Equilibrium and Kinetic Analysis of the Unusual Binding Behavior of a Highly Immunogenic Gluten Peptide to HLA-DQ2[†]

Jiang Xia,[‡] Ludvig M. Sollid,[§] and Chaitan Khosla*,[‡,||,⊥]

Departments of Chemistry, Chemical Engineering, and Biochemistry, Stanford University, Stanford, California 94305, and Institute of Immunology, Rikshospitalet University Hospital, University of Oslo, Oslo, Norway

Received October 21, 2004; Revised Manuscript Received January 9, 2005

ABSTRACT: HLA-DQ2 predisposes an individual to celiac sprue by presenting peptides from dietary gluten to intestinal $CD4^+$ T cells. A selectively deamidated multivalent peptide from gluten (LQLQPF-PQPE<u>L</u>PYPQPE<u>L</u>PYPQPE<u>L</u>PYPQPQPF; underlined residues correspond to posttranslational Q → E alterations) is a potent trigger of DQ2 restricted T cell proliferation. Here we report equilibrium and kinetic measurements of interactions between DQ2 and (i) this highly immunogenic multivalent peptide, (ii) its individual constituent epitopes, (iii) its nondeamidated precursor, and (iv) a reference high-affinity ligand of HLA-DQ2 that is not recognized by gluten-responsive T cells from celiac sprue patients. The deamidated 33-mer peptide efficiently exchanges with a preloaded peptide in the DQ2 ligand-binding groove at pH 5.5 as well as pH 7.3, suggesting that the peptide can be presented to T cells comparably well through the endocytic pathway or via direct loading onto extracellular HLA-DQ2. In contrast, the monovalent peptides, and the nondeamidated precursor, as well as the tight-binding reference peptide show a much poorer ability to exchange with a preloaded peptide in the DQ2 binding pocket, especially at pH 7.3, suggesting that endocytosis of these peptides is a prerequisite for T cell presentation. At pH 5.5 and 7.3, dissociation of the deamidated 33-mer peptide from DQ2 is much slower than dissociation of its constituent monovalent epitopes or the nondeamidated precursor but faster than dissociation of the reference high-affinity peptide. Oligomeric states involving multiple copies of the DQ2 heterodimer bound to a single copy of the multivalent 33-mer peptide are not observed. Together, these results suggest that the remarkable antigenicity of the 33-mer gluten peptide is primarily due to its unusually efficient ability to displace existing ligands in the HLA-DQ2 binding pocket, rather than an extremely low rate of dissociation.

Celiac sprue (also known as celiac disease or gluten-sensitive enteropathy) is an immune disease of the small intestine caused by the ingestion of gluten proteins from common dietary ingredients such as wheat, rye, and barley (*1*). The disease has a strong HLA[1] association; a majority (>90%) of patients possess the DQ2 (*DQA1*05/DQB1*02*) allele with DQ8 (*DQA1*03/DQB1*0302*) being the most common allele in the remaining patients (*2*). $CD4^+$ T cells isolated from the small intestine of celiac sprue patients, but not of controls, recognize gluten peptides in which selected glutamine residue(s) have been deamidated into glutamic acid residue(s) by transglutaminase 2 (TG2) mediated modifications (*2*). The binding of these antigenic peptides to DQ2 or DQ8, followed by their presentation to $CD4^+$ T cells, plays a key role in disease development.

Within the past few years, a number of T cell epitopes have been identified from gliadin and, to a lesser extent, glutenin proteins in wheat gluten (*2*−*6*). Antigen presenting cells (APCs) in the gut encounter these epitopes in the context of relatively stable products of peptic and pancreatic protease digestion in the upper small intestine. For example, a Pro- and Gln-rich 33-mer peptide (LQLQPFPQPQLP-YPQPQLPYPQPQLPYPQPQPF) from α_2 gliadin is particularly interesting (*7*). Through a combination of (i) its high resistance to breakdown by luminal proteases and intestinal brush-border enzymes, (ii) its high affinity for human TG2 (*7*), (iii) its natural propensity to adopt a type II polyproline helical conformation in solution (the same conformation that is adopted by peptides bound to MHC class II molecules) (*7*−*9*), and (iv) its multivalent character (it contains six partly overlapping copies of three epitopes), it is a potent trigger of T cell proliferation from small intestinal biopsy samples of all celiac sprue patients tested thus far. Indeed, the deamidated 33-mer, LQLQPFPQPE<u>L</u>PYPQPE<u>L</u>PYPQPE<u>L</u>P-YPQPQPF (Gln → Glu changes underlined), has a substantially higher binding affinity to DQ2 ($IC_{50} \sim 0.3\ \mu M$) than

[†] This work was supported by a grant from the NIH (R21 DK65965 to C.K.) and by grants from the Research Council of Norway and Rikshospitalet to L.M.S.

* Address correspondence to this author. Phone/Fax: 650-723-6538. E-mail: ck@chemeng.stanford.edu.

[‡] Department of Chemistry, Stanford University.

[§] Rikshospitalet University Hospital, University of Oslo.

[||] Department of Chemical Engineering, Stanford University.

[⊥] Department of Biochemistry, Stanford University.

[1] Abbreviations: MHC, major histocompatibility complex; HLA, human leukocyte antigen; HPSEC, high-performance size exclusion chromatography; APC, antigen presenting cells; deamidated 33-mer, LQLQPFPQPELPYPQPELPYPQPELPYPQPQPF; Boc, *tert*-butyloxycarbonyl; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; PAM, phenylacetamidomethyl; PBS, phosphate-buffered saline.

10.1021/bi047747c CCC: $30.25
Published on Web 02/16/2005

any of the three individual minimal epitopes PFPQPELPY, PQPELPYPQ, and PYPQPELPY contained in it ($IC_{50} \sim 4$ μM) (*10*). The TG2-treated 33-mer binds to HLA-DQ2 with a higher pH optimum than other high-affinity DQ2 binders and can be presented to T cells by live as well as glutaraldehyde-fixed cells, suggesting that endosomal as well as extracellular loading of HLA-DQ2 by this peptide is feasible (*10*). Therefore, understanding the mechanistic basis for binding of the 33-mer to HLA-DQ2 could provide fundamentally new insights into celiac sprue pathogenesis. From a practical perspective, such insights could be translated into the development of pharmacological agents to block gluten-mediated enteropathy in celiac sprue patients.

Here we have followed earlier precedents for investigating HLA−ligand interactions by utilizing the recombinant DQ2 heterodimer, synthetic fluorescent peptide ligands, and high-performance size exclusion chromatography (*11*−*13*) to explore the differences between the binding of the 33-mer peptide and representative monovalent peptides. Our findings highlight the fundamental differences between this gluten-derived peptide and other high-affinity ligands of HLA-DQ2.

