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Deamidation and cross-linking of gliadin peptides by transglutaminases and the relation to celiac disease

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Abstract

Activation of small intestinal gluten-reactive $CD4^+$ T cells is a critical event in celiac disease. Such cells predominantly recognise gluten peptides in which specific glutamines are deamidated. Deamidation may be catalysed by intestinal tissue transglutaminase (TG2), a protein which is also the main autoantigen in celiac disease. Our aim was to study how the two main catalytic activities of transglutaminase—deamidation and transamidation (cross-linking) of an immunodominant gliadin epitope—are influenced by the presence of acceptor amines in the intestinal mucosa, and thereby contribute to further elucidation of the pathogenetic mechanisms in celiac disease. We prepared monoclonal antibodies, reacting specifically with the non-deamidated epitope QPFPQPQLPYPQPQ-amide and/or the deamidated epitope QPFPQPELPYPQPQ-amide. A solid phase immunoassay combined with gel filtration chromatography was used to analyse deamidation and cross-linking of these peptides to proteins. Our results show that QPFPQPQLPYPQPQ-amide was deamidated when incubated with purified TG2, with fresh mucosal sheets and with mucosal homogenates. Of other transglutaminases tested, only *Streptoverticillium* transglutaminase was able to generate the deamidated epitope. A fraction of the non-deamidated epitope was cross-linked to proteins, including TG2. The results suggest that intestinal TG2 is responsible for generation of the active deamidated epitope. As the epitope often occurs in a repeat structure, the result may be cross-linking of a deamidated, i.e., activated cell epitope. Alternatively, the deamidation may occur by reversal of the cross-linking reaction. The results provide a basis for the suggestion that binding of a peptide to a protein, in connection to its modification to a T cell epitope, might be a general explanation for the role of TG2 in celiac disease and a possible mechanism for the generation of autoantigens.

Keywords: Celiac disease; Transglutaminase; Gliadin peptide; Cross-linking; Deamidation; Mucosal protein

1. Introduction

Celiac disease (CD) is a chronic inflammatory intestinal disorder, induced by dietary wheat gluten and related proteins in barley and rye. The disease is characterised by a lesion in the small intestine showing villous atrophy, crypt hyperplasia and a chronic inflammatory response. The intestine is infiltrated both with $CD8^+$ cytotoxic T cells (mainly intraepithelial) and $CD4^+$ T helper cells (mainly subepithelial).

The pathogenesis of CD is only partially known. Patients with the disease have circulating antibodies against tissue transglutaminase (TG2) [1] and possibly other endogenous antigens. Activation of gluten-reactive $CD4^+$ cells in a DQ2 or DQ8 restricted way seems to be a critical event in the development of the disease [2] but it is not known which mechanisms are involved in the progression of the disease.

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Wheat gluten contains about equal parts of alcohol soluble gliadins and alcohol insoluble glutenins. These proteins have a special structure, being rich in glutamine (about 30%) and proline (about 15%), clustered either as polyglutamine stretches or in repeating structures mainly containing glutamine and proline. It is well known that peptide bonds, where proline participates with the imino group, are only susceptible to digestion with few proteolytic enzymes, and it has been recently demonstrated that a 33-mer in a particular alpha-gliadin is resistant to enzymatic hydrolysis [3].

In recent years, several epitopes activating T cells isolated from the intestinal mucosa of CD patients have been identified. They occur mainly in gliadins of the alpha and gamma-type [4–9]. Interestingly, the indigestible 33-mer contains several repeats of some of these epitopes, one of which has been characterised as immunodominant [3]. To be active, most of the epitopes need to be deamidated at a specific glutamine residue, a reaction that has been shown to occur in vitro by treatment with purified TG2 [10,11]. There is also indirect evidence that TG2 is able to deamidate in vivo [12]. Furthermore, by its specificity, TG2 preferentially generates active epitopes on gliadins [5,13–15].

TG2 is involved in CD in at least two ways. The enzyme catalyses a calcium-dependent acyl transfer reaction, where the gamma-carboxamido group of peptide bound glutamine serves as an acyl donor [16]. A thiol group of the active site is acylated by the acyl donor, generating a transient covalently linked intermediate. The second part of the reaction, a deacylation of the enzyme, occurs either via hydrolysis (water as acceptor, deamidation) or aminolysis (primary amines as acceptors, transamidation) and is rate-limiting. If the acceptor is a peptide-bound lysine, the result is cross-linking to the peptide.

Cross-linking of a gliadin T cell epitope to transglutaminase may help the production of anti-TG2 by B cells [17]. Such a binding has been demonstrated using a labelled epitope peptide added to recombinant human TG2, and has been suggested to be due to temporary binding to the active site of the enzyme or to lysines in the protein [13,14]. Evidence for cross-linking was also provided by Ciccocioppo [18]. If gliadin peptides also bind to other proteins, a similar mechanism may be involved in generation of antibodies against other endogenous proteins [17].

In this paper, we studied the deamidation and cross-linking activities of transglutaminase. Using synthetic peptides containing the immunodominant gliadin epitope together with monoclonal antibodies specific for the deamidated form of the epitope or for both forms, we characterised both the deamidation and cross-linking to intestinal proteins. We demonstrated that the epitope was deamidated in an environment similar to the intestinal wall, albeit at a rather low rate. The lower rate was explained by concomitant binding to a multitude of proteins.

2. Materials and methods

2.1. Synthetic epitope peptides

Synthetic alpha-gliadin peptides containing the immunodominant motif in a deamidated (QPFPQPELPYPQPQ-amid) (PepE) and a non-deamidated form (QPFPQPQLPYPQPQ-amide) (PepQ) were obtained from Innovagen, Sweden and Schafer-N, Denmark, respectively.

