High Selectivity of Human Tissue Transglutaminase for Immunoactive Gliadin Peptides: Implications for Celiac Sprue†

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ABSTRACT: Celiac Sprue is an HLA DQ2 (or DQ8)-associated autoimmune disorder of the human small intestine that is induced by dietary exposure to wheat gliadin and related proteins from barley, rye, and possibly other food grains. Recently, tissue transglutaminase (tTGase)-catalyzed deamidation of gliadin peptides has been shown to increase their potency for activating patient-derived, gliadin-specific T cells, suggesting that tTGase plays a causative role in the onset of an inflammatory response to toxic food grains. To dissect the molecular recognition features of tTGase for gluten derived peptides, the regioselectivity and steady-state kinetics of tTGase-catalyzed deamidation of known immunogenic peptides were investigated. The specificity of recombinant human tTGase for all immunogenic peptides tested was comparable to and, in some cases, appreciably higher than the specificity for its natural substrate. Although each peptide was glutamine-rich, tTGase exhibited a high degree of regioselectivity for a particular glutamine residue in each peptide. This selectivity correlated well with Q → E substitutions that have earlier been shown to enhance the immunogenicity of the corresponding gliadin peptides. The specificity of tTGase toward homologues of PQPQLPY, a sequence motif found in immunodominant gliadin peptides, was analyzed in detail. Remarkably, the primary amino acid sequences of wheat-, rye-, and barley-derived proteins included many single-residue variants of this sequence that were high-affinity substrates of tTGase, whereas the closest homologues of this sequence found in rice, corn, or oat proteins were much poorer substrates of tTGase. (Rice, corn, and oats are nontoxic ingredients of the Celiac diet.) No consensus sequence for a high-affinity substrate of tTGase could be derived from our data, suggesting that the secondary structures of these food-grain peptides were important in their recognition by tTGase. Finally, under steady-state turnover conditions, a significant fraction of the tTGase active site was covalently bound to a representative high-affinity immunogenic gliadin peptide, suggesting a common mechanism by which cells responsible for immune surveillance of the intestinal tract recognize and generate an antibody response against both gliadin and tTGase. In addition to providing a quantitative framework for understanding the role of tTGase in Celiac Sprue, our results lay the groundwork for the design of small molecule mimetics of gliadin peptides as selective inhibitors of tTGase.

Celiac Sprue is an HLA DQ2 (or DQ8)-associated autoimmune disorder of the human small intestine that is induced by dietary exposure to wheat gliadin[1] and related proteins from barley, rye, and possibly other food grains (*2*, *3*). HLA-mediated presentation of gliadin-derived peptides to T cells leads to T cell stimulation as well as the secretion of specific antibodies against gliadin (*4*−*6*) and tissue transglutaminase (tTGase) (*7*). The inflammatory response leads to a flattening of the intestinal villi and disruption of the surface epithelium, the hallmarks of Celiac Sprue. In turn, this produces clinical symptoms, such as chronic diarrhea, malabsorption of nutrients, weight loss, abdominal distension, anemia, and fatigue, and a substantially enhanced risk of osteoporosis and intestinal malignancies (lymphoma and carcinoma) (*8*, *9*).

Although much remains to be clarified regarding the pathogenesis of Celiac Sprue, there is mounting evidence that tTGase may play a key role in this process (Figure 1). Specifically, tTGase-catalyzed deamidation of gliadin peptides has been shown to increase their potency for activating patient-derived, gliadin-specific T cells (*10*−*14*). The biological relevance of this deamidation process has been highlighted by two recent reports in which a short segment of α-gliadin was identified as the immunodominant epitope, accounting for most of the stimulatory activity of dietary gluten on intestinal and peripheral CD4+ T lymphocytes derived from patients (*12*, *14*). In both studies, regiospecific deamidation of this peptide by tTGase was essential for its recognition by T cells. In the immunodominant peptides

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[1] Footnotes: [1]Gliadin is the alcohol-soluble fraction of wheat gluten. It is composed primarily of three major proteins, α-, *β*-, and *ω*-gliadin. The primary amino acid sequences of gliadins derived from different strains of wheat show slight variations. For more information on gliadin structure, see a review by Wieser (*1*).

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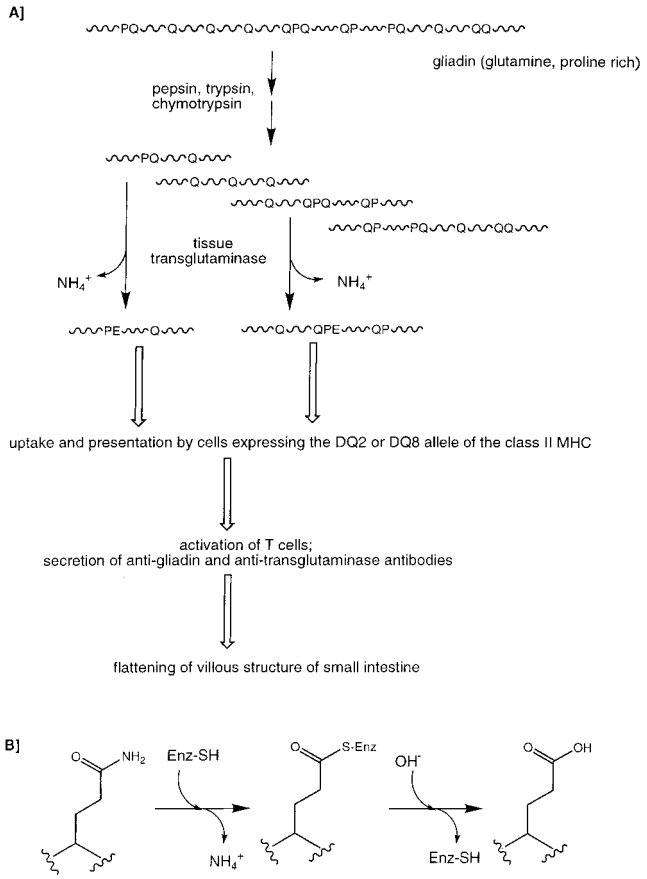

