized glutathione (GSH and GSSG) followed by trichloroacetic acid/ acetone precipitation and alkylation of all free cysteine residues with iodoacetamide (IAM, +57Da). After separation by SDS-PAGE, any disulfides present were reduced with DTT and alkylated with iodoacetic acid (IAA, +58Da) giving a mass difference of one Da between reduced and oxidized cysteine residues. Oxidation was then quantified by mass spectrometry analysis of trypsin digested TG2 samples.

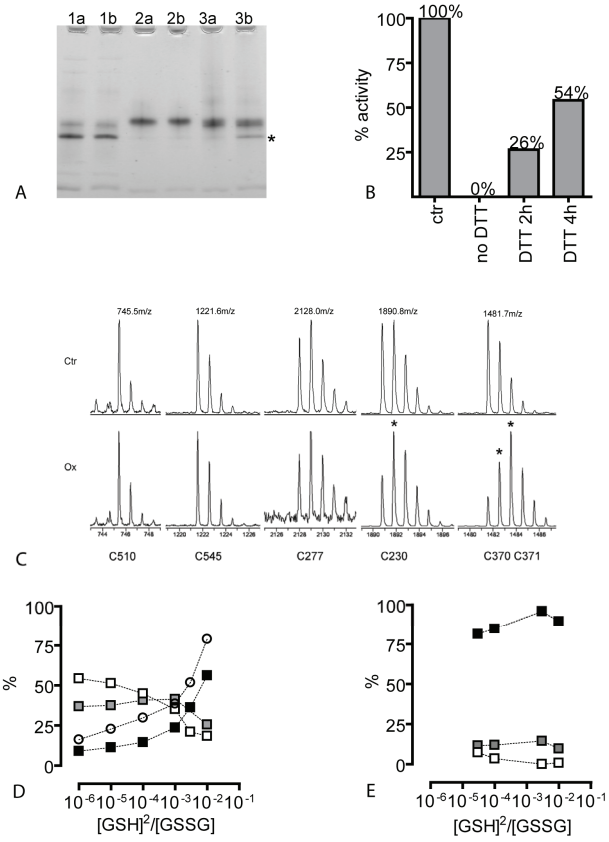

Figure 3 Oxidation of TG2 A*) TG2 conformation visualized by nPAGE after pre-treatment with 0mM DTT (a) or 30mM DTT (b)for 30min room temperature followed by 1h incubation with 500µM GTP and 1mM Mg^{2+}. 1= fresh enzyme, 2= inhibitor bound enzyme [17], 3= enzyme oxidized by prolonged gel filtration. Closed conformation recovered by DTT treatment is indicated by *.* ***B****) DTT treatment of oxidized TG2 (20mM DTT, 4°C, 2h or 4h) could partially recover enzymatic activity (% deamidation after 60min compared to activity of fresh enzyme (ctr) using 20µM DQ2-α-II and 0.1µg/µl enzyme).* ***C****) MALDI-ToF mass spectra revealing the isotopic envelope of IAM and IAA labeled tryptic peptides harboring the indicated cysteine residues from control and oxidized (0.24mM GSH/2mM GSSG) samples. Mass shift due to oxidation indicated as *.* ***D****) Quantification of oxidation of C370 and C371 upon GSH/GSSG titration. Relative amount of reduced C370C371 (■), reduced C371 and oxidized C370 (■) and oxidized C370C371 (□) after 3h incubation at 30°C. Activity (○) is given as % deamidation 2h after addition of*

100 µM DQ2-α-II and 5mM Ca^{2+} to oxidized samples (30min and 1h not shown). ***E****) Oxidation after 30min at 37°C in the presence of 250µM DQ2-α-II and 5mM Ca^{2+}.*