2.2. Monoclonal antibodies

The monoclonal antibodies (MC01 and MC02) used in this study were raised against a synthetic peptide (KLQPFPQPELPYPQPQ-amide) (E9) (Innovagen) containing the immunodominant motif. The peptide was coupled onto PPD (Tuberculin) (Statens Serum Institut (SSI), Denmark) at a 5:1 molar ratio (peptide/PPD) using glutaraldehyde as a homobifunctional coupling reagent. Mice (CF1xBalb/c, female) were pre-immunized with two human doses of BCG-vaccine (rendering them TH-immune to PPD), and 1 month later received antigen (corresponding to 25 μg of PPD) absorbed onto $Al(OH)_3$ and emulsified with Incomplete Freunds Adjuvant (IFA) (SSI, Denmark). Mice were boosted every 2 weeks with antigen without IFA. After four immunizations, fusion was performed, preceded by iv-boost with antigen without adjuvant. The fusion protocol was as described by Köhler and Milstein [19] with minor modifications [20]. Positive clones (tested in ELISA with E9 coupled onto the polystyrene plate) were retested with KLQPFPQPQLPYPQPQ-amide (Q9) and two clones were selected: MC01, which reacted only with E9, and MC02, which also reacted with Q9. The positive clones were re-cloned until monoclonality was secured. Supernatants from the cultured lymphocytes were used directly without further purification.

2.3. Transglutaminases

Guinea pig liver transglutaminase (GPLT) (T 5398, Sigma-Aldrich) was stored in frozen aliquots (5 mg/ml) until use. Factor XIII (Haematologic Technology, USA) was activated by incubation with 5-μg (0.8 unit) thrombin (Enzyme Research Laboratories, USA). *Streptoverticillium* transglutaminase was prepared by dissolving 500-mg Activa WM (Ajinomoto, Germany), containing 1% transglutaminase in carrier in 10 ml H_2O. The solution was filtered through a Cameo filter 25 AS (Frisenette, Denmark) with a pore size of 0.22 μm. Transglutaminase from *Phytophtora cactorum* was delivered in a partially purified form (kind gift from Thomas Lykke Sørensen, Novozymes, Denmark).

2.4. Small intestinal mucosa

Pigs were anaesthetised, the middle part of duodenum was removed, and the mucosal layer stripped from the

underlying layers. For deamidation studies, the fresh mucosa was cut into 20–30 mg pieces and immediately incubated with PepQ as described below. Other mucosal pieces were kept at −20 °C. For experiments with homogenate, frozen mucosa was thawed, then homogenized (Ultra-Turrax T8, IKA-werke, Germany) for 10 s on ice in 5 mM Tris–HCl at the indicated pH, containing 0.1 mM EDTA, aprotinin 10 μg/ml and leupeptin 10 μg/ml.

2.5. TR-FIA

A solid phase immunoassay (TR-FIA) using Europium (Eu^{3+}) as label was used for discriminating between PepE and PepQ, for identification of deamidation rate and for identification of the cross-linked epitope peptide. For the specificity and sensitivity experiments, 96-well microtiter plates (MaxiSorp FlouroNunc plates, Nunc, Denmark) were coated (37 °C, 2 h) (coating buffer 0.1 M sodium carbonate pH 8.3) with either 150 μl PepQ, 150-μl PepE or a mixture of these at the indicated concentrations. In the deamidation- and cross-linking experiments, the wells were coated with fractions obtained by gel chromatography of the incubated samples. The wells were blocked with bovine serum albumin (BSA) (Fluka, Switzerland) (1 mg/ml) and washed after each step in PBS with 0.05% Tween (PBS/Tween). The monoclonal antibody (MC01 or MC02) was diluted 10-fold in PBS/Tween and 200 μl was added per well before incubation (37 °C, 30 min). 150 μl of biotinylated rabbit anti-mouse IgG (E0464, DakoCytomation A/S, Denmark) diluted 1:100 in PBS/Tween was further added and the plates were incubated (room temperature, 30 min). 150 μl of Eu^{3+}-labelled 1 μg/ml streptavidin (Wallac, Finland) in Delfia assay buffer (Wallac) was then added (room temperature, 60 min). Finally, 180 μl Delfia enhancement solution was added (room temperature, 10 min) and the Eu^{3+}-fluorescence read on a Wallac 1420 D fluorometer. Samples were run in triplicate except for screening of chromatographic fractions.

2.6. Deamidation experiments

2.6.1. Incubation

All samples were incubated (37 °C, 30–60 min) in a reaction volume of 100 μl, and the reaction was stopped by heating (98 °C, 15 min). The diluted sample was filtered (Ultrafree-MC 0.22 μm filter, Millipore Corporation, USA) and 160 μl was chromatographed by gel filtration.

2.6.2. Chromatography

The chromatography of the incubated samples was performed on Superdex Peptide HR 10/30 (Pharmacia Biotech AB, Sweden) run on a Smart FPLC System (Pharmacia) in 0.1 M $NaHCO_3$ pH 8.3. The chromatography peak fractions containing the epitope peptide were used for coating of immunoplates. The quantitative recovery of the peptide was determined by its absorbance and the grade of deamidation was determined in a TR-FIA as described above by use of the monoclonal antibody MC01.

2.6.3. Deamidation by purified transglutaminases

PepQ (0.2 mg/ml) was incubated with either GPLT, factor XIIIa, transglutaminase from *Streptoverticillium*, or transglutaminase from *P. cactorum* at the indicated concentrations in 5 mM Tris–HCl, 5 mM $CaCl_2$ pH 6.5 except in experiments where the influence of 5 mM EDTA or pH 7.3 or 8.0 was investigated.

2.6.4. Deamidation by small intestinal mucosal pieces

PepQ (0.2 mg/ml) was incubated with fresh mucosal pieces from pig intestine in 5 mM Tris–HCl pH 6.5 containing 5 mM $CaCl_2$, 0.15 M NaCl and 0.1 mM valine-pyrrolidide (kind gift of J.J. Holst, University of Copenhagen, Denmark). In some experiments, 5 mM EDTA was used instead of 5 mM $CaCl_2$. After incubation, the sample was centrifuged (2000×*g*, 5 min) and the supernatant analysed for deamidated epitope as described above. The specific transamidating TG2 activity in the mucosa was quantified by the microtiter assay described below on a homogenised mucosal piece that had not been incubated.