EXPERIMENTAL PROCEDURES

DQ2 Expression and Purification. The soluble extracellular domains of the DQ2 α and β chains were coexpressed in High Five cells using a pAcAB3 baculovirus expression system and were affinity-purified using the anti-DQ2 mAb 2.12.E11 (*14*). The sequence QLQPFPQPELPY was fused to the N-terminus of the DQ2 β chain by a 15-residue linker (GAGSLVPRGSGGGGS), which includes a thrombin site (*15*). A complementary Fos/Jun leucine zipper pair was engineered at the C-terminal ends of α and β chains, respectively, with intervening factor Xa proteolysis sites, to increase the heterodimer stability during protein expression, analogous to earlier designs by Teyton and co-workers (*16*) and Wucherpfennig and co-workers (*17*).

The concentration of HLA-DQ2 was determined by a UV−vis spectrometer at 280 nm using the absorption coefficient factor 75 700 cm^{-1} M^{-1} as calculated from the contents of tyrosine, tryptophan, and cystine in the DQ2 sequence (*18*). DQ2 protein with covalently linked αI peptide occupying the binding groove was first treated with ∼2% (w/w) thrombin in pH 7.3 PBS at 0 °C for 1 h before binding experiments.

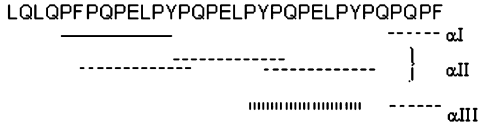
Peptide Synthesis, Labeling, and Purification. All peptides used in this study were synthesized using Boc/HBTU chemistry starting from *N*-α-*t*-Boc-L-aminoacylphenylacetamidomethyl (PAM) resin. Peptides were labeled at their N-termini while still attached to the resin with a 2.5-fold molar excess of 5- (and 6-) carboxyfluorescein succinimidyl ester and 100 μL of diisopropylethylamine as the catalyst in dimethylformamide as the solvent. Following cleavage of the peptidyl resin in trifluoroacetic acid/trifluoromethanesulfonic acid/thioanisole [TFA/TFMSA/thioanisole, 10:1:1 (v/v/v)] for 4 h, the crude peptides were precipitated in cold ether and dissolved in 1:1 (v/v) acetonitrile/water. The peptides were purified by reverse-phase HPLC on a semi-preparative C18 column using a water−acetonitrile gradient in 0.1% (v/v) TFA. The identity of the peptides was confirmed by electrospray mass spectrometry and analytical reverse-phase HPLC. The peptides were lyophilized and stored at −20 °C before use.

Peptide stock solutions in 10 mM PBS with 0.02% sodium azide were stored in aliquots at 4 °C. The quality of the stock solutions was checked periodically by analytical reverse-phase HPLC. The concentrations of fluorescein-labeled peptide stock solutions were determined by a UV−vis spectrometer at 495 nm using the absorption coefficient factor 80200 cm^{-1} M^{-1} provided by the manufacturer of 5- (and 6-) carboxyfluorescein succinimidyl ester.

Peptide Exchange Assay. For peptide exchange experiments, the DQ2 heterodimer with covalently linked αI peptide was pretreated with thrombin [40:1 (w/w)] for ∼1 h on ice. The resulting protein was incubated with fluoresceinated peptides at the indicated concentrations in a 1:1 mixture of PBS buffer (10 mM P_i, 150 mM NaCl, pH 7.3, supplemented with 0.02% NaN_3) and McIlvaine's citrate−phosphate buffer with defined pH values at 37 °C. In this citrate−phosphate buffer system, the final composition of the pH 5.5 buffer is 24 mM citrate, 55 mM P_i, and 75 mM NaCl, whereas the pH 7.3 buffer is composed of 6.5 mM citrate, 90 mM P_i, and 75 mM NaCl. Peptide binding was measured by HPSEC. Aliquots of 20 μL were injected onto a TSK-gel G3000SWxl size exclusion column or BioSep 3000 size exclusion column and eluted with the above-mentioned PBS buffer at 1 mL/min. The DQ2−peptide complex elutes at 8.5 min, with free peptides emerging ∼2 min later. The fluorescence signal was recorded using an in-line Shimadzu RA35 fluorescent detector with excitation wavelength set at 495 nm and emission detection set at 520 nm. The signal was converted into concentration of bound fluoresceinated peptide by comparing the peak area with those of peptide standards. The quantum yields of the free and bound peptides were comparable, as judged from peak area quantification.

Peptide Dissociation Assay. For dissociation experiments, DQ2−fluoresceinated peptide complexes were prepared by incubating thrombin-treated DQ2 (3−5 μM) with 10−20 μM fluoresceinated peptides at pH 5.5 for 25 h. Previous experiments with HLA-DR4 showed that peptide concentration in the range of 10−100 μM does not affect dissociation kinetics (*19*). The buffer composition was a 1:1 mixture of 10 mM PBS buffer and pH 5.1 or 7.3 McIlvaine's citrate−phosphate buffer (*20*), with the final pH measured to be 5.5 or 7.3. The reaction mixture was then cooled to 4 °C and stored at 4 °C until use. Under these conditions we also investigated the effect of competing unlabeled peptide on DQ2 dissociation kinetics. For example, in the presence of a 10-fold excess of unlabeled 33-mer (a high-affinity ligand), the dissociation constant of the fluorescent 33-mer was comparable and so was its biphasic dissociation behavior. Thus, it appears that the on-rates of the peptides explored in this study are slow enough so as to not influence corresponding off-rate measurements.

Excess free peptide was separated from the complex with a small-scale Bio-Rad spin column on ice. Spin columns were packed with Sephadex G-50 superfine medium and blocked with 1% BSA solution to minimize the binding of DQ2 to the column. Spin columns were then washed with pH 7.3 PBS buffer and stored at 4 °C until use. The DQ2−fluoresceinated peptide complex was eluted from the column with a final volume of about 230 μL. The residual amount of coeluted free peptide was typically less than 10% of the DQ2−fluoresceinated peptide complex. The solution was



Binding pocket	P_{-2}	P_{-1}	P_1	P_2	P_3	P_4	P_5	P_6	P_7	P_8	P_9
E αI	L	Q	P	F	P	Q	P	E	L	P	Y
Minimal αI			P	F	P	Q	P	E	L	P	Y
Minimal αII			P	Q	P	E	L	P	Y	P	Q
Minimal αIII			P	Y	P	Q	P	E	L	P	Y
Reference	A	A	I	A	A	V	K	E	E	A	F

FIGURE 1: The deamidated 33-mer gluten peptide and its constituent epitopes. The peptide (α_2 gliadin 56−88) contains six overlapping copies of three DQ2 restricted T cell epitopes.

then divided into two 115 μL aliquots in nonbinding tubes, where pH values were adjusted to pH 5.5 or 7.3.

Dissociation experiments were started by incubating the DQ2−fluoresceinated peptide complex at 37 °C. The dissociation kinetics was followed by injecting 10−15 μL aliquots into the HPSEC column at different time points. The amount of remaining DQ2−fluoresceinated peptide complex was quantified by measuring the fluorescence peak associated with DQ2.