We found that three cysteine residues, C230, C370 and C371, were more susceptible to oxidation than other solvent exposed cysteine residues (Fig 3C). The active site C277 was not found to be oxidized under these conditions (Fig.3C). Interestingly, C370 was found to participate in two disulfide bonds, either with C230 or its neighbor C371 (data not shown). C370 was consistently more oxidized than C371 (quantified by MSMS of 1481 m/z, data not shown). This was observed in TG2 oxidized both in the presence and absence of GSH/GSSG and can therefore not simply be due to stable C370-GSH adduct formation (no C370-GSH adduct could be observed in MALDI-ToF). Thus, it is likely that a fraction of C370 is engaged in a disulfide bond with C230 while another fraction is occupied in a vicinal disulfide bond with C371. Quantifying the percentage of free versus oxidized C370 and C371 upon GSH/GSSG titration suggested that the C370-C230 disulfide is formed at less oxidizing conditions (Fig. 3D, grey squares, only C370 oxidized) than the vicinal disulfide bond between C370 and C371 which increasingly dominated under very oxidizing conditions (Fig.3D, white squares, both C370 and C371 oxidized).

To address whether the presence of these disulfides influenced enzymatic activity, peptide substrate and Ca^{2+} was added to samples of oxidized TG2 and deamidation was quantified after various time points. The level of deamidation was found to correlate with the percentage of reduced C370 and C371 still present upon addition of substrate, suggesting that these cysteine residues must be in a reduced form in the active enzyme (Fig.3D). On the other hand, the crystal structure harboring the vicinal disulfide suggested that this bond could be a part of the transient thioesther enzyme-substrate intermediate. As TG2 was reported to have a high steady-state active site occupancy [12], GSH/GSSG titration was performed in the presence of saturating amounts of peptide substrate (250µM DQ2-α-II, K_M = 70µM [12]) and 5mM Ca^{2+}. This should allow for isolation of thioester complexes. However, no accumulation of the vicinal disulfide bond was observed upon enzymatic turnover (Fig.3E). Thus, the vicinal disulfide bond is not likely to be a part of the active state of the enzyme.

To further establish the role of these three cysteine residues, recombinant TG2 mutants were expressed (*C230A*, *C370A* and *C371A*). While the activity of *C230A* and *C370A* were

comparable to wild type (WT) enzyme, *C371A* was inactive (data not shown). If the vicinal disulfide bond would be critical for oxidative inactivation of TG2, both *C370A* and *C371A*, who cannot form this bond, should be protected from inactivation. As *C371A* already was inactive, only *C370A* was used for further experiments. Surprisingly, also *C370A* was inactivated over time upon oxidation with GSH/GSSG. However, this could be due to rapid glutathionylation of C230 which might result in steric hindrance of the active site (data not shown). Further, *C230A* was also inactivated upon oxidation. Again, enzymatic activity correlated with the relative amount of reduced C370 and C371. Interestingly, formation of the vicinal disulfide bond in *C230A* mutant required stronger oxidizing conditions than in wild type enzyme (Fig.4A-C). Thus, although C230 is not essential for vicinal disulfide bond formation in the presence of GSH/GSSG, it seems to facilitate formation of this disulfide bond in the wild type enzyme.

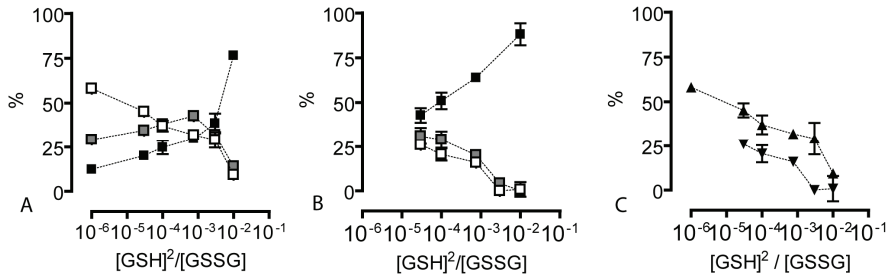

Figure 4 Differential oxidation of TG2 mutants *WT (**A**) and C230A (**B**) oxidation after 1h at 37°C showing relative amount of reduced C370C371 (■), reduced C371 and oxidized C370 (■) and oxidized C370C371 (□). **C**) Comparison of WT (▲) and C230A (▼) oxidized C370C371. Values given are means with SD as error bars.*