2.6.5. Deamidation by mucosal homogenate

PepQ (0.2 mg/ml) was incubated with mucosal homogenate (40 mg/ml) in 5 mM Tris–HCl pH 6.5 with or without addition of GPLT. Before incubation, 5 mM $CaCl_2$ and 0.1 mM valine-pyrrolidide was added. The sample was then centrifuged (2000×*g*, 5 min) and the supernatant analysed for deamidated epitope as described above.

2.7. Determination of deamidation/transamidation at various lysine concentrations

The relation between deamidation and transamidation was measured by quantifying the amount of released ammonia in the absence (deamidation) or presence (transamidation) of lysine. Solpro 300 (proteolytically digested gluten) (Amylum Group, Belgium) 3.0 mg/ml was incubated (37 °C, 60 min) with GPLT (10 μg/ml) in the presence of various lysine concentrations in 3 mM Tris–HCl pH 6.5, 0.5 mM $CaCl_2$. Ammonia released was measured in a coupled assay essentially as described by Levitzki [21].

Deamidation of the epitope peptide was determined by incubating PepQ (0.2 mg/ml) with GPLT (6 μg/ml) in the presence of various lysine concentrations in 5 mM Tris–HCl, 5 mM $CaCl_2$ pH 7.3 followed by chromatography and TR-FIA with MC01 as described above. Lysine was removed by rechromatography of the peptide fractions before they were used for coating of immunoplates.

2.8. Cross-linking experiments

2.8.1. Incubation

All samples were incubated (37 °C, 60 min) in a reaction volume of 100 μl and the reaction stopped by heating (70 °C, 15 min). The diluted sample was then filtered and 50 μl applied to the Superose column.

2.8.2. Chromatography

The chromatography of the incubated samples was performed on Superose 12 PC 3.2/30 (Pharmacia) in 0.1 M $NaHCO_3$ pH 8.3 run on a Smart FPLC System. All chromatography fractions containing peptides with a higher molecular weight than the epitope peptide were used for coating of immunoplates, and the cross-linked epitope peptide in these higher molecular weight components was identified with TR-FIA, as described above, by use of the monoclonal antibody MC02.

2.8.3. Cross-linking to mucosal proteins

PepQ (0.2 mg/ml) was incubated with mucosal homogenate (40 mg/ml) in 5 mM Tris–HCl pH 7.3 with 5 mM $CaCl_2$ and 0.1 mM valine-pyrrolidide with or without GPLT (0.1–0.2 mg/ml). In some experiments, the proteolytic activity of the homogenate was further eliminated by heat inactivation (70 °C, 15 min). In some of these experiments the heat inactivated mucosal homogenate was dialysed (18 h, 4 °C) against 5 mM Tris–HCl pH 7.3 before incubation.

2.8.4. Cross-linking to chicken egg albumin and BSA

PepQ (0.2 mg/ml) was incubated with GPLT (0.1 mg/ml) and chicken egg albumin (A-5503, Sigma-Aldrich) (2 mg/ml) or BSA (2 mg/ml) in 5 mM Tris–HCl, 5 mM $CaCl_2$ pH 7.3.

2.9. Cross-linking to purified TG2

PepQ (0.2 mg/ml) was incubated (37 °C, 60 min) with GPLT (0.1 mg/ml) in 5 mM Tris–HCl pH 7.3, 5 mM $CaCl_2$. In order to investigate whether other sites besides the active site of TG2 were involved in cross-linking, high concentrations of heat-inactivated GPLT (0.5 mg/ml) were added to some of the incubations. In these cases we could observe a high molecular weight precipitate. The incubated samples were centrifuged (12,000×*g*, 10 min), and the resulting pellets were incubated (37 °C, 60 min) with Proteinase K (P-2308, Sigma-Aldrich) (0.1 mg/ml). The reaction was stopped by heating (70 °C, 15 min), the sample was filtered, gel-filtrated on the Superose column and the resulting fractions analysed for cross-linked epitope peptide in TR-FIA with MC02, as described above.

2.10. Transglutaminase assay

The transamidating activity of TG2 in the mucosal homogenate was quantified in a microtiter plate assay using Solpro 300 as solid phase substrate and Eu^{3+}-labelled streptavidin to quantify the 5-(biotinamido)pentylamine covalently linked to the plate by transglutaminase, essentially as described earlier [22]. Protein was determined by use of the Biorad Protein Assay (Bio-Rad Laboratories, USA) using BSA as a standard. The concentration of mucosal proteins in the assay was kept between 10 and 50 μg/ml. At these protein concentrations the measured activity is proportional to the added amount of mucosa.

3. Results

3.1. Characterisation of the assay

In TR-FIA, MC01 reacted specifically with PepE while MC02 reacted well with both PepE and PepQ. Sensitivity was tested by coating various concentrations of PepQ to microtiter plates. The result (Fig. 1A) shows a working range of 2 to 50 nM PepQ. By coating immunoplates using mixtures of PepQ and PepE with a fixed total concentration of 3 μM, we could demonstrate that MC01 was specific for the deamidated form with a sensitivity, corresponding to a working range of 0.3 to 1.5 μM (Fig. 1B). The method was able to detect as little as 1% of PepE in the mixture.

3.2. Deamidation

3.2.1. Purified transglutaminases

GPLT generated deamidated epitope peptide in a concentration-dependent manner (Fig. 2). When 5 mM EDTA replaced $CaCl_2$ during incubation, the deamidation was completely inhibited. As TG2 is a calcium-dependent enzyme, this further supports that the obtained results reflect the activity of TG2. The deamidation was pH-dependent. When tested at three different pH values (6.5; 7.3 and 8.0), the activity was highest at pH 6.5, even if the deamidation at pH 8.0 was substantial (data not shown). This is in accordance with previous reports [23] and further supports the specificity of the method.

