Effect of protein structure on deamidation rate in the Fc fragment of an IgG1 monoclonal antibody

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Abstract: The effects of secondary structure on asparagine (N) deamidation in a 22 amino acid sequence (369-GFYPSDIAVEWESNGQPENNYK-390) of the crystallizable (Fc) fragment of a human monoclonal antibody (Fc IgG1) were investigated using high-resolution ultra performance liquid chromatography with tandem mass spectrometry (UPLC/MS). Samples containing either the intact Fc IgG (~50 kD) ("intact protein"), or corresponding synthetic peptides ("peptide") were stored in Tris buffer at 37°C and pH 7.5 for up to forty days, then subjected to UPLC/MS analysis with high energy MS1 fragmentation. The peptide deamidated only at N₃₈₂ to form the isoaspartate (isoD₃₈₂) and aspartate (D₃₈₂) products in the ratio of ~4:1, with a half-life of ~3.4 days. The succinimide intermediate (Su₃₈₂) was also detected; deamidation was not observed for the other two sites (N₃₈₇ and N₃₈₈) in peptide samples. The intact protein showed a 30-fold slower overall deamidation half-life of ~108 days to produce the isoD₃₈₂ and D₃₈₇ products, together with minor amounts of D₃₈₂. Surprisingly, the D₃₈₂ and isoD₃₈₇ products were not detected in intact protein samples and, as in the peptide samples, deamidation was not detected at N₃₈₈. The results indicate that higher order structure influences both the rate of N-deamidation and the product distribution.

Keywords: deamidation; monoclonal antibody; IgG1; protein stability; secondary structure

Additional Supporting Information may be found in the online version of this article.

Abbreviations: D₃₈₂NN, peptide GFYPSDIAVEWESDGPENNYK; EIC, extracted ion chromatogram; +ESI, electrospray ionization in the positive ion mode; Fc IgG, crystallizable fragment (Fc) of a monoclonal antibody (IgG); isoD, isoaspartate; (isoD)₃₈₂NN, peptide GFYPSDIAVEWES(isoD)GQPENNYK; ND₃₈₇N, the peptide GFYPSDIAVEWESNGQPEDNYK; N(isoD)₃₈₇N, the peptide GFYPSDIAVEWESNGQPE(isoD)NYK; NNN, the peptide GFYPSDIAVEWESNGQPENNYK, corresponding to tryptic fragment G₃₆₉-K₃₉₀ of Fc IgG; Su succinimide, cyclic imide; an intermediate in deamidation; Su₃₈₂NN, the peptide GFYPSDIAVEWES(Su)GQPENNYK; UPLC/MS, ultra performance liquid chromatography with tandem mass spectrometry.

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Introduction

Therapeutic antibodies are one of the fastest growing segments of the pharmaceutical industry.^{1,2} To maintain potency and minimize immunogenicity, antibodies and other protein drugs must be protected from physical and chemical degradation during manufacturing and storage. One of the most common routes of chemical inactivation of proteins is deamidation at asparagine (N) residues, a hydrolytic reaction that can influence the activity of endogenous proteins as well as protein drug products.^{3–5} In neutral to basic solution, deamidation proceeds via the nucleophilic attack of the N + 1 nitrogen of the protein backbone on the carbonyl group of the N side chain, forming a cyclic imide (succinimide, Su) intermediate. Hydrolysis then occurs at either of the succinimide carbonyl groups to form the aspartate- (D) and isoaspartate- (isoD) containing products, which differ from the parent sequence both in charge (acidic vs. neutral) and mass (+1 amu). Protein primary sequence strongly influences deamidation rate. For example, in unstructured model peptides (37°C, pH 7.4), deamidation half-lives for the NG sequence are typically on the order of 1 day, while half-lives at NV and NI sequences of more than 200 days have been reported.⁶ Secondary structure also influences deamidation rate,^{4,7–10} but its effects have not been as clearly elucidated.

Various analytical methods have been used to detect deamidation in proteins.¹¹ Ion-exchange chromatography (IEC) exploits the difference in charge of the acidic degradation products and the parent molecule,^{12,13} and is routinely used in the pharmaceutical and biotechnology industry. Tryptic digestion with reversed-phase LC/MS analysis has been used to identify sites of deamidation and to quantify deamidation rates.^{14,15} More recently, electron capture dissociation (ECD) in Fourier transform mass spectrometry (FTMS) has been used to distinguish D- and isoD-containing forms in tryptic digests based on their signature fragmentation patterns.^{16,17} The enzyme protein isoaspartyl methyl transferase (PIMT) repairs isoD residues *in vivo*, and has been used to quantify isoD levels *in vitro*; PIMT detection kits are available commercially (e.g., IsoQuant®, Promega, Madison, WI).