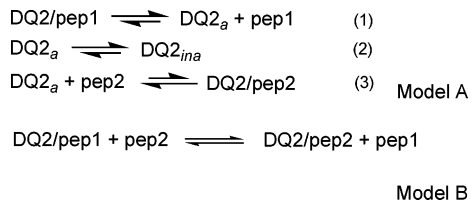
RESULTS

Preparation and Analysis of Thrombin-Treated αI-DQ2. To generate recombinant DQ2 that is suitable for biochemical studies, we first tested the thrombin cleavage efficiency at the VPRG sequence in the engineered linker between the antigenic αI peptide and the DQ2 β chain. Purified recombinant αI-DQ2 was incubated with ∼2% (w/w) thrombin at 0 °C (on ice), 25 °C (room temperature), or 37 °C for 5 min, 30 min, 1 h, and 2 h. Reactions were quenched with protease inhibitor cocktail and stored at 4 °C until analysis by SDS−PAGE. Complete cleavage was observed after 30 min on ice (Figure 2A). LC-MS analysis revealed a new peak (MW 2610 Da), corresponding to the molecular weight of the αI peptide plus residual linker RDSGQLQPFPQPELPYGAGSLVPR (Figure 2B).

To assess the feasibility of preparing DQ2 heterodimers bound to alternative ligands, we estimated the rate of dissociation of αI peptide from the DQ2 heterodimer binding groove at 0 and 25 °C. The endogenous (unlabeled) ligand was replaced with fluoresceinated LQPFPQPELPY, and the fluorescent peptide−DQ2 complex was separated on a gel filtration column. Loss of fluorescence from the resulting αI−DQ2 complex was monitored by HPSEC (see Experimental Procedures for details). Dissociation of the fluorescent peptide from DQ2 was extremely slow at 0 °C and did not occur to a significant extent over 10 min even at 25 °C. These results suggested that negligible dissociation would occur in the course of preparing reagents for subsequent assays (∼25 min on ice) or during HPSEC analysis (∼9 min at room temperature). Also, in the course of thrombin treatment at 0 °C for 1 h, >95% of the endogenous (unlabeled) ligand still remains bound, yielding a homogeneous system.

Peptide Exchange Calibration Studies. Using the thrombin-treated DQ2 heterodimer, we investigated the kinetics of peptide exchange. For this, a fixed amount of DQ2 and varying amounts of incoming peptides were used. The initial rate of the peptide exchange reaction showed saturation at a peptide:DQ2 ratio of ∼10 (Figure 3). Furthermore, even with a large excess of a high-affinity peptide (e.g., 33-mer:DQ2 = 30), at most 20% of the DQ2 protein remains bound by fluorescent peptide after 5 days, suggesting that the deactivation step is favorable because ∼80% of DQ2 fails to bind to the fluorescent peptide (data not shown). This suggests that the mechanism of peptide exchange most likely follows a multistep model (model A in Scheme 1) instead of a concerted one-step peptide displacement model (model B in Scheme 1), since a one-step mechanism would predict >90% of peptide exchange at equilibrium in the context of a large excess of fluorescent peptides (Scheme 1). Most studies with MHC proteins support a multistep model for ligand exchange (*11*) although examples of direct displacement through a two-peptide intermediate have been reported (*12*). The low occupancy of exogenous strong-binding peptides even under excess peptide conditions indicates that the DQ2 deactivation step (reaction 3 in model A, Scheme 1) is favorable and may reflect DQ2 aggregation.

Scheme 1: Two Possible Mechanisms of the Peptide Exchange



Deamidated 33-mer Gluten Peptide Is Highly Effective at Exchanging with Preloaded DQ2 Ligands, Especially at pH 7. It has been estimated that, at pH 4.9, the deamidated 33-mer gluten peptide (LQLQPFPQPELPYPQPELPYPQPELPYPQPQPF) has an IC_{50} of ∼0.3 μM, substantially lower than the IC_{50} of LQPFPQPELPY (4 μM) and that of the nondeamidated 33-mer gluten peptide (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF) (4.2 μM) (*10*). The peptide AAIAAVKEEAF, designed to conform to the DQ2 binding motif, is used as the tight-binding non-gluten reference DQ2 ligand. We compared the peptide exchange kinetics of fluorescein-conjugated forms of LQPFPQPELPY, the deamidated 33-mer peptide, and the reference peptide at neutral as well as acidic pH values (Figure 4). At pH 7.3 the deamidated 33-mer peptide displaced 11% of the endogenous αI ligand in 300 min at a peptide concentration of 11 μM and a DQ2 concentration of 0.72 μM. In contrast, only 1% of the endogenous ligand bound to DQ2 was displaced by fluoresceinated LQPFPQPELPY at the same pH on a similar time scale. More remarkably, the steady-state occupancy of DQ2 by fluoresceinated AAIAAVKEEAF was also relatively low under comparable conditions, even though the IC_{50} value of this peptide is estimated to be lower than that of the deamidated 33-mer peptide (E. Bergseng, unpublished observation).

Like most other MHC class II ligands studied thus far, binding of both the αI peptide LQPFPQPELPY and the reference AAIAAVKEEAF peptide to DQ2 improved at pH 5.5 as compared to pH 7.3 (Figure 3). In sharp contrast, the exchange characteristics of the deamidated 33-mer were similar at both pH values. Together, these findings suggest that the multivalent peptide associates with the DQ2 ligand-