Transglutaminase from *Streptoverticillium* also deamidates PepQ in a concentration-dependent manner (Fig. 3). The deamidation rate was, however, lower than with TG2, as an approximately 20–40 times higher *Streptoverticillium* transglutaminase concentration was needed to achieve a comparable deamidation rate. In contrast to this, Factor XIIIa and transglutaminase from *P. cactorum* generated minor or no deamidated epitope, even at high enzyme concentrations (Fig. 3).

3.2.2. Small intestinal mucosal pieces

After incubation of PepQ with a mucosal piece, the fluorescence of the epitope peptide peak increased significantly when analysed in the deamidation assay (Table 1), showing that the epitope was deamidated during the incubation. In the presence of EDTA, no deamidation of

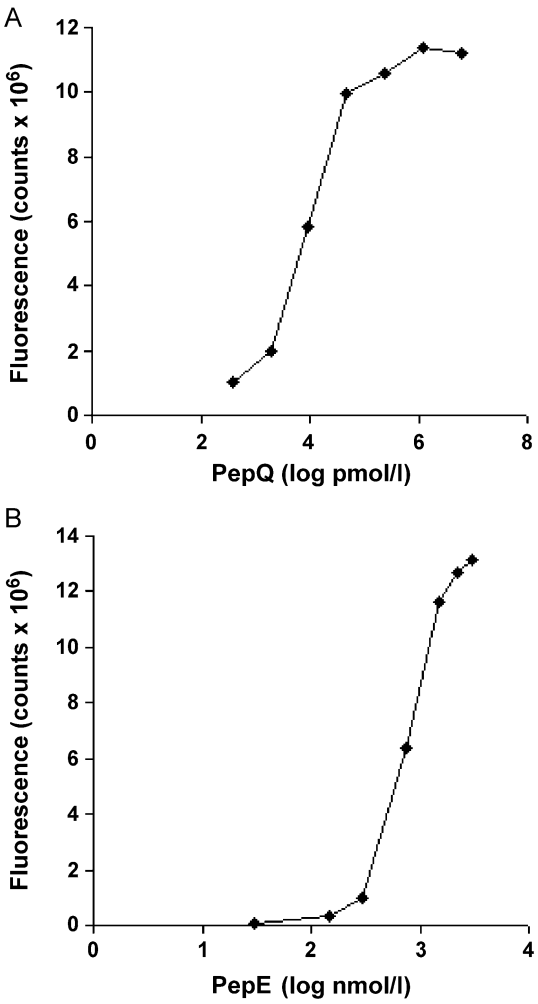

Fig. 1. (A) Detection limit for PepQ. Microtiter plates were coated with PepQ at concentrations between 0.38 and 6100 nmol/l and was measured by TR-FIA using the monoclonal antibody MC02. The data were corrected for a buffer control sharing approximately 3000 counts. (B) TR-FIA fluorescence at various proportions of PepQ and PepE. Microtiter plates were coated with PepQ mixed with increasing amounts of PepE (0.03 to 3 μmol/l). The total peptide concentration was kept constant at 3 μmol/l. PepE was determined by TR-FIA using the monoclonal antibody MC01. The data were corrected for a buffer control sharing approximately 2000 counts.

the epitope peptide could be detected. As the activity of mucosal TG2 is known to decrease during incubation, the specific transglutaminase activity was measured in a non-incubated biopsy taken from the same mucosal piece. From the measured specific activity, it was calculated that the initial transamidating activity in the mucosal pieces approximately corresponds to the activity that can be measured in 2–10 μg of pure GPLT [22]. We can therefore conclude that the deamidation by transglutaminase in the mucosal piece was significantly lower than expected from our data obtained with GPLT in the absence of amine acceptors, where this amount of enzyme would give an antibody reactivity higher than 7×10^6 fluorescence counts (Fig. 2). This is probably explained by cross-linking with primary

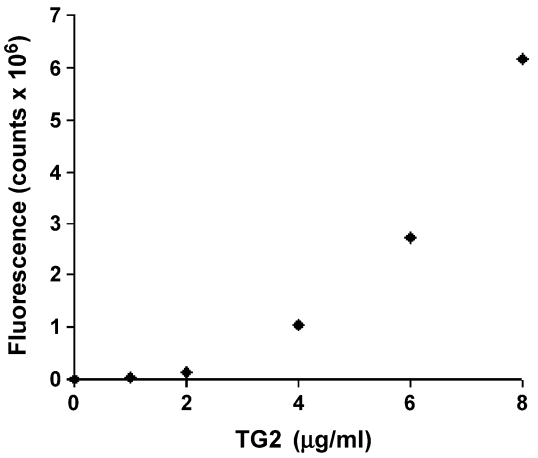

Fig. 2. TG2 catalysed deamidation. PepQ (0.2 mg/ml) was incubated (60 min) with GPLT in increasing concentrations (0, 1, 2, 4, 6, 8 μg/ml). After gel filtration on Superdex Peptide HR 10/30, the pooled peptide peaks were used for coating of immunoplates and quantified in TR-FIA using the monoclonal antibody MC01 which reacts specifically with PepE. The data were corrected for a buffer control sharing approximately 2000 counts.

amines in high or low molecular weight compounds of the intestinal mucosa.