The effect of calcium on oxidation is challenging to address as TG2 is prone to extensive self cross-linking. To circumvent this, Ca^{2+} titration was performed in the presence of saturating amounts of peptide substrate. In these experiments, increasing amounts of Ca^{2+} seemed to protect against oxidation (Fig.5A). As the lack of oxidation could both be due to binding of Ca^{2+} and/or catalytic turnover of the enzyme, Ca^{2+} titration was performed in the absence of substrate using a TG2 active site mutant (Fig.5B). Again, Ca^{2+} clearly protected against oxidation with a dramatic change around 1-3 mM Ca^{2+} which is close to the K_a of Ca^{2+} [12]. It is important however to keep in mind that TG2 active site mutants show impaired GTP binding and will not readily

assume a closed conformation in nPAGE. They might therefore differ from wild type enzyme also in other aspects [15]. Notably, the presence of saturating amounts of substrate in the absence of Ca^{2+} also had a slight protective effect against oxidation (data not shown).

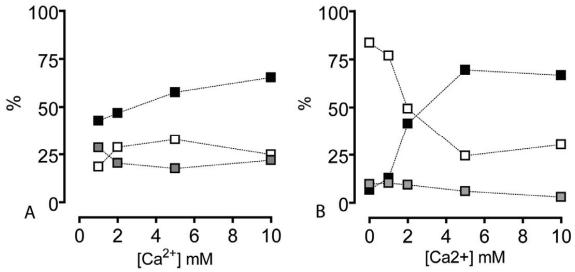

Figure 5 Effect of Ca^{2+} on oxidation *A) The effect of Ca^{2+} on oxidation of WT enzyme (30min at 37°C) in the presence 250µM DQ2-α-II.* ***B)*** *The effect of Ca^{2+} on oxidation of C277S (3h at 30°C).Graphs show relative amount of reduced C370C371 (■), reduced C371 and oxidized C370 (■) and oxidized C370C371 (□).*

In summary, these data demonstrate that oxidative inactivation of TG2 involves a cysteine triad consisting of C230, C370 and C371 and seems independent of the active site C277. The vicinal disulfide bond reported by Pinkas *et.al* does not seem to be part of the active conformation of the enzyme, rather the opposite. The presence of substrate and high amounts of Ca^{2+} could protect against oxidation of TG2 in these experimental settings. While the adjacent cysteine pair is found in several TGase isoforms (TG1, TG4, TG5 and TG7), C230 is unique for TG2. This residue seemed to play a role in the formation of the vicinal disulfide and could itself form a disulfide with C370. However, the order of events and the mechanism underlying the formation and breakage of these bonds cannot be elucidated from the current data. We can only speculate whether C230 might facilitate breakage of the vicinal bond under reducing conditions or alternatively induce enzyme inactivation under relatively mild oxidative conditions. Further, it is not clear whether the vicinal disulfide bond alone is responsible for "locking" the enzyme in an open conformation. However, this conformation can clearly be induced by oxidation, independent of active site occupancy. Further experiments are required to elucidate these remaining questions.

DISCUSSION

Transglutaminase 2 in celiac disease

Several TGases are implicated in human diseases [1]. Not surprisingly, TG2 is the most frequently suggested culprit, spanning a long list from Parkinson and Alzheimer disease, arthrosclerosis and cancer to gluten sensitive diseases like CD. Its functional role is however often not established [78].

The main function of TG2 in CD is on the other hand well characterized, where specific deamidation of gluten T-cell epitopes is pivotal to initiate and maintain a pathogenic immune response towards gluten. Epitopes preferentially targeted by TG2 are more frequently recognized by T cells from the small intestine of CD patients than other epitopes [140]. Further, stimulation of small intestinal biopsies with non-deamidated gluten peptides give rise to T-cell lines specific for deamidated gluten peptides as a result of TG2 activity *in situ* [139]. Thus, the importance of TG2 enzymatic activity in CD is undisputable, and inhibition of this enzyme is an attractive therapeutic option. However, several aspects of TG2 biology are incompletely characterized. This prevents us from fully understanding the role of TG2 in CD, which is a prerequisite for intervention.