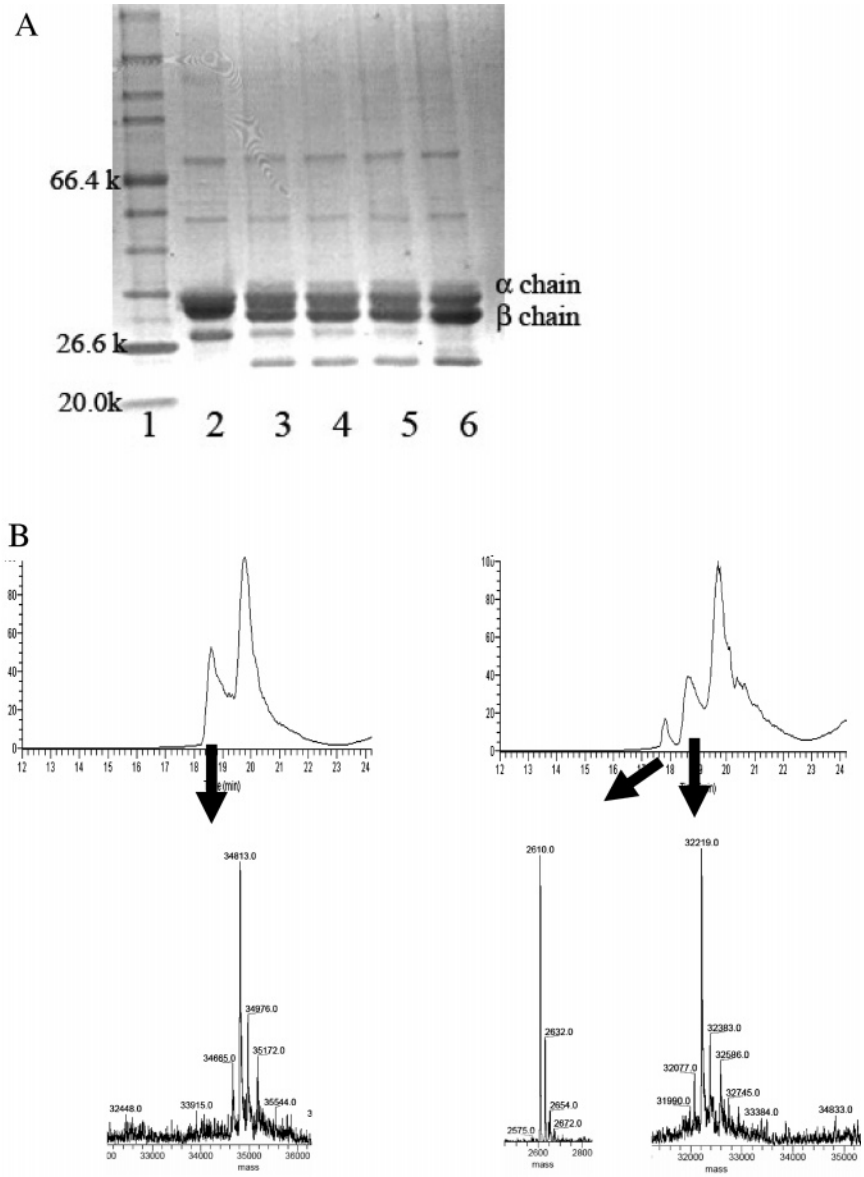


FIGURE 2: (A) SDS gel of thrombin cleavage reaction: lane 1, protein molecular marker; lane 2, uncleaved DQ2; lanes 3−5, cleavage reaction 5 min after mixing of thrombin and DQ2 on ice, after 30 min on ice, and after 1 h on ice, respectively; lane 6, cleavage for 2 h at room temperature. The band slightly above 26.6 kDa in the uncleaved DQ2 sample (lane 1) represents the major impurity from the protein purification. Following thrombin cleavage, this band migrates between 26.6 and 20.0 kDa in lanes 2−6. (B) Total ion counts of uncleaved DQ2 HPLC trace (left) and thrombin-cleaved DQ2 HPLC trace (right) vs retention time. In the left panel, the uncleaved DQ2 β chain (retention time 18.6 min, molecular mass 34 815 Da) predominates, whereas in the right panel, the cleaved DQ2 β chain (retention time 18.6 min, molecular mass 32219 Da) predominates. A trace amount of mass 34 833 Da, corresponding to the uncleaved β chain, is observed in the right panel. A new peak appears in the cleaved DQ2 trace with a retention time of 17.7 min and a molecular mass of 2610 Da; this corresponds to the cleaved peptide.

binding site via a different strategy as compared to the two monovalent peptides. Moreover, the difference between these binding strategies is inadequately reflected in IC_{50} values, which were measured at pH 5.5 and reflect only the dissociation behavior of peptide from the ligand-binding groove of DQ2.

In ligand exchange experiments using a limited amount of peptide and a large excess of DQ2 protein (22-fold in excess), the deamidated 33-mer gluten peptide retains its exceptional capacity for ligand exchange, especially at pH 7.3. As shown in Figure 5, the deamidated 33-mer peptide and the reference peptide AAIAAVKEEAF have similar ligand exchange efficiency at pH 5.5; however, 30% of the deamidated 33-mer peptide was bound to DQ2 at pH 7.3 as compared to only 14% in the case of AAIAAVKEEAF. Under steady-state conditions, the percentage of the monovalent peptide LQPFPQPELPY was considerably lower, both under acidic conditions (15%) and especially under neutral conditions (2% after 10 h and decreasing rapidly thereafter). Preliminary studies suggest that a slightly longer peptide (PQPELPYPQPELPY) does not show a 33-mer peptide-like capacity for efficient ligand exchange, whereas even longer analogues (PQPELPYPQPELPYPQPELPY and PFPQPELPYPQPELPYPQPELPYPQPQP) resemble the 33-mer peptide with respect to ligand exchange (data not shown).

To test the hypothesis that the multivalent 33-mer gluten peptide is more effectively loaded onto DQ2 due to its ability to coordinate multiple copies of the DQ2 heterodimer, we searched for multimeric DQ2 peaks by HPSEC. Within the limits of detection, no fluorescent or UV absorbing peaks

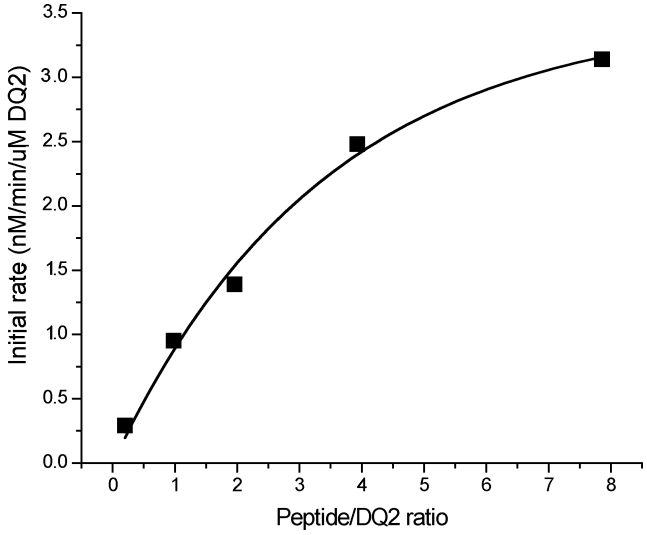


FIGURE 3: Initial rates of fluorescent αI gliadin epitope to DQ2 at various peptide:DQ2 concentration ratios at 37 °C. Reaction initial rates were converted to concentration of peptide-bound DQ2 by comparing to a fluorescent αI peptide standard solution. An exponential curve was fit to the data. The best fit equation is $Y = 3.5[1 - \exp(-0.29x)]$.

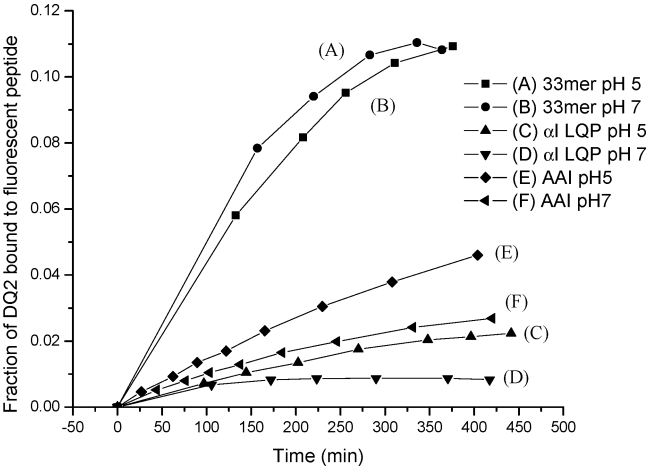


FIGURE 4: Exchange reactions of the deamidated 33-mer peptide (denoted as 33-mer), the αI gliadin epitope (denoted as αI LQP), and the tight-binding reference peptide AAIAAVKEEAF (denoted as AAI peptide) binding to DQ2 under conditions of excess peptide (peptide:DQ2 ratio > 12). All incubations were performed at 37 °C and either pH 5.5 or pH 7.3. Fluorescent signals corresponding to the DQ2 protein were compared to a fluorescent peptide standard to obtain the concentration of the fluorescent peptide−DQ2 complex and then normalized by the concentration of DQ2 to obtain the fraction of the fluorescent peptide−DQ2 complex in total DQ2 population.

were observed in the molecular mass range of >64 kDa (data not shown).