3.3. Relation between deamidation and cross-linking

3.3.1. Mucosal homogenate

To further characterise the influence of primary amines in the mucosa, we studied the effect of a mucosal homogenate on the deamidation activity of GPLT. The results of a representative experiment are shown in Table 2. PepQ incubated with mucosal homogenate alone (40 mg/ml estimated to contain 4 μg/ml of endogenous TG2) resulted

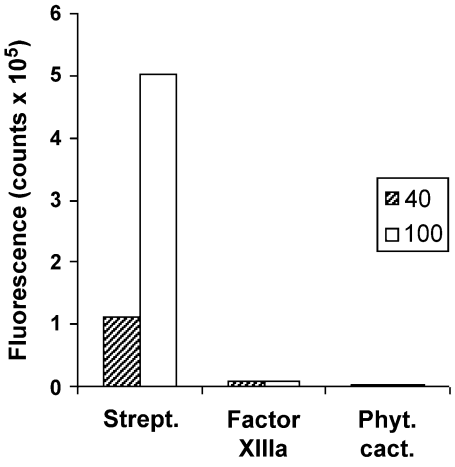

Fig. 3. Deamidation of PepQ by various transglutaminases. PepQ (0.2 mg/ml) was incubated (60 min) with either Factor XIIIa or transglutaminases from *Streptoverticillium* or *P. cactorum* at two different concentrations (40 and 100 μg/ml). After gel filtration, the pooled peptide peaks from each experiment were used for coating of microtiter plates, and the deamidated fraction of PepQ was determined in a TR-FIA using the monoclonal antibody MC01 which reacts specifically with the deamidated peptide. The data were corrected for a buffer control sharing approximately 8000 counts.

Table 1
Deamidation of PepQ by pig small intestinal mucosa

	Biopsy weight (mg)	PepQ incubated with biopsy (counts×10^3)
$CaCl_2$	21	214
EDTA	14	13

PepQ (0.2 mg/ml) was incubated (60 min) with fresh mucosal tissue and the product chromatographed. The peptide peak was analysed for deamidated peptide in TR-FIA using the monoclonal antibody MC01. For details, see Section 2. Six biopsies from four different pigs were analysed in four independent experiments. A representative biopsy is shown in the table together with a biopsy from the same intestine incubated in the presence of EDTA. The control values, i.e., PepQ incubated without a mucosal biopsy (16×10^3 counts) and a mucosal biopsy incubated without PepQ (2×10^3 counts), were not subtracted. The specific transamidating TG2 activity in the mucosal piece was 18×10^6 counts/min/mg protein as measured in a non-incubated "sisterbiopsy". The original non-deamidated peptide PepQ was recovered after incubation, i.e., it was not degraded by hydrolysis as shown by the fact that the chromatographic pattern of the peptide peak was essentially unchanged as was the antibody reactivity when tested with MC02.

in only a low level of deamidation of the epitope peptide. Addition of GPLT to the homogenate increased deamidation, but still to a significantly lower level than the deamidation rate obtained with PepQ and GPLT in the absence of primary amines. The result strongly supports the suggestion that the presence of primary amines in the mucosal homogenate inhibits deamidation. However, the result also shows that deamidation catalysed by TG2 does actually occur in an environment approaching physiological conditions, in spite of a high protein concentration.

3.4. Purified transglutaminases

To study further the relation between deamidation and transamidation with respect to purified transglutaminases, proteolytically digested gluten was incubated with or without various concentrations of lysine. The reaction rate in this experiment was measured by the rate of liberation of ammonia. The results, shown in Table 3, demonstrate that the maximal transamidation rate for TG2 was about four times higher than the deamidation rate (in the absence of lysine). When measured in TR-FIA using MC01, we could demonstrate that TG2 deamidation of PepQ was substantially inhibited by lysine (Fig. 4). Notably, however, significant TG2 deamidation could be registered even at high concentrations of this amino acid.

Table 2
Influence of mucosal homogenate on deamidating activity of GPLT

Homogenate (counts×10^3)	GPLT 10 μg/ml+homogenate (counts×10^3)	GPLT 10 μg/ml (counts×10^3)
25	423	7516

PepQ (0.2 mg/ml) was incubated with either mucosal homogenate (40 mg/ml estimated to contain 4 μg/ml of endogenous TG2), GPLT 10 μg/ml, or a mixture of these. The product was chromatographed and the peptide peak was analysed for deamidated peptide in TR-FIA using the monoclonal antibody MC01. The original non-deamidated peptide PepQ was recovered after incubation, i.e., it was not degraded by hydrolysis as the chromatographic pattern of the peptide peak was essentially unchanged as was the antibody reactivity when tested with MC02.

Table 3
Relation between deamidation and transamidation measured at different lysine concentrations

Lysine (mM)	0	20	40	60	80	100
Released NH_3	0.152	0.393	0.500	0.591	0.617	0.609

Solpro (3.0 mg/ml) was incubated with GPLT (10 μg/ml) in the presence of different lysine concentrations (0, 20, 40, 60, 80, 100 mM). The released ammonia was measured in a coupled assay: 210 μl of the sample was mixed with 100 μl 0.5 M triethanolamine–HCl pH 8.0 containing 1.82 mM ADP and 35 mM oxoglutarate. 10 μl NADH (5 mg/ml) and 20 μg glutamate dehydrogenase were added and the rates of NADH oxidation were followed for 20 min at 339 nm. Blanks incubated for 0 min were withdrawn from the results. Data are given as change in absorbance at 339 nm.

3.5. Cross-linking

3.5.1. Mucosal homogenate

We suggest that the inhibition of deamidation by mucosal proteins is due to cross-linking by TG2. To further study the nature of this cross-linking, a series of experiments was performed. Fig. 5 shows the resulting chromatography following incubation of PepQ with a mucosal homogenate and GPLT. When the elution fractions were analysed in TR-FIA with MC02, a broad peak with a molecular weight of approximately 80,000 kDa was seen, as well as a minor but significant labelling peak corresponding to higher molecular weight proteins. The same result was obtained irrespective of whether fresh or heat inactivated mucosa was used for the experiments. When PepQ was incubated with fresh homo-

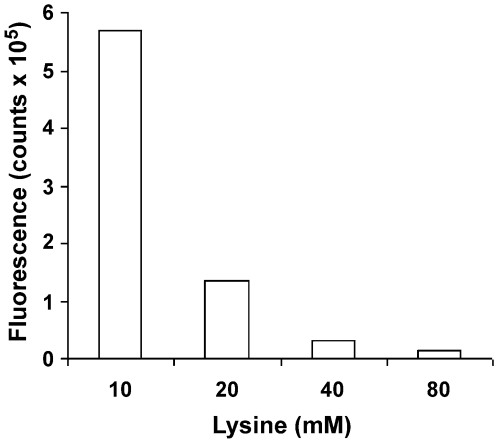

Fig. 4. Deamidation of PepQ in the presence of lysine. Deamidation of the epitope peptide was determined by incubating PepQ (0.2 mg/ml) with GPLT (6 μg/ml) in the presence of various lysine concentrations (10, 20, 40 and 80 mM) in 5 mM Tris–HCl, 5 mM $CaCl_2$ pH 7.3 followed by chromatography and TR-FIA with MC01 which reacts specifically with the deamidated peptide. The data were corrected for a buffer control sharing approximately 2000 counts.