Enzymatic activity

It is not clear how gluten peptides can enter the celiac lesion and whether the peptides are restricted to cellular compartments of APCs or if they “flow freely” in the lamina propria. Even though the location remains unknown, the peptides are likely to encounter active TG2 at sites with ample amounts of primary amines. Transglutaminase-mediated deamidation has long been considered an unspecific side-reaction. Notably, the relative amount of deamidation has been demonstrated to increase upon decreasing pH [27], an observation which could be explained as follows: The TG2-substrate thioester intermediate formed upon acylation of the enzyme will be attacked by a primary amine or a water molecule resulting in a transamidated or deamidated product. Transamidation is believed to occur through a general base catalyzed mechanism [19, 27] where the primary amine group of incoming acyl-acceptors is suggested to be deprotonated by a histidine residue, presumably the catalytic triad member H335. Deprotonation enables the

amine to perform a nucleophihlic attack. A decrease in pH results in increased protonation of the histidine which would result in less deprotonated amine accessible for transamidation [19, 27]. The rate of deamidation was on the other hand not influenced by pH [27]. Notably, H335 is also required to deprotonate the active site thiol group upon enzyme acylation and thioester formation. Increased protonation of H335 due to changes in pH should therefore also reduce the overall activity. The enzymatic activity of TG2 was however unchanged over a pH range of 6.0-8.0 suggesting that a basic amino acid residue different from H335 must be responsible for acyl-acceptor depronondation prior to the nucleophilic attack [19].

Recently, deamidation was also suggested to be a substrate dependent event, as selective deamidation and transamidation of two different glutamine residues within the same protein was observed [160]. In paper I we demonstrated that the ratio of deamidation to transamidation can be substrate dependent also for peptides, as good peptide substrates (e.g. -QXP-) were more prone to transamidation while poorer substrates (e.g. -QXF-) were less prone to transamidation whilst more or less retaining their level of deamidated. This could suggest that the enzyme-substrate complex of a good substrate differs from that of a poor substrate. Indeed, the most recent crystal structure of TG2 describe an exceptionally good fit of the preferred TG2 substrate motif -QXP(hydrophobic)- to the active site of the enzyme [17]. Once bound to the active site, the “fit” of a peptide substrate might influence the accessibility for incoming primary amines and hence the level of transamidation whereas small and abundant water molecules easily can gain access independent of the substrate sequence. Notably, an unusual set of slow-binding TG2 inhibitors were found to display differential properties depending on the substrate, suggesting that different substrates also might induce slight differences in enzyme conformation [161].

We also demonstrated that TG2 can cleave iso-peptide bonds between two peptide substrates, introducing an indirect route for deamidation. This was primarily observed for readily transamidated, good peptide substrates at high concentrations of active enzyme. This suggests that TG2 might be able to release not only gluten peptides cross-linked to small biogenic amines, but also peptides cross-linked to for example ECM proteins [24, 162]. In conclusion, work presented in this thesis together with previous reports clearly demonstrates that deamidated gluten T-cell epitopes can accumulate under most assay conditions. However, the *in vivo*

implications are not clear until the level and regulation of TG2 activity is characterized in a biological setting.

Regulation of enzymatic activity

Siegel *et.al* [47] recently demonstrated that TG2 in the ECM is inactive during homeostasis. Although several mechanisms can explain this observation, oxidation is an attractive alternative considering that the active site of TG2 is a cysteine residue and that the protein has no reported disulfide bridges in its native state. Lai *et al* demonstrated reversible inactivation of TG2 by calcium-dependent nitrosylation of several cysteine residues [163]. In fact Telci and co-workers recently reported that both TG2 activity and ECM deposition can be modulated by nitrosylation although it was not clear whether this was due to cysteine and tyrosine nitrosylation [72]. Folk and co-workers reported both calcium dependent and calcium independent reversible, oxidative inactivation of TG2 independent of the active site cysteine [157-159] indicating that TG2 activity in the ECM might be regulated by multiple mechanisms.