Comparative Analysis of Dissociation of the Deamidated 33-mer Gluten Peptide and Its Constituent Epitopes from the DQ2 Binding Pocket. Kinetic analysis of the dissociation of fluoresceinated LQPFPQPELPY from the binding groove of DQ2 revealed biphasic dissociation behavior at 37 °C (Figure 6). The fast phase has a short half-life of ∼2 h and comprises about one-third of the total peptide−DQ2 population, whereas the slow phase has a much longer half-life of about 20 h and leads to dissociation of the remaining two-thirds of the bound peptide. Overall, 50% of the peptide dissociated from DQ2 in ∼4 h. Similar biphasic dissociation behavior was observed for most other peptides at pH 5.5 and 7.3. The weighted preexponential factors of both phases, as well as the calculated dissociation rate constants, are reported in Table 1. The observation that the full-length αI peptide LQPFPQPELPY binds stronger than the minimal αI epitope PFPQPELPY is consistent with the previous observation that P_{-1} and P_{-2} amino acids are involved in peptide−DQ2 binding (*21*). Also, in our present study, the contribution, if any, of the N-terminal fluorescein moiety to DQ2 binding has not been assessed.

Two features are noteworthy regarding the fluoresceinated deamidated 33-mer peptide. First, a relatively small population (23−27%) of this peptide dissociates during the fast phase, especially at pH 7.3 (i.e., the pH corresponding to the extracellular milieu of an antigen presenting cell). Second, the dissociation constant corresponding to the slow phase is significantly smaller than the corresponding parameter for each of the monovalent gluten peptides. For example, at pH 7.3, the slow off-rate of LQPFPQPELPY is 6.5-fold faster than the deamidated 33-mer peptide. As a result of both of these features, the average half-life of the deamidated 33-mer peptide on DQ2 is considerably longer than any of the constituent epitopes, an important feature of highly immunogenic peptides (*22*). Our measurements demonstrate that previously reported IC_{50} values, although less detailed or specific, correlate well with the dissociation half-life of the peptide−DQ2 complexes (i.e., the lower the IC_{50} value, the longer the dissociation half-life, indicative of a tighter binding ligand) (*10*). Since the dissociation of fluoresceinated AAIAAVKEEAF from DQ2 was not significant even after 7 days, accurate dissociation kinetics could not be measured for this peptide.

Comparing the Deamidated 33-mer vs Its Nondeamidated Precursor. The observation that the deamidated 33-mer dissociates considerably slower than any of the monovalent peptides is consistent with the hypothesis that the deamidated 33-mer peptide possesses a secondary binding site on HLA-DQ2. As a first step toward interrogating the molecular recognition features at this secondary binding site, the ligand exchange and dissociation kinetics of its nondeamidated precursor, fluorescein−LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF, were measured at pH 5.5 and 7.3. The nondeamidated 33-mer is selectively transformed by transglutaminase 2 into the high-affinity DQ2 ligand characterized above. As might be expected on the basis of the well-established importance of Glu residues in DQ2 epitopes (*9*, *22*), the nondeamidated peptide showed a considerably lower affinity for DQ2, as judged by both exchange and dissociation assays. Under excess DQ2 conditions, only 5% of total peptide bound to DQ2 at either pH 5.5 or pH 7.3 (data not shown), whereas the half-life of the peptide−DQ2 complex was 7-fold shorter than its deamidated counterpart at pH 7.3 (Table 1). The pH dependence of dissociation of the two 33-mer peptides was strikingly different. At pH 5.5 the complex between DQ2 and the nondeamidated peptide is extremely unstable (half-life of <30 min), whereas the deamidated peptide binds stably to the DQ2 under identical conditions. Destabilization of the complex between DQ2 and the nondeamidated peptide can also be achieved at pH 7.3 by increasing the citrate concentration in the buffer (Table 1), suggesting a possible involvement of the divalent metal

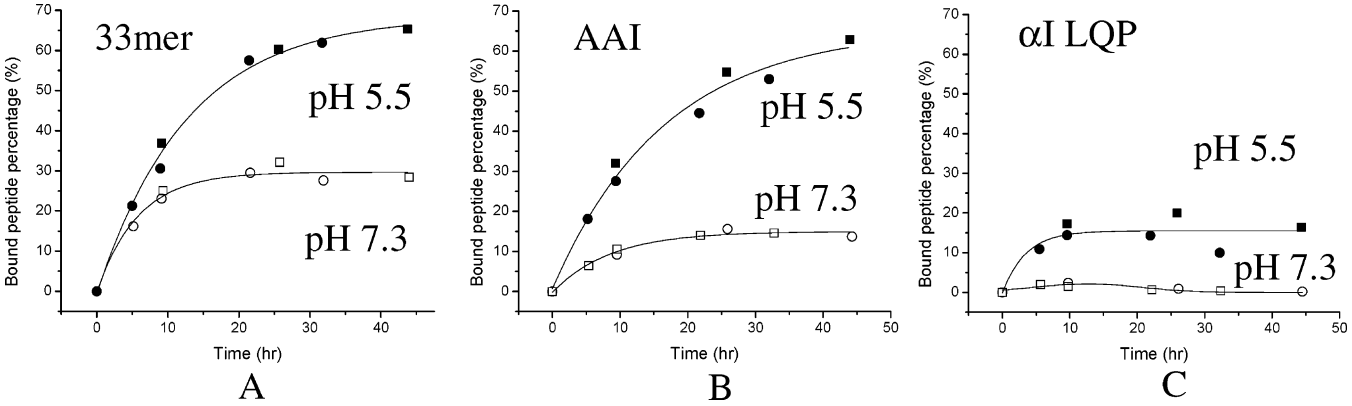


FIGURE 5: Exchange reaction with (A) the deamidated 33-mer gluten peptide (33-mer), (B) the reference peptide AAIAAVKEEAF (AAI), and (C) the LQPFPQPELPY (αI) peptide (αI LQP). These experiments were performed under conditions of 6 μM DQ2 solution and 0.27 $\pm$ 0.03 μM fluorescent peptides at 37 °C and at either pH 5.5 or pH 7.3. Open circles and squares (○ and □) represent data from pH 7.3, whereas closed circles and squares represent data from pH 5.5; circles or squares show data from two independent sets of experiments; the lines correspond to exponential fits of the data, except in the case of LQPFPQPELPY (C) at pH 7.3.