FIGURE 1: Role of tTGase in Celiac Sprue pathogenesis. (A) Proteolytic fragments of gliadin are deamidated by tTGase. These deamidated fragments are presented to T cells by antigen-presenting cells expressing the DQ2 (or DQ8) allele of the MHCII heterodimer. This leads to activation of T and B cells, secretion of anti-gliadin and anti-tTGase antibodies, and the eventual destruction of the small intestinal villous structure. (B) Mechanism of deamidation of a peptide-borne glutamine residue by tTGase. The active site of the enzyme has a cysteine thiol that forms a transient thioester intermediate during the catalytic cycle.

PQPQLPYPQPQLPY (from α-2 gliadin) and QLQPF-PQPQLPY (from α-9 gliadin), identified by Sollid and co-workers (*12*), deamidation occurred at Q11 and Q9 (underlined), respectively, whereas the peptide QLQPFPQPQLP-YPQPQS, identified by Anderson and co-workers (*14*) was deamidated at Q9 (underlined). Thus, in each case, it appeared that the deamidation activity of tTGase was directed toward the core sequence PQPQLPY.

The finding that a single gliadin-derived peptide is a substrate of tTGase and is potentially responsible for initiating the T cell response associated with Celiac Sprue may have implications for both prevention and treatment of the disease (*15*). However, before such possibilities can be realized, the relationship between tTGase specificity and immunodominance must be established in quantitative terms. More generally, the recognition features of tTGase for peptides derived from gliadins and related food-grain proteins remain to be systematically investigated. Here, we investigate the catalytic and allosteric regulatory properties of tTGase as they pertain to the pathogenesis of Celiac Sprue.

EXPERIMENTAL PROCEDURES

Expression and Purification of tTGase. The cDNA encoding human tissue transglutaminase (*16*), a gift from P. J. A. Davies, was cloned into pET-28a (Novagen, Madison, WI) between the *Nde*I and *Hind*III sites. The resulting plasmid, pJLP4, was expressed in BL21(DE3) cells (Novagen). A 5 mL inoculum was grown at 37 °C for 12−16 h in the presence of 50 μg/mL kanamycin. The preculture was used to inoculate 1 L of LB medium, also containing 50 μg/mL kanamycin. After growth at 37 °C to a stage where A_{600} = 0.6, the culture was induced by the addition of IPTG (1 μM) and incubated at 20 °C for 20 h.

Purification of Recombinant Human tTGase. Escherichia coli cells expressing recombinant tTGase were centrifuged at 3700 rpm for 20 min. All chemicals were from Sigma (St. Louis, MO) unless otherwise noted. The pellet was resuspended in a buffer containing 50 mM NaH_2PO_4 (Mallinckrodt, Phillipsburg, NJ) and 300 mM NaCl (pH 8.0) (buffer A; Mallinckrodt) and lysed in a French press. After centrifugation at 42 000*g* at 4 °C for 45 min, the supernatant containing the tTGase was harvested and applied to a column containing 0.75 mL of Ni−NTA resin (Qiagen, Valencia, CA). The column was rinsed with buffer A until no protein was detected in the wash by Bradford assay (Bio-Rad, Hercules, CA). tTGase was then eluted in a buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, and 150 mM imidazole (pH 8.0). Fractions containing tTGase were pooled and exchanged into buffer B (20 mM Tris HCl (Gibco Invitrogen Corporation, Carlsbad, CA), 1 mM DTT, and 1 mM EDTA (pH 7.2)). The protein was purified further on a HiTrap Q column using a 0−1 M NaCl gradient in buffer B. tTGase eluted at ∼300 mM NaCl and was exchanged into buffer B with 150 mM NaCl. Glycerol was added to a final concentration of 10%, and the protein was flash-frozen in liquid nitrogen and stored as aliquots of 500 μL at −80 °C.

Kinetic Analysis of tTGase. Although the natural reaction catalyzed by tTGase is a transamidation reaction, in this study, the reaction being monitored is the deamidation reaction (i.e., the conversion of glutamine into glutamic acid). tTGase-catalyzed deamidation activity was measured via a coupled assay previously developed by Keillor and Day (*17*), where glutamate dehydrogenase (GDH; Biozyme, San Diego, CA) is used to couple the ammonium ion from the tTGase-catalyzed deamidation to α-ketoglutarate to yield glutamate. The concomitant oxidation of NADH to NAD^+, as shown in the following reaction, can be spectrophotometrically monitored at 340 nm:

$$\text{NADH} + \text{NH}_4^+ + \text{α-ketoglutarate} \rightarrow \text{glutamate} + \text{NAD}^+ + \text{H}_2\text{O}$$

The standard assay, optimized for human tTGase, utilized the following freshly prepared stock solutions: (1) MOPS buffer solution: a 5 × stock of 200 mM MOPS, 5 mM $CaCl_2$ (Mallinckrodt), 1 mM Na_4EDTA, and 10 mM α-ketoglutarate (pH 7.2); (2) NADH stock solution: 1.25 mM NADH; (3) GDH stock solution: 0.25 units of GDH/μL in a MOPS buffer; and (4) 300 mM benzyloxycarbonyl-glutaminyl-glycine (Z-Gln-Gly; Sigma) in water. A 100 μL portion of the NADH stock solution was combined with 64 μL of the MOPS buffer solution, 36 μL of the GDH stock solution, a specified amount of Z-Gln-Gly, and water to a final volume of 420 μL. The reaction was started by adding tTGase (50 μg in a final volume of 80 μL) to the assay mixture, and

NADH consumption was monitored at 30 °C for 20 min. The reaction velocity was calculated from the linear portion of the progress curve, assuming a molar extinction coefficient for NADH of 6220 M^{-1} cm^{-1}. To confirm that the reaction rate is independent of the GDH concentration, Z-Gln-Gly was held at a constant concentration while the GDH concentration was varied between 1 and 9 units/reaction. The rate of NADH oxidation was independent of the GDH concentration at 7 units/reaction, but 9 units/reaction was chosen to allow for sufficient GDH activity under all conditions. The concentration of tTGase was determined by densitometry with bovine serum albumin (Calbiochem, San Diego, CA) as the reference.