We have demonstrated that oxidative inactivation of TG2 at low concentrations or in the absence of calcium correlate with the oxidation of three cysteine residues C370, C371, C230 but was not found to involve the active site C277. Oxidation of TG2 resulted in loss of enzymatic activity and loss of ability to assume a closed conformation in the presence of GTP. The loss of activity correlated with disulfide bond formation between C370-C371 and C230-C370 where formation of C230-C370 seemed to facilitate formation of the vicinal bond. Further studies are however required to confirm this. While an adjacent cysteine pair is found in several TGases, C230 is only found in TG2 but is conserved in several species. It is therefore tempting to speculate whether oxidative inactivation of TG2 differs from that of other TGase isoforms.

Loss of enzymatic activity correlated with the loss of reduced C370 and C371. The vicinal disulfide bond is not present in native, active enzyme and did not accumulate upon enzymatic turnover. Thus, the vicinal disulfide bond does not seem to be part of the active form or conformation of the enzyme. One can question whether the reported crystal structure of TG2 in an open conformation harboring this disulfide bond might not represent the true active form of the enzyme. However, the conformational differences between active and inactive enzyme could

be subtle as ligand free enzyme (i.e. not bound to Ca^{2+}, GTP or substrate), enzyme with the active site occupied by inhibitors or iodoacetamide (J.Stamnæs, unpublished observation) and oxidized enzyme can all assume an open conformation as resolved by nPAGE, suggesting that multiple variations of this conformation exists. Notably, crystallization of the "open conformation" was performed in the absence of reducing agents in contrast to crystallization of the closed, GDP bound conformation where 5mM DTT was included in every step [16, 17]. Incorporation of DTT in the work up process could perhaps confirm whether the reported open conformation indeed represents the true TG2-substrate thioester complex conformation.

In line with previous findings, we observed that saturating amounts of Ca^{2+} and saturating amounts of substrate could protect TG2 from oxidation [158, 164], suggesting that a temporary or local increase in Ca^{2+} and substrate concentrations might prevent an otherwise rapid, oxidative inactivation of TG2 upon deposition in the ECM. This might also be relevant in CD if unusually high amounts of good peptide substrates such as gluten peptides would continuously be present together with TG2 in the lamina propria. However, further studies must be conducted to determine if oxidation indeed is the cause for inactivation of ECM associated TG2 *in vivo*.

The curious case of cell surface TG2

CD IgA autoantibodies towards TG2 are found in deposits in the endomysium, hence their original description as anti endomysial antibodies. These antibodies also react with the myofibroblast-derived extracellular matrix localized underneath the epithelium in the small intestine. They give no staining of cell surfaces, similar to most commercially available monoclonal anti-TG2 antibodies [141]. Thus, while TG2 is readily recognized in the cytosol or ECM it seems to be poorly recognized in its cell surface context, suggesting that csTG2 displays a shielded or altered conformation compared to ECM TG2. The lack of cell surface staining would also indicate that any auto-reactive B cells in CD recognizing csTG2 must have been efficiently deleted.

The isolation and characterization of the monoclonal antibody 6B9 from a mouse immunized with intact human T cells introduced a tool for specific targeting of csTG2 [73]. Indeed, 6B9 did not recognize ECM associated TG2, but gave abundant cell surface staining of most cell types.

This antibody was subsequently used in several studies, implicating csTG2 in innate immune responses towards gluten and more specifically in transepithelial migration of $CD8^+$ T cells [73, 103]. However, endocytosis of csTG2-gliadin complexes on dendritic cells could not be demonstrated by use of 6B9. Further, TG2 could not be detected in luminal IgA-antigen complexes of CD patients using 6B9 [101, 165]. We wished to utilize this antibody as a tool to study the conformation of csTG2 to shed light on the lack of autoreactivity towards this TG2 conformation in CD. Despite thorough effort we were not able observe binding of 6B9 to recombinant human TG2 or to isolate TG2 in immunoprecipitation experiments. Surprisingly, we found that 6B9 rather recognized CD44, an abundant cell surface proteoglycan well known to be implicated in for example lymphocyte migration [166]. The original characterization of 6B9 as specific for csTG2 was founded on the identification of a single TG2 derived tryptic peptide from an immunoprecipitated band of 80kDa. Notably, TG2 was recently shown to interact with heparan sulfate side chains, which could explain a potential co-immunoprecipitation of TG2 with CD44 [68].