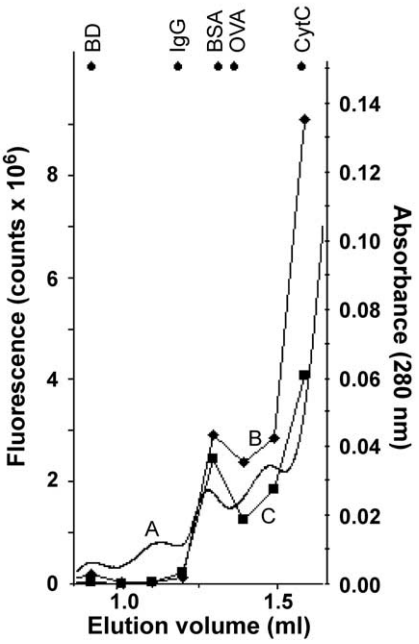

Fig. 5. Cross-linking of PepQ to mucosal proteins. PepQ (0.2 mg/ml) was incubated with mucosal homogenate (protein concentration 4 mg/ml) and GPLT (0.1 mg/ml) prior to chromatography. The chromatographic fractions were used for coating of immunoplates and cross-linked PepQ was identified in TR-FIA using the monoclonal antibody MC02. For details, see Section 2. A: absorbance at 280 nm; B: antibody reactivity of non-dialysed homogenate; C: antibody reactivity of dialysed homogenate. BD: blue dextran; IgG: immunoglobulin G; BSA: bovine serum albumin; OVA: chicken egg albumin; CytC: cytochrome *C*.

genate without addition of GPLT, the epitope peptide was still cross-linked, although to a lower extent. In several experiments of this type, we could show that the fractions containing cross-linked peptide had no immunoreactivity when analysed with MC01. This fits the assumption that the critical glutamine residue was involved in cross-linking. The possible influence of low molecular weight primary amines was studied by dialysis experiments (Fig. 5). When the homogenate was dialysed before incubation with PepQ, the fluorescence counts were slightly decreased, indicating that some of the registered high molecular weight compounds were due to the involvement of monoamines and diamines removed during dialysis.

3.6. Purified proteins

3.6.1. Chicken egg albumin and BSA

To further study the extent of cross-linking of PepQ to proteins, several purified proteins were studied. The results for chicken egg albumin and BSA are presented in Fig. 6 and clearly show that, in the presence of GPLT, PepQ was cross-linked to these proteins. The high molecular weight peaks seen both in the presence of chicken egg albumin and BSA were only present after incubation with GPLT, and represent the polymerised protein which has also incorporated the epitope peptide.

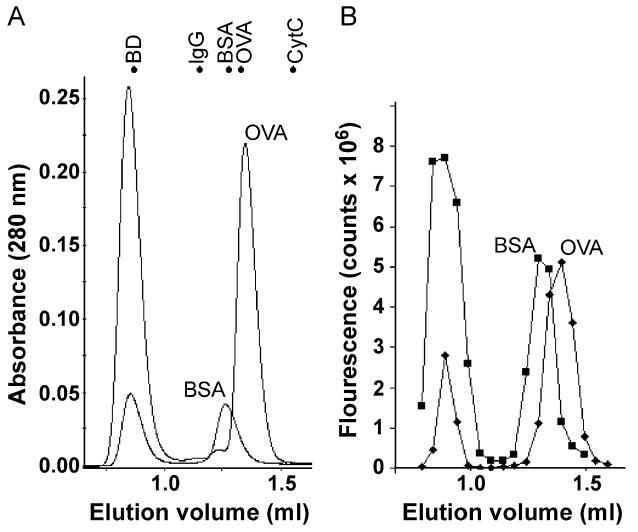

Fig. 6. Cross-linking of PepQ to chicken egg albumin and BSA. PepQ (0.2 mg/ml) was incubated with chicken egg albumin (2 mg/ml) or BSA (2 mg/ml) in the presence of GPLT (0.1 mg/ml). (A) Absorbance at 280 nm. (B) Antibody reactivity with MC02. The two experiments were run in parallel and the results superimposed. BD: blue dextran; IgG: immunoglobulin G; BSA: bovine serum albumin; OVA: chicken egg albumin; CytC: cytochrome *C*.

3.6.2. TG2

It has earlier been reported that gliadin peptides bind to TG2 [13,14,18]. To further analyse this reaction, we performed a series of experiments similar to those with BSA and chicken egg albumin. Gel filtration of active GPLT alone revealed a dominating peak with a molecular weight higher than the monomeric form (approximately 130,000