The effects of Ca^{2+}, GTP, and DTT on tTGase enzyme kinetics were determined using the coupled assay previously described. A 4 × 4 plot was obtained for Ca^{2+} by varying the concentration of Ca^{2+} between 2 and 5 mM while Z-Gln-Gly was varied between 3.6 and 36 mM. Similarly, a 4 × 4 plot was obtained for the nonhydrolyzable GTP analogue GMP−PNP (Boehringer Mannheim, Indianapolis, IN) in the concentration range of 5−60 μM for GMP−PNP and 6−36 mM for Z-Gln-Gly. The data were plotted as $1/V$ versus 1/[S], and the $k_{cat,app}$ and $K_{M,app}$ were determined from the y and x intercepts. From this, the activation or inhibition constants were calculated according to the formulas $k_{cat,app} = k_{cat}(1 + [Ca^{2+}]/k_a)$, $K_{M,app} = K_M/(1 + [Ca^{2+}]/K_a)$, and $K_{M,app} = K_M(1 + [GMP-PNP]/K_i)$. The effects of DTT on kinetics were also determined by preparing tTGase in buffer B without DTT. This tTGase was then used in the coupled assay with Z-Gln-Gly.

The parameters k_{cat} and K_M were measured for known immunogenic gliadin peptides by replacing Z-Gln-Gly in the coupled assay with the peptide. The gliadin peptides used included QLQPFPQPQLPYPQPQS, QLQPFPQPELPYPQPQS, PQPQLPYPQPQLPY, PQPELPYPQPQLPY, PQPQLPYPQPELPY, QLQPFPQPQLPY, QLQPFPQPELPY, and PQQPQQSFPQQQRP (*10*−*12*, *14*). As a comparison, a natural substrate for tTGase, the γ-fibrinogen peptide (TIGEGQQHHLG (*18*)), was also used in the kinetic assay. To analyze the structural basis of tTGase specificity for the core PQPQLPY sequence, several derivatives of this peptide (Table 2) were also assayed. The peptides, synthesized at the Stanford Protein and Nucleic Acid facility, were purified via preparative HPLC and verified to be >90% pure by analytical HPLC and mass spectrometry. The following gradient was used on a Vydac 214MS5415 column for all HPLC experiments: 0−25% of B over 5 min, 25−40% of B over 40 min, 40−100% of B over 1 min, 100% of B for 8 min, 100−0% of B over 1 min, and 0% of B for 7 min. In each instance, the remainder of the percentage was made up of solution A (solution A = H_2O/0.1% TFA (J. T. Baker, Phillipsburg, NJ) and solution B = 80% acetonitrile (Mallinckrodt)/0.1% TFA). The parameters k_{cat} and K_M were calculated by fitting V versus [S] data to the Michaelis−Menten equation.

Regioselectivity of tTGase. Because most peptides assayed in this study contained multiple glutamine residues, the regioselectivity of tTGase-catalyzed deamidation was monitored by quenching the enzyme reaction at 1−5 turnovers, 10−50 turnovers, and >100 turnovers (as determined by k_{cat} data) via the addition of iodoacetamide to a final concentration of 20 mM. The reaction mixture was applied to a preparative reversed-phase HPLC column. Fractions collected from HPLC were submitted to the Stanford Mass Spectrometry Facility for MS/MS identification of the deamidated glutamine residue(s) in each peptide using an electrospray instrument (Finnigan, San Jose, CA).

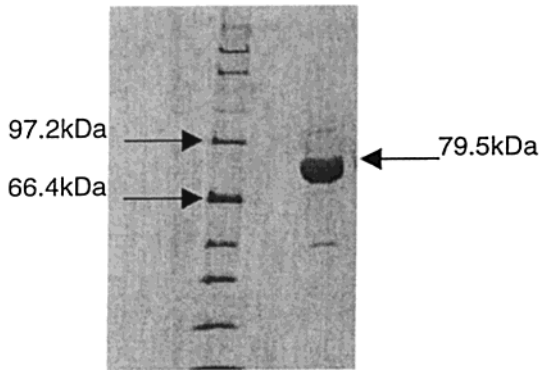

FIGURE 2: Purified recombinant human tissue transglutaminase (for details, see text).