Our data speak strongly against any reactivity of mAb 6B9 towards TG2. Thus, functions ascribed to csTG2 using this antibody should be revised. Perhaps more importantly, also negative findings using this antibody should be reconsidered. Although not likely to be an indispensable player, a substantial body of literature indicates that csTG2 mainly is present in structures involved in cell adhesion and migration. Known to be massively up-regulated upon differentiation of professional APCs like DCs, internalization of active csTG2 and gluten by professional APCs still represents an attractive but unproven route for efficient uptake of gluten in CD. Further effort should therefore be made to characterize not only csTG2 abundance but also its state of activity.

Gluten sensitive diseases and transglutaminase redundancy

TG2 is not the only isoform implicated in gluten sensitive diseases. Its closest relatives TG3 and TG6 are now regarded as the main autoantigens in dermatitis herpetiformis (DH) and gluten ataxia (GA) where at least in DH the production autoantibodies depends on the intake of gluten. Typically, DH patients have antibody populations which primarily recognize TG2, are cross-reactive with TG2 and TG3 or which have high avidity (and are thus seemingly specific) for

TG3. A similar situation was reported for TG6 in GA. Two mechanisms can explain the involvement of TG3 and TG6. The “specific” antibody populations could result from epitope spreading where TG3 and TG6 are merely passive targets of the immune response. However, as the antibody production is gluten dependent (and assumingly also T-cell dependent) these B-cells must retain sufficient affinity for TG2 to be able to internalize TG2-gliadin complexes required to obtain T-cell help from gluten reactive T cells. Alternatively, if these antibodies are directed against TG3 and TG6, active participation from these isoforms is required, similar to TG2 in CD. We show that TG3 and TG6 are indeed able to accommodate gluten peptides as substrates and that TG3 and TG6 can form thioester and iso-peptide linked complexes with gluten, although less efficiently than TG2. Nevertheless, the activity of TG3 and TG6 could be of relevance for DH and GA in an *in vivo* setting where little is known about activity and regulation of any of the TGase isoforms. A prerequisite for active involvement of TG3 and TG6 is however their expression at sites where they can encounter gluten; that is either in the small intestine or in cells migrating to the small intestine. Although preliminary results seem to indicate intestinal expression of both TG3 and TG6 (D.Aeschlimann, personal communication) further studies must be conducted to confirm this.

Comparisons of transglutaminase isoform by *in vitro* experiments does not necessary reflect their relation *in vivo*. Nevertheless, observing that several TGase isoforms can perform similar tasks supports the notion that the fine regulation of TGase activity *in vivo* to a large extend derives from the differential expression of the various isoforms [1]. The fact that TG2 knock out mice have a seemingly unaffected and surprisingly normal phenotype despite the vast number of functions ascribed to TG2 could suggest a certain functional redundancy among the transglutaminases [167, 168]. Indeed, transglutaminase activity has been reported in TG2 knock out mice at sites where such activity normally is ascribed to TG2 [169]. Some TGase isoforms (at least TG3) also seem to be up-regulated in TG2 knock out mice [2]. It is tempting to speculate whether some of the functions of TG2 therefore can be accounted for by other isoforms. This has however not yet been reported. More importantly, the presence (and perhaps increased expression and activity) of other TGase isoforms should be taken into consideration before conclusions are drawn from studies in TG2 knock out mice [78]. Similarly, one should be cautious when defining TG2 biology through the use of non-isoform specific substrates and inhibitors [170].