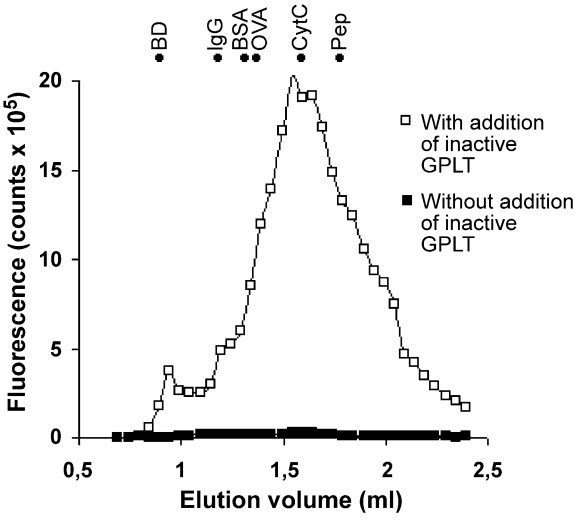

Fig. 7. Cross-linking of PepQ to purified TG2. PepQ (0.2 mg/ml) was incubated with GPLT (0.1 mg/ml) with or without addition of heat-inactivated GPLT (0.5 mg/ml). After centrifugation the pellets were digested with proteinase K, gel-filtered on the Superose column, and the resulting fractions analysed for cross-linked epitope peptide in TR-FIA with MC02. The antibody reactivity without addition of inactive GPLT has a maximum of approximately 25 000 counts. BD: blue dextran; IgG: immunoglobulin G; BSA: bovine serum albumin; OVA: chicken egg albumin; CytC: cytochrome *C*; Pep: epitope peptide.

kDa) whereas previous inactivation of GPLT with EDTA and heating gave an even higher aggregation state. These high molecular weight peaks were only obtained when GPLT was incubated without the epitope peptide. In contrast, incubation of active GPLT with the epitope peptide resulted in disappearance of most of the enzyme from the chromatogram, indicating precipitation due to polymerisation, substantiated by the observation that when a substantial amount of inactivated transglutaminase was added to the incubation, a visible precipitate was seen. Analysis of this precipitate for cross-linked epitope peptide after digestion with proteinase K showed that it was highly antibody reactive with MC02 (Fig. 7), showing that the epitope peptide was bound to GPLT (inactive and active) as observed for other proteins. In control experiments without addition of high amounts of inactive GPLT, the antibody reactivity was low (Fig. 7), demonstrating that most of the registered epitope peptide was cross-linked to the inactivated GPLT.

4. Discussion

In this paper, it is demonstrated that TG2, when present in the intestinal mucosa, can deamidate an immunodominant peptide epitope. In the presence of primary amines, this reaction is inhibited, although not completely, and cross-linking to intestinal proteins seems to dominate. This result provides a basis for the suggestion that binding of a peptide to a protein in connection to its modification to a T cell epitope might be a general explanation for the role of TG2 in celiac disease and a possible mechanism for the generation of autoantigens.

A short segment, PQPQLPY, was previously identified as a core sequence of the immunodominant alpha-gliadin epitope, which is responsible for most of the stimulatory activity on intestinal and peripheral $CD4^+$ T lymphocytes [4,5]. Deamidation of the underlined glutamine by TG2 was shown to be important for recognition by the T cells. To further characterise this deamidation, we generated a monoclonal antibody (MC01) reacting specifically with the deamidated form of the epitope. The antigen used for immunisation was C-terminally amidated with the aim to increase the probability of obtaining an antibody specific for the carboxyl group of the antigenically important glutamic acid. The epitope peptide furthermore contained an extra N-terminal lysine, of importance for binding the peptide to PPD, thereby increasing the immunogenecity. Another of the generated antibodies (MC02) reacted both with the non-deamidated and the deamidated epitope peptide.

The concentrations of non-deamidated and deamidated epitope peptides were estimated following their coating on microtiter plates. Prior to immunoassay, the incubated samples were fractionated by gel filtration, thus enabling specific measurements on either the epitope peptide (for the deamidation assay) or on compounds with higher molecular weight (allowing assay of possible cross-linking). The specificity of antibody MC01 was demonstrated by coating with a constant concentration of PepQ plus PepE, but with varying proportions of the two compounds. The method turned out to be highly sensitive. Thus, MC02 was capable of detecting as little as 1 nM epitope peptide, whereas MC01 could detect as little as 1% deamidation under conditions where the total concentration of PepQ was 3 μM. This approach has, to the best of our knowledge, not been previously described.

The applicability of the developed TR-FIA was demonstrated using purified TG2 from guinea pig liver, which in the absence of primary amines (except 5 mM Tris) was shown to generate deamidated epitopes. We also examined the epitope deamidating ability of other enzymes. Generation of the deamidated epitope was low or non-detectable following incubation with either Factor XIIIa or a microbial transglutaminase from *P. cactorum*. This lack of specific deamidation of an immunodominant epitope strengthens the suggestion [14,15,23] that there is a connection between the specificity of TG2 and generation of DQ2 binding epitopes. The connection is, however, not absolute as a transglutaminase from *Streptoverticillium* was shown to be able to generate the active epitope, although at a lower rate.

Whereas the transamidation reaction has been demonstrated to increase with increasing pH, the pH dependence of the deamidation in the absence of a competing amine is expected to be independent of pH [23]. Our results, demonstrating that the enzymatic deamidation was more efficient at lower pH in the range pH 6.5 to pH 8.0, might be explained by competition with the primary amine of the buffer component Tris–(hydroxymethyl)aminomethan, having a pK_a of 8.1. Thus, the presence of 50 mM Tris–HCl at pH 7.3 resulted in a significantly lower deamidation rate in relation to that exhibited at 5 mM Tris–HCl pH 7.3. Interestingly, buffers containing 50–100 mM Tris–HCl (pH 7.3) have frequently been used in studies on the kinetics of transglutaminase, but the possible influence of Tris–HCl has to our knowledge not previously been discussed.

In the small intestine, TG2 is mainly localized extracellularly in lamina propria [10,24] (Skovbjerg et al., manuscript submitted) and shows increased levels in patients with CD [25]. It is generally believed that immunologically active peptide fragments access the subepithelial tissue via a leaky epithelium. To mimic the celiac situation, we prepared epithelial sheets including the subepithelial connective tissue from pig small intestine and incubated the tissues with non-deamidated epitope peptide in an organ culture similar model. This model allows access of the epitope peptide to the subepithelial tissue at the same time as endogenous TG2 is allowed to act in an environment approximating the in vivo situation. This type of experiment demonstrated that an immunodominant gliadin peptide could in fact be deamidated in this near

physiological environment, in line with other indirect observations [12–15].