Determination of Active Site Occupancy. Peptide PQPQLPYPQPQLPY was acetylated by ^{14}C-labeled acetic anhydride (54 mCi/mmol; ICN, Irvine, CA). The reaction was initiated by mixing 3.4 μL of *N*-methylimidazole, 39.6 μL of tetrahydrofuran (THF), and 71.6 μL of PQPQLPYPQPQLPY (2.0 mg/mL and suspended in H_2O) with 0.9 μL of acetic anhydride, 40.4 μL of THF, and 1.7 μL of 2,6-lutidine. The reaction was allowed to run for 10 min at 24 °C (room temperature) and was stopped by adding 14.3 μL of 5 M NaOH (J. T. Baker). The reaction was then mixed with 3 mL of H_2O with 0.1% TFA and applied to a C18 Sep-Pak column (Waters, Milford, MA). The column was equilibrated with 3 mL of H_2O with 0.1% TFA, and the peptide was eluted with 4 mL of 80:20 acetonitrile/H_2O with 0.1% TFA. The yield of [^{14}C]-acetyl-PQPQLPYPQPQLPY was 96%; the balance of the peptide was assumed to remain unreacted by analogy with the results from a similar reaction between nonradioactive acetic anhydride and PQPQLPYPQPQLPY. The eluate was concentrated to 800 μL, of which 100 μL was incubated at 30 °C with 25 μL of a MOPS buffer solution (as described previously) and 3 μL of tTGase (6.6 μg/μL). After 2, 4, and 6 min, 10 μL aliquots of the reaction mixture were removed, mixed with 5 μL of an SDS sample buffer (New England Biolabs, Beverly, MA), and subjected to SDS−PAGE. Alternatively, 25 μL of the reaction mixture was brought to pH 5.5 by the addition of 3 μL of 0.5 M HCl. A 10 μL portion of this sample was mixed with 5 μL of an SDS sample buffer and subjected to SDS−PAGE. This gel was stained and dried, and the radioactivity associated with tTGase was quantified on a Packard InstantImager.

RESULTS

Expression, Purification, and Assay Development. Under optimal expression conditions, the yield of purified tTGase is 5−8 mg/L. By use of the two-step purification process, a Ni−NTA column followed by Hi-Trap Q column, the purity of recombinant tTGase was >90% (Figure 2). Optimal conditions for Z-Gln-Gly deamidation in the coupled assay of Keillor and Day (*17*) (originally developed with guinea pig transglutaminase) were established for use with human tTGase. The reaction conditions were modified to maximize

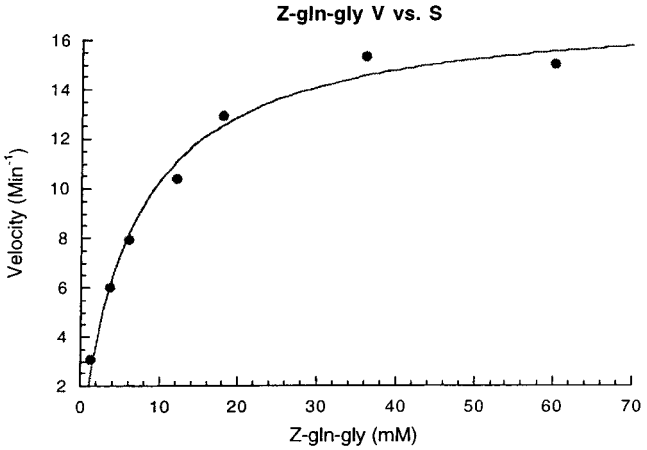

FIGURE 3: Kinetic data for Z-Gln-Gly plotted on a *V* versus [S] plot. (●) Experimental data (−) Michaelis−Menten best fit.

k_{cat} for tTGase with Z-Gln-Gly as the reference substrate. A comparison of reaction velocities in 200 mM MOPS, 100 mM Tris HCl, and 200 mM phosphate buffers (all at pH 7.0) showed that the k_{cat} in 100 mM Tris HCl was 26% lower than that in 200 mM MOPS, whereas tTGase had no detectable activity in 200 mM phosphate buffer. The activity of tTGase in 200 mM MOPS buffer was measured in the pH range of 6.0−7.8, and was found to be optimal at pH 7.2 (data not shown). Measurement of *V* versus [Z-Gln-Gly] (Figure 3) revealed a k_{cat} of 17.2 min^{-1} and a K_M of 6.8 mM; these parameters compare well with those measured by Keillor and Day (k_{cat} = 34.0 min^{-1}, K_M = 4.1 mM) for guinea pig transglutaminase (*17*).

Dependence on Ca^{+2}*, GTP, and DTT.* Previous experiments have shown that tTGase activity is dependent on Ca^{2+} (*19*). Moreover, the enzyme also has GTPase activity (*20, 21*). To quantify the dependence of tTGase activity on Ca^{2+} and the nonhydrolyzable GTP analogue GMP−PNP, their concentrations were varied together with those of Z-Gln-Gly to generate 4 × 4 plots (Figure 4A,B). Analysis of these data shows that Ca^{2+} affects both the k_{cat} and K_M of tTGase, with a k_a of 1.3 ± 0.3 mM and a K_a of 1.0 ± 0.3 mM. (k_a and K_a represent the noncompetitive and competitive components of activation, respectively; see the Experimental Procedures section for definitions.) GMP−PNP, on the other hand, is a competitive inhibitor of tTGase activity with a K_i of 20 ± 5 μM. It should be noted that earlier studies with guinea pig liver and rat liver transglutaminases showed that GTP was a noncompetitive inhibitor of those enzymes (K_i = 90−150 μM) (*22*). The reason for this discrepancy is unclear and may be due to either the different sources of the enzyme or the different inhibitors used.

Kinetic Analysis of Immunogenic Gliadin Peptides. Several gliadin-derived peptides have been identified as immunogenic in assays involving ex vivo organ culture systems or T cell clones derived therefrom (*10*−*14*). In each case, deamidation of these peptides by guinea pig tTGase was shown to enhance their immunogenicity. For assessment of the relative specificity of tTGase for these and other biologically relevant peptides, steady-state kinetic data was collected for each peptide and fit to the Michaelis−Menten equation. It should be noted that although the natural reaction catalyzed by tTGase involves transamidation, in this study, the kinetics of the deamidation reaction were measured. (Because the primary goal of this study was to probe the specificity of human tTGase toward gliadin peptides and because the specificity constant (k_{cat}/K_M) only incorporates kinetic parameters up to the first irreversible step (ammonia release, which is common to both the deamidation and transamidation pathways), the substrate specificity of the two pathways can be expected to be indistinguishable.)