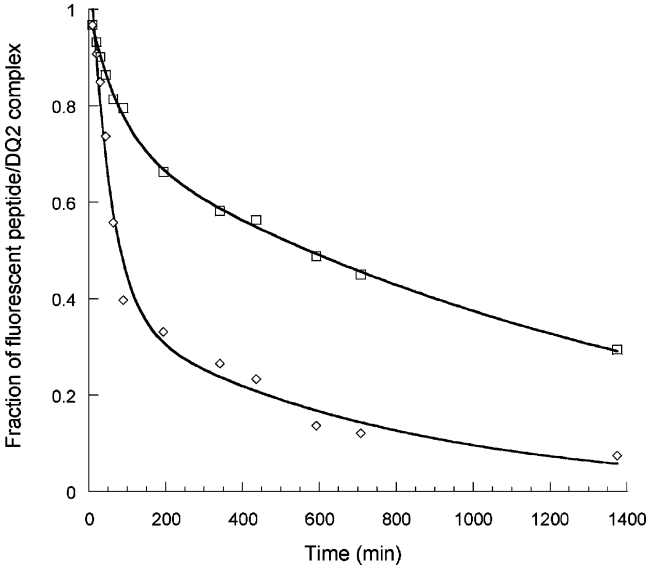


FIGURE 6: Representative kinetic curve for dissociation of the fluorescent αI gliadin peptide from DQ2 at pH 5.5 (□, upper line) and pH 7.3 (◇, lower line). Double exponential decay equations were fit to the data.

Table 1: Dissociation Kinetic Parameters of Triply Deamidated 33-mer, Monovalent Peptides, and the Reference Strong Binding Peptide AAIAAVKEEAF[a]

peptide	pH	$T_{50\%}$ (min)[b]	A_f (%)[c]	k_f (min^{-1})	A_s (%)[d]	k_s (min^{-1})
deamidated	5.5	4500	27	0.018	73	1.2×10^{-4}
33-mer	7.3	5000	23	6.8×10^{-3}	77	8.7×10^{-5}
	5.5[e]	3000	23	0.01	77	1.9×10^{-4}
nondeamidated	5.5	27	82	0.04	18	4.3×10^{-4}
33-mer	7.3	710	42	3.8×10^{-3}	58	3.2×10^{-4}
	7.3[f]	44	68	0.054	32	6.3×10^{-4}
αI	5.5	560	27	0.012	73	6.7×10^{-4}
	7.3	230	68	5.4×10^{-3}	32	5.7×10^{-4}
minimal αI	5.5	90	31	0.028	69	3.5×10^{-3}
	7.3	70	73	0.013	27	2.8×10^{-3}
minimal αII	5.5	880	24	0.015	76	4×10^{-4}
	7.3	900	74	1.0×10^{-3}	26	2×10^{-4}
minimal αIII	5.5	130	54	7.4×10^{-3}	46	2.3×10^{-3}
	7.3	80	88	0.01	12	1.8×10^{-3}
reference	5.5	≫7000				$<1 \times 10^{-5}$
	7.3	≫7000				$<1 \times 10^{-5}$

[a] The dissociation kinetics were fit into the exponential double decay function: $Y = A_f \exp(-k_f x) + A_s \exp(-k_s x)$ using Kaleidagraph 3.5. [b] $T_{50\%}$ is defined as the apparent first half-life when 50% of the overall fluorescent DQ2 signal has disappeared; i.e., $x = T_{50\%}$ and $Y = 0.5(A_f + A_s)$. [c] A_f (%) is defined as the proportion of the fast phase in the total population of the peptide−DQ2 complex. A_f (%) $= A_f/(A_f + A_s) \times 100$. [d] A_s (%) is defined as the proportion of the fast phase in the total population of the peptide−DQ2 complex. A_s (%) $= A_s/(A_f + A_s) \times 100$. [e] Measured at pH 5.5 with 20 μM unlabeled deamidated 33-mer added. [f] Measured at pH 7.3 with [citrate] = 13 mM and [P_i] = 170 mM; unless unspecified, reactions were conducted at pH 5.5 with [citrate] = 24 mM and [P_i] = 55 mM or at pH 7.3 with [citrate] = 6.5 mM and [P_i] = 90 mM.

ions in the architecture of the secondary binding site on HLA-DQ2.

DISCUSSION

The interaction between selected Pro-rich gluten peptides and HLA-DQ2 is of critical importance in the pathogenesis of celiac sprue, an inheritable lifelong disease of the small intestine. Here we have developed a DQ2−peptide binding assay using preloaded DQ2 and high-performance size exclusion chromatography to monitor the peptide exchange and dissociation kinetics of a reference tight-binding peptide AAIAAVKEEAF as well as several physiologically relevant gluten peptides.

As with some other class II MHC heterodimers, to maximize peptide occupancy, the DQ2 α/β heterodimer must be kept in a receptive (bound) state before exchange reactions are initiated (*23*). Our preloaded DQ2−αI protein proved to be an appropriate system for such measurements. The temperature-dependent dissociation kinetics of the preloaded αI gliadin peptide from the DQ2 groove not only secures the integrity of the DQ2−peptide complex at low temperature but also provides a large proportion of receptive DQ2 molecules at 37 °C. Thus, in an exchange experiment involving excess peptide, >10% of the receptor could bind to an incoming ligand (Figure 4), whereas under excess DQ2 conditions, as much as 65% of a strong-binding peptide was bound to DQ2 (Figure 5).

The 33-mer peptide from α_2 gliadin, LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF, is emblematic of the problem with dietary gluten in celiac sprue patients (*7*). In contrast to peptides derived from other dietary protein sources, this peptide is highly resistant to pancreatic and intestinal surface proteolysis, allowing it to persist for substantially longer durations in the mammalian small

intestine. It is an excellent substrate for human transglutaminase 2 (TG2), an extracellular enzyme expressed in small intestinal tissue that is also the primary target of the celiac autoantibody response. TG2 deamidates selected Gln residues, thereby unmasking epitopes such as the αI, αII, and αIII gliadin epitopes (Figure 1). The resulting mixtures of deamidated peptides are potent triggers of disease-specific CD4 T cells derived from intestinal biopsies of all celiac sprue patients tested thus far.

An example of a deamidated peptide, LQLQPFPQPELPYPQPELPYPQPELPYPQPQPF, harbors the deamidated αI, αII, and αIII gliadin epitopes (Figure 1) and is highly effective at triggering T cell proliferation in an HLA-DQ2 restricted fashion (*10*). Moreover, the deamidated 33-mer peptide binds to cell-surface DQ2 with considerably greater avidity than any of its constituent monovalent epitopes. Here we have investigated the mechanistic logic for this remarkable and physiologically crucial molecular recognition event. Our studies have revealed that the deamidated 33-mer peptide distinguishes itself from other ligands, including the tight-binding reference peptide, AAIAAVKEEAF, in several important ways that presumably contribute to its exceptional immunogenicity.