The alpha-epitope used in this study was blocked at the C-terminal end by an amide group, thus rendering the epitope resistant to hydrolysis by intestinal carboxypeptidases. Having a non-protected N-terminal, the epitope can still potentially be cleaved by the microvillar enzyme DPP IV. To avoid degradation, the DPPIV inhibitor valine-pyrrolidide, which efficiently inhibits this hydrolysis [26], was added to incubations where mucosal biopsy or homogenate was used. The efficiency of this inhibition was verified in the chromatographic runs that preceded the immunoassay.

The relation between deamidation and transamidation was studied in a pure system with gliadin as acyl donor in the presence of either H_2O or varying concentrations of lysine, assuming that an increased catalytic rate (measured as production of ammonia) by increasing concentrations of a primary amine is due to transamidation. By this approach, we found a ratio between deamidation and transamidation of 1:4 for TG2 at optimal lysine concentrations. The finding that significant deamidation of PepQ was registered even at high concentrations of lysine strengthens the suggestion that deamidation actually takes place in the intestinal environments, under conditions where the concentration of primary amines, especially peptide-bound lysine, is expected to be high.

Even if transglutaminase from *Streptoverticillium* has a low deamidation ratio compared to TG2, then its ability to generate the deamidated alpha-epitope has a certain practical interest, as *Streptoverticillium* transglutaminase is used as a cross-linker in the food industry. The result also opens the possibility that deamidation of the epitope might be undertaken by other transglutaminases, such as transglutaminase in fish [27] or other microbial or vegetable transglutaminases. We have earlier compared the transamidating activities of various transglutaminases with gliadin as substrate [22] and found the specific transamidating activity of *Streptoverticillium* transglutaminase to be 45% of the specific activity of TG2. The specific transamidating activity of Factor XIIIa was almost 10 times higher than that of TG2, while the specific transamidating activity of *P. cactorum* transglutaminase was 70% of that of TG2. The last two enzymes thus have a considerable transamidating activity with gliadin as substrate, but either without the specificity for the critical glutamines in the immunodominant motif or without deamidating ability.

Incubation of BSA and chicken egg albumin with epitope peptide and active transglutaminase clearly demonstrated that the epitope peptide bound to these proteins, as the binding profile in both cases, follows the protein profile. Corresponding experiments with TG2 were more intriguing. The peak profile of TG2 after gel filtration is known to vary considerably depending on the circumstances, probably due to autocatalytic cross-linking [28]. Very little absorbance in the gel chromatographies could be detected after incubation of the epitope peptide with either active GPLT or a mixture of active and inactive GPLT. The precipitate, visible after addition of large amounts of (inactive) GPLT, was shown to contain the epitope peptide. This demonstrates that the epitope peptide is cross-linked to GPLT independent of a native active site conformation, and is in accordance with our demonstration of binding also to other proteins and with recent findings on TG2 of Fleckenstein [13]. From our data, it cannot be decided whether a transient covalent acyl intermediary GPLT gliadin-peptide complex, as earlier suggested [13,14], is also generated.

Patients with CD have circulating antibodies against TG2, which is considered to be the dominating autoantigen [1], including the reactivity earlier ascribed to endomysium and reticulin. However, not all anti-TG2 activities of patient sera are absorbed by guinea pig or monoclonal TG2 [1,24,29,30], pointing to the existence of other submucosal autoantigens. In addition, some patients have autoantibodies against the cytoskeletal protein actin [31,32] and possibly other proteins [32].

The binding of the epitope peptide to TG2 supports the hypothesis of Sollid et al. [17] that T cell immune response to gliadin would drive antibody responses towards TG2 that is cross-linked to gliadin T cell epitopes. The cross-linking occurs, as demonstrated in this paper and recently by others [13], also outside the active site of TG2. Cross-linking is therefore expected to occur to all proteins having epsilon-amino groups that are reactive with the gliadin epitope. That this really is the case is demonstrated in this paper by its binding to BSA and chicken egg albumin. This strengthens the hypothesis [17] that gliadin also drives antibody responses to proteins other than TG2. The importance of posttranslational protein modifications in antigen recognition and autoimmunity has recently been reviewed [33]. Sjöström et al. [6] suggested that the intestinal immune system is tolerant to non-deamidated peptides and that a process leading to deamidation of gliadins could contribute to break of tolerance. However, the use of the particular glutamine residue in the gliadin epitope for cross-linking instead of deamidation may argue against this suggestion. Interestingly, the studied immunodominant epitope occurs in an oligomerised non-digestible form [3] having more than one deamidation/transamidation site, thus potentially allowing simultaneous cross-linking and deamidation by transglutaminase. Alternatively, the isopeptidase activity of TG2 may release the bound epitope in its deamidated form by hydrolysis after uptake. Further studies are needed to elucidate the details on this mechanism.

It has been reported that the prevalence of autoimmune disorders in celiac disease is related to the duration of exposure to gluten [34], although this result has been challenged [35]. The importance of the introduction of gluten in relation to breast feeding has also been under investigation. Interestingly, food supplementation with gluten-containing foods before the age of 3 months was shown to be associated with significantly increased islet

autoantibody risk [36]. In addition, autoantibodies specific to diabetes mellitus type 1 have in one case been reported to disappear following change to a gluten-free diet [37]. Furthermore, patients with gluten ataxia have been shown to have antibodies against Purkinje cells [38]. It has been suggested that the up-regulation of TG2 in CD may generate additional antigenic neo-epitopes by cross-linking or deamidating viral, nutritional or endogenous proteins, and thereby contribute to initiation of autoimmune diseases [39]. Cross-linking with TG2 has also been suggested to be involved in autoantigen modifications during apoptosis or cellular injury [40]. However, in none of these cases the mechanism for generation of neo-epitopes was specified. It can be speculated that, under conditions where undigested gliadin peptides have access to the circulation due to a barrier disturbance, these peptides can potentially be cross-linked to proteins in organs where TG2 is available for the reaction. In this way, gliadin peptides may also be an initiating factor for other autoimmune diseases.

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