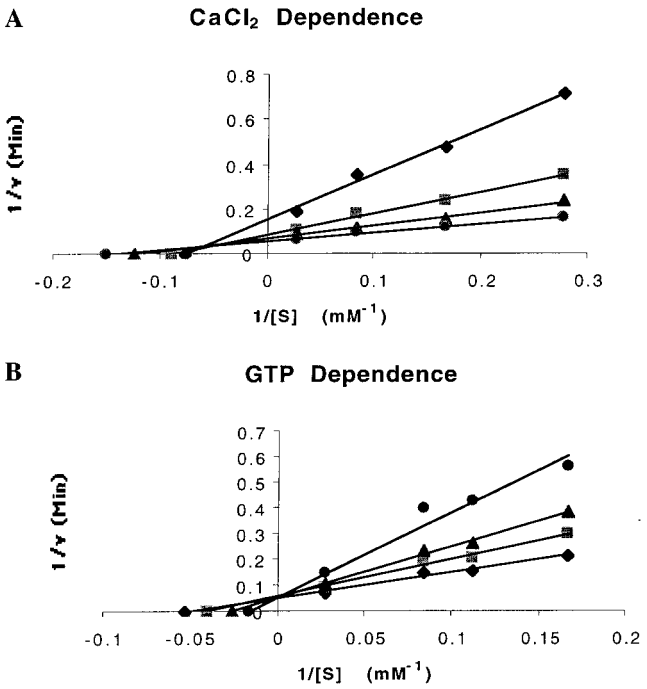

FIGURE 4: (A) Activation data for $CaCl_2$ plotted on a 4 × 4 1/*V* versus 1/[S] plot: (◆) 2 mM $CaCl_2$, (■) 3 mM $CaCl_2$, (▲) 4 mM $CaCl_2$, and (●) 5 mM $CaCl_2$. (B) Inhibition data for GMP−PNP plotted on a 4 × 4 1/*V* versus 1/[S] plot: (●) 60 μM GMP−PNP, (▲)30 μM GMP−PNP, (■) 15 μM GMP−PNP, and (◆) 5 μM GMP−PNP.

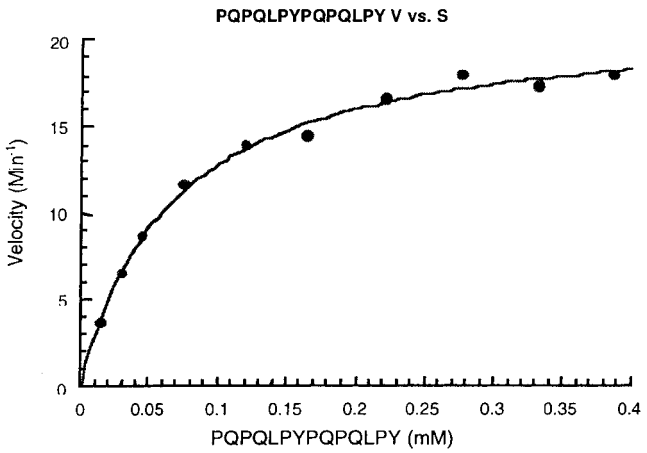

FIGURE 5: Kinetic data for PQPQLPYPQPQLPY plotted on a *V* versus [S] plot: (●) experimental data, (−) Michaelis−Menten best fit.

Figure 5 shows sample *V* versus [S] data for PQPQLPYPQPQLPY, and Table 1 summarizes the kinetic constants for all peptides. Several points are noteworthy. First, tTGase has comparable specificity for immunogenic gliadin peptides and its natural substrate (TIGEGQQHHLG; the cross-linking site of γ-fibrinogen (*18*)). In particular, the overall specificity (k_{cat}/K_m) of tTGase is appreciably higher toward the immunodominant peptides PQPQLPYPQPQLPY (*12*) and QLQPF-

Table 1: Summary of Kinetic Data

	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M ($min^{-1}\,mM^{-1}$)
Z-Gln-Gly	17 ± 0.6	6.8 ± 0.8	2.5
TIGEGQQHHLG	29 ± 1.9	0.5 ± 0.1	63
QLQPFPQPQLPYPQPQS	7 ± 0.9	0.03 ± 0.01	260
QLQPFPQPELPYPQPQS[a]			<1.6
PQPQLPYPQPQLPY	21 ± 0.5	0.07 ± 0.01	300
PQPELPYPQPQLPY[a]	14 ± 1.2	0.1 ± 0.02	140
PQPQLPYPQPELPY[a]	51 ± 5	0.4 ± 0.1	116
QLQPFPQPQLPY	23	0.35	66
QLQPFPQPELPY[a]			<0.6
PQQPQQSFPQQQRP	30	0.5	61

[a] These peptides are synthetic forms of the deamidated products of the gliadin-derived peptides shown in this table.

Table 2: Kinetic Data for PQPQLPY and Derivatives

	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M ($min^{-1}\,mM^{-1}$)
PQPQLPY	28 ± 1.7	0.3 ± 0.1	82
PQPQLPF	28 ± 0.6	0.4 ± 0.03	72
PRPQLPY	9.9 ± 0.5	0.4 ± 0.05	24
PQPQQPY	29 ± 2.0	0.4 ± 0.08	66
SQPQLPY	27 ± 1.2	0.3 ± 0.04	96
PQPQLSY	27 ± 1.2	0.3 ± 0.03	87
PQPNLPY[a]	−	−	<4
PQPQ[a]	−	−	<1
PQPQQPP[b]	−	−	1
PQPQAPS[b]	−	−	7

[a] No tTGase activity was detectable in the presence of these substrates up to their solubility limits. The reported k_{cat}/K_M values were estimated from the background (uncatalyzed) rate of NADH oxidation and from the concentration of each peptide under saturation conditions and are upper limits for k_{cat}/K_M. [b] Although tTGase activity could be detected in the presence of these substrates, neither substrate was able to saturate the enzyme. The reported k_{cat}/K_M values were estimated from the slopes of the V versus [S] curves.