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Celiac disease is one of the best characterized and best understood human, immunological diseases. The major genetic (HLA) and environmental (gluten) factors are known and the immune response and pathogenesis is by now fairly well characterized. The disease itself is not associated with a dramatic increase in mortality but serves as an important model to understand immune mediated diseases and autoimmunity as a whole. Yet, there are several unanswered questions; in particular how and why the disease is initiated. The combined effort of numerous research groups world wide will undoubtedly make advances in the following years, and functional studies of recently identified disease associated genes are expected to shed new light on the disease pathogenesis.

More than a decade after the identification of TG2 as the celiac disease auto-antigen, followed by the discovery of its pivotal role in gluten modification, little is known for sure about the activity, conformation, localization and function of TG2 in the celiac lesion. Throughout these years, a steadily increasing number of functions have been ascribed to this single enzyme and yet it seems to be completely dispensable in animal models. This apparent discrepancy can only contribute to confound our understanding of TG2 biology.

This thesis has aimed to delineate aspects of TG2 biochemistry important to understand its role in CD and has provided new information on enzymatic activity and regulation thereof, enzyme abundance and potential redundancy among the TGase family members in GSDs. Extrapolation of biochemical properties to biological systems always requires caution but they are nevertheless pivotal to define a framework of possibilities and limitations for later *in vivo* studies.

But what lies ahead? With fear of exaggerating, I feel that much of past and current research on TG2 falls within two major groups. The first consists of a large body of literature primarily describing biochemical properties like conformation, activity and ligand binding. The second group forms an ever-increasing list of studies reporting a putative role for TG2 somewhere somehow in complex biological systems, often lacking any mechanistic explanation. With a few recent exceptions [45, 47], the “in-between” studies systematically addressing issues like binding

partners, cellular localization and regulation of activity have so far been scarce or little convincing. I think many questions can be answered from such studies. They will however require highly specific tools where the development of isoform specific inhibitors and antibodies will be pivotal. I hope and believe that future efforts will enable us to draw lines between all the scattered dots made up by past and present literature on TG2. Hopefully, a picture we all can agree upon will emerge.

In celiac disease, a better understanding of TG2 biology should allow for specific targeting and inhibition of TG2 enzymatic activity, thereby introducing a long sought pharmacological therapy for this disease.

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Erratum

Thesis

p. 4 line 4: *"the Norwegian Research Council"* was changed to "*the Research Council of Norway"*

p. 5, the following abbreviations were added; *"APC, antigen presenting cell; CE, capillary electrophoresis; LIF, laser induced fluorescence; PDI, protein disulfide isomerase."*

p. 9 figure legend, line 3: *"(light green and light blue)"* was changed to *"(light green and light yellow)"*

p. 21 line 2: *"In contrast, Deiterich et.al [148] reported no difference in the inhibitory capacity of IgA purified from CD patients and control subjects. Purified anti-TG2 autoantibodies from CD patients exerted a dose dependent inhibition of transamidation but were unable to completely block activity. The authors questioned the in vivo relevance of this inhibition due to the high level of residual activity."*

was changed to

"Dieterich et.al [148] reported no difference in the inhibitory capacity of total IgA purified from CD patients and control subjects. Affinity purified anti-TG2 autoantibodies from CD patients exerted a dose dependent inhibition of transamidation (up to 80% inhibition). This was however deemed insufficient to block biologically active deamidating activity."

p. 31 line 6: *(>50%~15min)* was changed to *(data not shown)*

p. 32 figure legend, line 2: *C277A* wash changed to *C277S*

p. 36 line 11 from bottom: *"Their staining pattern corresponds to with the ECM of the epithelial substratum and with endothelial cells of small capillaries"* was changed to *"These antibodies also react with the myofibroblast-derived extracellular matrix localized underneath the epithelium in the small intestine."*

p. 39 line 10 "*, followed by discovery*..." was changed to "*, followed by the discovery*…"

Dots and commas were corrected throughout the thesis

Paper I

p. 1806, section 2.4: should read "Separations were performed at 20 kV at 25 °C with the electro-osmotic flow running from the anode to the cathode"

I

This article is removed.

II

This article is removed.

III

This article is removed.