Ordinarily, antigens are presented to T cells by class II MHC proteins via a pathway involving endocytosis of the extracellular antigen, proteolytic processing in the lysosome, antigen loading into the MHC binding groove, and export of the peptide−MHC complex onto the surface of the antigen presenting cell (*24*). In an attempt to mimic the vacuolar environment of the cell, some of our assays were performed under acidic conditions (pH 5.5). It has also been suggested that MHC class II heterodimers can directly bind to T cell antigens on the surface of antigen presenting cells (*25*). To mimic this mechanism of antigen presentation, we also performed assays under neutral pH conditions (pH 7.3). Our equilibrium and kinetic analysis supports the proposal that T cell presentation of the deamidated 33-mer peptide by DQ2 can occur with comparable efficiency via the endocytic or extracellular binding pathway. The efficiency of peptide loading is especially striking at pH 7.3, when compared to monovalent epitopes or even a tight-binding reference peptide whose ability to exchange preloaded ligands in the DQ2 binding pocket drops significantly under neutral pH conditions (Figures 4 and 5).

Comparative kinetic analysis of dissociation of the deamidated 33-mer gluten peptide and other peptides (Table 1) has also highlighted its unusual properties. In all cases biphasic dissociation kinetic behavior was observed, a phenomenon that has been well documented in the literature for other MHC class II proteins (*26*−*32*). It is generally accepted that such biphasic behavior is due to two different isomeric forms of DQ2−peptide complexes (*26*−*32*). The deamidated 33-mer gluten peptide is different in its DQ2 dissociation properties in two important ways. First, its dissociation rate is much slower than either of the monovalent αI, αII, or αIII peptides. This ensures that, once loaded, the deamidated 33-mer has a relatively long half-life on the DQ2 binding site. Since our experiments have ruled out the possibility of DQ2 oligomerization on the multivalent 33-mer peptide, an unknown secondary binding site on this peptide may be responsible for its slower dissociation from the DQ2 binding groove. This is reminiscent of earlier observations for murine MHC class II I-E^k, where elongating a peptide ligand also increased the binding ability to MHC class II protein (*33*). Second, in contrast to monovalent gluten peptides, the 33-mer peptide−DQ2 complex is comparably stable under acidic and neutral pH conditions. The much slower dissociation kinetics under neutral pH conditions presumably ensures accumulation of peptide−DQ2 complexes on the surface of antigen presenting cells, facilitating stimulation of T cells to a larger extent.

Comparison of the dissociation kinetics of the deamidated 33-mer and its nondeamidated precursor provides some insights into the secondary binding site for this long peptide on HLA-DQ2. Consistent with earlier structural and biological evidence highlighting the importance of a Gln → Glu alteration in a DQ2 epitope (*9*, *22*), the nondeamidated peptide has lower affinity for HLA-DQ2 as judged by both exchange and dissociation assays. While alternative hypotheses cannot be ruled out, this reduction in affinity is consistent with the existence of the secondary binding site on DQ2. Surprisingly, the nondeamidated 33-mer peptide showed a 26-fold increase in dissociation rate when the pH was reduced from 7.3 to 5.5. This pH-dependent destabilization could also be mimicked by doubling the citrate concentration from 6.5 to 13 mM at pH 7.3. We therefore speculate a possible role for a divalent metal ion in stabilizing the secondary binding site. Citrate anions chelate divalent metal ions, which could play a role in fashioning a motif on the DQ2 surface for peptide binding. For example, a carboxylate cluster is observed in the crystal structure of DQ2, comprising Glu 85, Glu 88, Asp 110, and Asp 142 on the DQ2 α chain. Divalent metal ions such as Ca^{2+} or Mg^{2+} could coordinate into this anionic cave and may give rise to additional coordination sites for backbone oxygen atoms at the C-terminus of the incoming peptide. Consistent with the prediction that shorter peptides would lack the ability to bind to this secondary binding site, the citrate effect is not observed for any of the monovalent peptides. Why the citrate effect does not affect binding of the deamidated 33-mer peptide is unclear at present. It could either be due to the dominance of the primary binding site in the overall interaction between this peptide and DQ2 or it could reflect the possibility that a secondary Glu residue in the deamidated 33-mer enhances the metal binding affinity of the secondary binding site.

The deamidated 33-mer gluten peptide also appears to utilize a different strategy for DQ2-mediated presentation in comparison to typical high-affinity MHC antigens, as exemplified here by the peptide AAIAAVKEEAF. AAIAAVKEEAF maintains good binding ability mainly due to its extremely slow dissociation from the DQ2 binding site. Physiologically, once such a peptide binds to HLA-DQ2, it presumably never falls off. In contrast, the deamidated 33-mer gluten peptide binds comparably well to DQ2, yet it has a measurable dissociation constant. It achieves its goal of efficient MHC loading primarily through extraordinarily efficient exchange kinetics.

ACKNOWLEDGMENT

We thank Harden McConnell and Craig Beeson for helpful discussions and Harden McConnell for permission to use the HPSEC apparatus.