PQPQLPYPQPQS (*14*) from gliadin as compared to its cognate substrate. Second, the enhanced selectivity of tTGase toward gliadin peptides primarily results from a low K_M, indicating that gliadin peptides are high-affinity ligands for the substrate binding site of tTGase. Third, tTGase-catalyzed deamidation of gliadin peptides appears to be highly regioselective, as illustrated by the lack of activity of tTGase against the peptide products of tTGase (QLQPFPQPELPYPQPQS and QLQPFPQPELPY), in which single glutamine residues were replaced by glutamic acid. Direct evidence for this regiospecificity is provided in the following paragraphs. Finally, the products of tTGase-catalyzed deamidation do not inhibit the enzyme ($K_{i,product}$ > 0.3 mM for QLQPFPQPELPYPQPQS and QLQPFPQPELPY; data not shown).

Most of the high-affinity substrates shown in Table 1 contain the consensus sequence PQPQLPY. To dissect the structural basis for specificity toward this consensus sequence, we analyzed tTGase activity against a series of single-residue substitutions of this peptide (Table 2). The choice of these residue changes was based on an exhaustive analysis of the amino acid sequences of all known gliadins, hordeins, and secalins in the NCBI database. (Gliadins, hordeins, and secalins are homologous families of food-grain proteins in wheat, rye and barley, respectively, that are known causative agents of the inflammatory response associated with Celiac Sprue.) Every naturally occurring point variation of PQPQLPY in these storage proteins was synthesized and assayed. They include PQPQLPF (gliadin), PRPQLPY (gliadin), PQPQQPY (secalin), SQPQLPY (gliadin), and PQPQLSY (gliadin). As summarized in Table 2, the activity of tTGase is fairly tolerant to point substitutions in the consensus sequence by neutral residues. In contrast, replacement of the reactive glutamine with asparagine (as in PQPNLPY) or further truncation of the consensus sequence (as in PQPQ) results in the abolition of tTGase activity. The latter observation is consistent with the data in Table 1, where the peptide QLQPFPQPELPYPQPQS is not recognized by tTGase, even though it contains a second PQPQ motif.

Given the ability of tTGase to recognize derivatives of PQPQLPY, the amino acid sequences of storage proteins in rice, corn, and oats were searched for sequences that contained similar sequences to PQPQLPY. By use of the current data to determine appropriate substitutions, two sequences present in rice gluten, PQPQQPP and PQPQAPS (NCBI accession numbers AAK20052 and BAB21167, respectively), were identified as potential tTGase substrates. Although tTGase did show measurable deamidation activity on these two peptides, its specificity is more than an order of magnitude lower for these peptides as compared to PQPQLPY (Table 2).

Direct Evidence for Regioselectivity of Deamidation. The high specificity for a particular glutamine residue in the tTGase-catalyzed deamidation of immunogenic gliadin peptides is remarkable, considering that these peptides are glutamine-rich. To obtain direct evidence for this regioselectivity, we incubated the gliadin peptides from Table 1 with tTGase for 1−5, 10−50, or >100 turnovers before quenching with iodoacetamide. Analysis of the deamidation products at different points along the progress curve allowed for the determination of the relative preference of individual deamidation sites in peptides containing multiple glutamine residues. For example, Figure 6 shows the results of LC−MS analysis for PQPQLPYPQPQLPY. Peak A corresponds to the original peptide, the primary product; peak B is PQPQLPYPQPELPY; and the final product, peak C, is PQPELPYPQPELPY. As summarized in Table 3, in all cases where the PQPQLPY consensus sequence was present, the second glutamine residue was regiospecifically deamidated. In contrast, the immunogenic peptide PQQPQQSFPQQQRP was deamidated at two positions. Both sites lie within the PQQQ motif; the preferred site of deamidation is the third glutamine residue in this motif, whereas the first glutamine residue is the secondary site of deamidation. Notably, no deamidation of either the PQQP or the PQQS motif was observed. Similarly, as predicted from the results of Table 1, the immunodominant peptide QLQPFPQPQLPYPQPQS was deamidated at a unique single position (underlined in Table 3) where the LPY sequence followed. The terminal PQPQS motif was unmodified.

Determination of Steady-State Active Site Occupancy. Previous studies have shown that the immune response elicited in Celiac Sprue is at least partially directed against tTGase via the secretion of anti-tTGase antibodies (*7*). The reason for this autoimmune response against tTGase is unclear, but the formation of relatively stable, covalent tTGase−gliadin peptide complexes upon dietary exposure to gluten may lead to the misidentification of tTGase as a non-self-entity by the immune system. To test whether

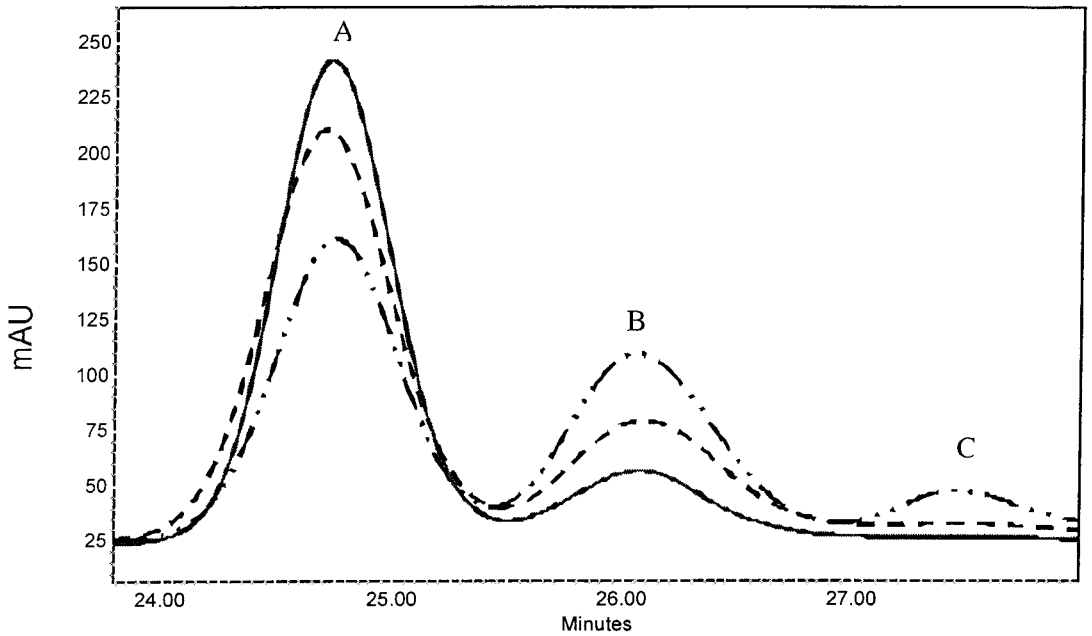

FIGURE 6: LC−MS monitoring of the deamidation reaction for PQPQLPYPQPQLPY. The original peptide PQPQLPYPQPQLPY (peak A) gets regioselectively deamidated to yield PQPQLPYPQPELPY (peak B). PQPQLPYPQPELPY is then further deamidated to yield PQPELPYPQPELPY (peak C): (solid) 1−5 turnovers, (dashed) 10−50 turnovers, and (dot-dashed) over 100 turnovers.

Table 3: Summary of Deamidation Sites

gliadin peptide	deamidation product 1	deamidation product 2
QLQPFPQPQLPYPQPQS	QLQPFPQPELPYPQPQS	
PQPQLPYPQPQLPY	PQPQLPYPQPELPY	PQPELPYPQPELPY
QLQPFPQPQLPY	QLQPFPQPELPY	
PQQPQQSFPQQQRP	PQQPQQSFPQQERP	PQQPQQSFPEQERP

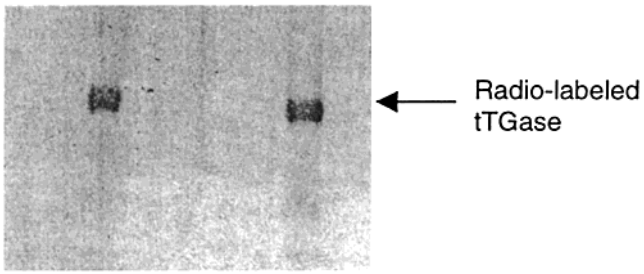

FIGURE 7: Detection of tTGase-bound covalent intermediate with ^{14}C-acetylated-PQPQLPYPQPQLPY.

tTGase−gliadin peptide adducts are likely to accumulate under ordinary steady-state turnover conditions in the human intestine, [^{14}C]-acetyl-PQPQLPYPQPQLPY was incubated with recombinant tTGase as described in the Experimental Procedures section. (In a control experiment, the specificity of tTGase for PQPQLPYPQPQLPY and unlabeled acetyl-PQPQLPYPQPQLPY was found to be comparable; data not shown.) Figure 7 shows the labeling of tTGase by [^{14}C]-acetyl-PQPQLPYPQPQLPY (80 μM). By counting the radioactivity associated with each band, an average active site occupancy of 14% was calculated, which corresponds to a k_{on}/k_{off} of 2 mM^{-1}. Because tTGase is known to be associated with the intestinal epithelium (*23*), at typical physiological concentrations of 0.1−1.0 mM gliadin in the upper small intestine after a gluten-containing meal, a substantial fraction of this tTGase can be expected to be covalently associated with gliadin peptides. The abundance of the peptide−tTGase adduct was relatively unperturbed by pH between the pH range of 5.5−7.2 (data not shown).

DISCUSSION

Transglutaminases (also known as γ-glutamyl transpeptidases) are widely distributed enzymes in the human body (*24*). The catalytic mechanisms of prototypical transglutaminases have been well studied (*25*−*27*). Since the discovery that tissue transglutaminase (tTGase; also known as transglutaminase C) is a major autoantigen in Celiac Sprue (*7*), a growing body of results suggests that this enzyme is actively involved in the pathogenesis of the disease. Specifically, it has been suggested that tTGase catalyzes deamidation of gliadin peptides, which in turn enhances their immunogenicity as defined by their ability to induce T cell proliferation (*10*−*14*). To the extent that tTGase plays a causative role in the inflammatory process, it may be possible to attenuate inflammation associated with Celiac Sprue by inhibition of this enzyme. Understanding the molecular recognition features of tTGase is an important prerequisite for this purpose. Here, we have quantified and dissected the specificity of tTGase toward gliadin peptides. Our studies have focused on the recently identified immunodominant peptides from wheat gliadins, all of which contain the consensus sequence PQPQLPY.