REFERENCES

1. Trier, J. S. (1991) Celiac sprue, *N. Engl. J. Med. 325*, 1709−1719.
2. Sollid, L. M. (2002) Coeliac disease: dissecting a complex inflammatory disorder, *Nat. Rev. Immunol. 2*, 647−655.
3. Sjöström, H., Lundin, K. E. A., Molberg, Ø., Korner, R., McAdam, S. N., Anthonsen, D., Quarsten, H., Noren, O., Roepstorff, P., Thorsby, E., and Sollid L. M. (1998) Identification of a gliadin T-cell epitope in coeliac disease: general importance of gliadin deamidation for intestinal T-cell recognition, *Scand. J. Immunol. 48*, 111−115.
4. Arentz-Hansen, H., McAdam, S. N., Molberg, Ø., Fleckenstein, B., Lundin, K. E., Jorgensen, T. J., Jung, G., Roepstorff, P., and Sollid, L. M. (2002) Celiac lesion T cells recognize epitopes that cluster in regions of gliadins rich in proline residues, *Gastroenterology 123*, 803−809.
5. Vader, W., Kooy, Y., van Veelen, P., de Ru, A., Harris, D., Benckhuijsen, W., Pena, S., Mearin, L., Drijfhout, J. W., and Koning, F. (2002) The gluten response in children with celiac disease is directed toward multiple gliadin and glutenin peptides, *Gastroenterology 122*, 1729.
6. Vader, W., Stepniak, D. T., Bunnik, E. M., Kooy, Y. M. C., De Haan, W., Drijfhout, J. W., Van Veelen, P. A., and Koning, F. (2003) Characterization of cereal toxicity for celiac disease patients based on protein homology in grains, *Gastroenterology 125*, 1105.
7. Shan, L., Molberg, Ø., Parrot, I., Hausch, F., Filiz, F., Gray, G. M., Sollid, L. M., and Khosla C. (2002) Structural basis for gluten intolerance in celiac sprue, *Science 297*, 2275−2279.
8. Jardetzky, T. S., Brown, J. H., Gorga, J. C., Stern, L. J., Urban, R. G., Strominger, J. L., and Wiley, D. C. (1996) Crystallographic analysis of endogenous peptides associated with HLA-DR1 suggests a common, polyproline II-like conformation for bound peptides, *Proc. Natl. Acad. Sci. U.S.A. 93*, 734−738.
9. Kim, C. Y., Quarsten, H., Bergseng, E., Khosla, C., and Sollid, L. M. (2004) Structural basis for HLA-DQ2 mediated presentation of gluten epitopes in celiac disease, *Proc. Natl. Acad. Sci. U.S.A. 101*, 4175−4179.
10. Qiao, S.-W., Bergseng, E., Molberg, Ø., Xia, J., Fleckenstein, B., Khosla, C., and Sollid, L. M. (2004) Antigen presentation to celiac lesion-derived T cells of a 33-mer gliadin peptide naturally formed by gastrointestinal digestion, *J. Immunol. 173*, 1757−1762.
11. Kasson, P. M., Rabinowitz, J. D., Schmitt, L., Davis, M. M., and McConnell, H. M. (2000) Kinetics of peptide binding to the class II MHC protein I-E^k, *Biochemistry 39*, 1048−1058.
12. De Kroon, A. I., and McConnell, H. M. (1994) Kinetics and specificity of peptide-MHC class II complex displacement reactions, *J. Immunol. 152*, 609−619.
13. McFarland, B. J., and Beeson, C. (2002) Binding interactions between peptides and proteins of the class II major histocompatibility complex, *Med. Res. Rev. 22*, 168−203.
14. Quarsten, H., McAdam, S. N., Jensen, T., Arentz-Hansen, H., Molberg, Ø., Lundin, K. E. A., and Sollid, L. M. (2001) Staining of celiac disease-relevant T cells by peptide-DQ2 multimers, *J. Immunol. 167*, 4861−4868.
15. Kozono, H., White, J., Clements, J., Marrack, P., and Kappler, J. (1996) Production of soluble MHC class II proteins with covalently bound single peptides, *Nature 369*, 151−154.
16. Scott, C. A., Garcia, K. C., Carbone, F. R., Wilson, I. A., and Teyton, L. (1996) Role of chain pairing for the production of functional soluble IA major histocompatibility complex class II molecules, *J. Exp. Med. 183*, 2087−2095.
17. Kalandadze, A., Galleno, M., Foncerrada, L., Strominger, J. L., and Wucherpfennig, K. W. (1996) Expression of recombinant HLA-DR2 molecules. Replacement of the hydrophobic transmembrane region by a leucine zipper dimerization motif allows the assembly and secretion of soluble DR alpha beta heterodimers, *J. Biol. Chem. 271*, 20156−20162.
18. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) How to measure and predict the molar absorption coefficient of a protein, *Protein Sci. 4*, 2411−2423.
19. Belmares, M. P., Rabinowitz, J. D., Liu, W., Mellins, E. D., and McConnell, H. M. (2000) pH stability of HLA-DR4 complexes with antigenic peptides, *Biochemistry 39*, 14558−14566.
20. Dawson, R. M. C., Elliott, D. C., Elliott, W. H., and Jones, K. M. (1986) *Data for biochemical research*, 3rd ed., Clarendon Press, Oxford, U.K.
21. Hall, F. C., Rabinowitz, J. D., Busch, R., Visconti, K. C., Belmares, M., Patil, N. S., Cope, A. P., Patel, S., McConnell, H. M., Mellins, E. D., and Sonderstrup, G. (2002) Relationship between kinetic stability and immunogenicity of HLA-DR4/peptide complexes, *Eur. J. Immunol. 32*, 662−670.
22. Arentz-Hansen, H., Körner, R., Molberg, Ø., Quarsten, H., Vader, W., Kooy, Y. M., Lundin, K. E. A., Koning, F., Roepstorff, P., Sollid, L. M., and McAdam, S. N. (2000) The intestinal T cell response to alpha-gliadin in adult celiac disease is focused on a single deamidated glutamine targeted by tissue transglutaminase, *J. Exp. Med. 191*, 603−612.
23. Rabinowitz, J. D., Vrljic, M., Kasson, P. M., Liang, M. N., Busch, R., Boniface, J. J., Davis, M. M., and McConnell, H. M. (1998) Formation of a highly peptide-receptive state of class II MHC, *Immunity 9*, 699−709.
24. Watts, C. (2004) The exogenous pathway for antigen presentation on major histocompatibility complex class II and CD1 molecules, *Nat. Immunol. 5*, 685−692.
25. Santambrogio, L., Sato, A. K., Carven, G. J., Belyanskaya, S. L., Strominger, J. L., and Stern, L. J. (1999) Extracellular antigen processing and presentation by immature dendritic cells, *Proc. Natl. Acad. Sci. U.S.A. 96*, 15056−15061.
26. Sadegh-Nasseri, S., and McConnell, H. M. (1989) A kinetic intermediate in the reaction of an antigenic peptide and I-E^k, *Nature 337*, 274−276.
27. Sadegh-Nasseri, S., Stern, L. J., Wiley, D. C., and Germain, R. N. (1994) MHC class II function preserved by low-affinity peptide interactions preceding stable binding, *Nature 370*, 647−650.
28. Beeson, C., Anderson, T. G., Lee, C., and McConnell, H. M. (1996) Isomeric complexes of peptides with class II proteins of the major histocompatibility complex, *J. Am. Chem. Soc. 118*, 977−980.
29. Schmitt, L., Boniface, J. J., Davis, M. M., and McConnell, H. M. (1999) Conformational isomers of a class II MHC-peptide complex in solution, *J. Mol. Biol. 286*, 207−218.
30. Schmitt, L., Boniface, J. J., Davis, M. M., and McConnell, H. M. (1998) Kinetic isomers of a class II MHC-peptide complex, *Biochemistry 37*, 17371−17380.
31. Anderson, T. G., and McConnell, H. M. (1999) Interpretation of biphasic dissociation kinetics for isomeric class II major histocompatibility complex-peptide complexes, *Biophys. J. 77*, 2451−2461.
32. Rabinowitz, J. D., Liang, M. N., Tate, K., Lee, C., Beeson, C., and McConnell, H. M. (1997) Specific T cell recognition of kinetic isomers in the binding of peptide to class II major histocompatibility complex, *Proc. Natl. Acad. Sci. U.S.A. 94*, 8702−8707.
33. Srinivasan, M., Domanico, S. Z., Kaumaya, P. T., and Pierce, S. K. (1993) Peptides of 23 residues or greater are required to stimulate a high-affinity class II-restricted T-cell response, *Eur. J. Immunol. 23*, 1011−1016.

BI047747C