Parenthetically, our results shed some light on two unusual properties of human tTGase. First, although the protein encoded by the tTGase cDNA lacks a signal sequence and is not glycosylated (*16*, *28*−*30*), its primary biological role appears to be in the cross-linking of extracellular matrix proteins such as fibronectin, implying that the enzyme must somehow be translocated to the extracellular space of many cell types.The mechanism of this translocation process

remains a mystery (*24*). Second, in addition to catalyzing the formation of isopeptide bonds in the extracellular matrix, intracellular tTGase also plays the role of a G protein in signal transduction by the α-adrenergic receptor (*20*). The mechanism by which the same protein performs two very different functions in two different cellular milieus is unknown. Here, we have quantified the stimulatory effect of Ca^{2+} and the inhibitory effect of the nonhydrolyzable GTP analogue GMP−PNP on tTGase activity (Figure 3). In mammalian cells, the intracellular concentration of GTP is 200−500 μM (*31*), while the intracellular and extracellular concentrations of Ca^{2+} are <1 μM and 2.5−5.0 mM, respectively (*32*). Therefore, the abundance of GTP (K_i = 20 μM) and lack of Ca^{2+} (k_a = 1.0 mM and K_a = 1.3 mM) inside the cell should drastically reduce the tTGase activity and allow the protein to function as a GTPase. (Under apoptotic conditions, however, the intracellular concentrations of Ca^{2+} and GTP presumably undergo a reversal, thereby allowing the intracellular tTGase to play a catalytic role in a dying cell (*24*).) In contrast, tTGase activity can be expected to be high once the protein is exported into the extracellular space where GTP is negligible and the Ca^{2+} concentration is >1 mM. Thus, a quantitative analysis of the allosteric effects of both calcium and GTP confirm that the biological role of tTGase depends on the subcellular localization of the protein. (They do not, however, shed light on the mechanism of translocation of this enzyme.)

The primary goal of this study was to investigate the role of tTGase in Celiac Sprue. Toward this end, analysis of the substrate specificity of tTGase (Tables 1 and 2) revealed that variations in the catalytic efficiency (k_{cat}/K_M) are primarily a function of variations in K_M, with there being only modest changes in k_{cat}. Thus, gliadin peptides are excellent substrates for tTGase because their ground-state conformation appears to be well-matched to the substrate-binding pocket of the enzyme. Indeed, it is remarkable that not only are the immunodominant peptides from gliadin excellent substrates for tTGase (up to 5-fold higher specificity than for the natural substrate; Table 1) but that the deamidation products are appreciably better ligands for DQ2 (the disease associated class II MHC) than the parent gliadin peptides themselves (*10*−*14*). For example, the IC_{50} for binding to DQ2 was ∼7-fold lower for PQPELPYPQPELPY than that for PQPQLPYPQPQLPY (α-gliadin, residues 62−75) and was >10-fold lower for QLQPFPQPELPY than that for QLQPFPQPQLPY (α-gliadin, residues 57−68) (*12*). Similarly, in T cell activation assays, the γ-gliadin peptide PQQPQQSFPQQQRP (residues 139−152) became 50-fold more immunogenic following the deamidation of residues 148 and 150 [10]. Therefore, a more refined correlation between the specificity of tTGase for gliadin, hordein, and secalin peptides, the affinity of the corresponding products for DQ2, and possibly even the T cell activating potential of the resulting peptide−MHC complex could provide a valuable framework for understanding immunodominance at a quantitative level. Moreover, such structure−activity relationships might provide a quantitative and practical method for assessing the toxicity of various food grains and processed foods associated with the Celiac diet. For example, proteins present in rice gluten contain sequences such as PQPQQPP and PQPQAPS (NCBI accession numbers AAK20052 and BAB21167, respectively), although rice is not an inflammatory food grain for patients suffering from Celiac Sprue. Our results demonstrate that tTGase specificity for these rice gluten-derived peptides is more than 9-fold lower than that for the natural substrate and more than 45-fold lower than that for the best gliadin substrates. Thus, there appears to be a correlation between the nontoxic nature of food-grain peptides and their low specificity for tTGase. These results strengthen the argument that tTGase plays a causative role in the inflammatory process associated with Celiac Sprue.

Our assessment of active site occupancy of tTGase indicates that the immunodominant peptide PQPQLPYPQPQLPY can occupy a significant fraction of available tTGase active sites under steady-state conditions (Figure 7). This is in contrast to the behavior of typical intestinal proteases and peptidases, where acylation is rate-limiting and the steady-state concentration of the E−S covalent adduct is low (*33*). Stable occupancy of the active site of tTGase by gliadin peptides suggests a common mechanism for inducing an immune response against both gliadin and tTGase, two of the most diagnostic features of Celiac Sprue (*6*, *7*). Specifically, cells responsible for immune surveillance of the intestinal tract may be able to internalize these gliadin−tTGase complexes, leading to DQ2-mediated production of anti-gliadin and anti-tTGase antibodies and the onset of an inflammatory response. If so, understanding the precise cellular location and molecular consequences of the encounter between gliadin and tTGase could yield fundamentally new insights into the pathogenesis of Celiac Sprue.

In summary, this study has attempted to lay the groundwork for the design of small molecule mimetics of gliadin peptides as selective inhibitors of tTGase. Although the consensus sequence PQPQLPY from the immunodominant peptides was validated as a high-affinity substrate for TGase, no universal consensus sequence could be unambiguously derived from the results in Tables 1 and 2. This suggests that either peptides can adopt multiple high-affinity conformations in the TGase active site or that, notwithstanding primary sequence differences, the high-affinity gliadin peptides reported here adopt defined conformations in which a unique glutamine residue is presented to the active site cysteine of tTGase. The observed regiospecificity in Table 3 provides strong support for the latter hypothesis. If so, a better understanding of these three-dimensional conformational constraints should facilitate the design of tTGase inhibitors. In turn, such inhibitors might allow for a definitive evaluation of whether tTGase activity is necessary for stimulating the autoimmune response associated with Celiac Sprue.

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