Cumulative evidence from a number of monoclonal antibodies suggests that these proteins typically contain deamidation-prone sites: (i) in the complementarity determining regions (CDR), depending on sequence, and (ii) in several “hot spots” in the constant regions (Fc). Recent studies of deamidation in the Fc-domain of a humanized monoclonal antibody employed tryptic digestion and liquid chromatography/electrospray ionization mass spectrometry (HPLC/+ESI/MS).¹⁸ The results showed that the reaction is sensitive to antibody structure, with tryptic fragment G₃₆₉-K₃₉₀, (GFYPSDIAVEWESNGQPEN-NYK) showing rapid and structure-dependent deami-

dation at N₃₈₂ and N₃₈₇. Interestingly, the authors did not observe several expected deamidation products at these sites, including the isoD product at N₃₈₇ (i.e., isoD₃₈₇), the Asp product at N₃₈₂ (i.e., D₃₈₂), and the succinimide intermediate at N₃₈₇. The authors infer that the mechanisms of deamidation differ at the two sites, perhaps due to differences in secondary structure. This is an important observation and a potentially significant contribution to our understanding of the effects of secondary structure on chemical reactivity in proteins. However, it is also possible that the “missing” products were simply poorly resolved chromatographically and so were not detected. This is particularly problematic if the isoD and D variants co-eluted, because the two products have identical mass and cannot be resolved by the use of base ion chromatograms, as used by the authors. As a result, the effect of secondary structure on deamidation in this commercially important protein remains an open question.

The work reported here is a detailed study of deamidation at N₃₈₂ and N₃₈₇ in the Fc portion of a humanized IgG1 antibody. We have used ultraperformance liquid chromatography with electrospray ionization mass spectrometry (UPLC/+ESI/MS) in an attempt to achieve chromatographic resolution of deamidation products while maintaining relatively short analysis times. In addition, synthetic peptides corresponding to the tryptic fragment G₃₆₉-K₃₉₀ and its deamidation products have been used as analytical standards and as controls for the effects of structure on deamidation. The results demonstrate that both the product profiles and reaction kinetics differ for the synthetic peptide and the intact protein, supporting the hypothesis that higher order structure plays a significant role in deamidation in this system.

Results

Product identification

UPLC/+ESI-MS analysis of synthetic peptide standards. Four synthetic peptides (NNN, ND₃₈₇N, IsoD₃₈₂NN, NisoD₃₈₇N) were analyzed by UPLC/+ESI-MS to establish chromatographic elution times for use in identifying deamidation products. Approximate elution times were: IsoD₃₈₂NN = 36.0 min; NNN = 37.0 min; NisoD₃₈₇N = 37.0 min; ND₃₈₇N = 38.5 min. Baseline resolution was achieved for the IsoD₃₈₂NN and ND₃₈₇N peaks, and for the NNN and ND₃₈₇N peaks; resolution of the IsoD₃₈₂NN and NNN peaks was partial. The NNN and NisoD₃₈₇N peptides showed identical retention times and were not distinguishable chromatographically. This suggests that the NisoD₃₈₇N deamidation product may be masked by the NNN parent peak, and that careful analysis of NNN mass envelopes is warranted to establish the presence or absence of this product.

Figure 1. Deamidation products in the NNN synthetic peptide. Representative extracted ion chromatogram (EIC) (A) and molecular ion isotope envelopes (B) of IsoD₃₈₂NN (1), NNN (2) and D₃₈₂NN (3) peaks in the EIC for a sample of the NNN synthetic peptide stressed for 90 h at 37°C, pH 7.4. The EIC (A) shows the elution order, with IsoD₃₈₂NN eluting first followed by NNN and D₃₈₂NN. The isotope envelopes (B) show the +1 amu mass increase for the first and third peaks, consistent with deamidated forms of the NNN peptide.

Deamidation products in the NNN synthetic peptide. The EIC for a representative sample of the NNN synthetic peptide following 23 h of storage (37°C, pH 7.4) shows three well-resolved peaks eluting at ~36.2 min (Peak 1), 37 min (Peak 2), and 39.3 min (Peak 3) [Fig. 1(A)]. Figure 1(B) shows the molecular ion mass spectra of the first three of these peaks, presented in the order of elution. The second peak shows an *m/z* of 1273 [Fig. 1(B, 2)] and is thereby identified as the intact parent peptide (i.e., G₃₆₉-K₃₉₀). The first peak [Fig. 1(B, 1)] and the third peak [Fig. 1(B, 3)] both shown +1 amu shifts in their molecular isotope envelopes, consistent with singly deamidated products. Because the retention time of the first peak is comparable to that of the IsoD₃₈₂NN synthetic peptide, this peak is tentatively assigned to the isoD₃₈₂ deamidation product, suggesting that the third peak is the corresponding D₃₈₂ product. A fourth peak has an *m/z* of 1264 (~41 min, -17 amu; not shown) and so is tentatively identified as the succinimide intermediate at position 382 (i.e., Su₃₈₂NN).

The site of deamidation was confirmed using the daughter ions (i.e., *b*- and *y*'-ions) formed during high energy MS1 analysis of each of these peaks (see Fig. 2). Figure 2(A,C) show *y*'₁₀, *y*'₁₂, *y*'₁₃, and *y*'₁₉ ions with a

mass increase of +1 amu, consistent with deamidation in these fragments and which could have occurred at the N₃₈₂, N₃₈₇, or N₃₈₈ sites. However, Figure 2(A,C) show no mass changes in the *y*'₆, *y*'₄, and *y*'₃ ions, which excludes deamidation at the N₃₈₇ and N₃₈₈ sites. Thus, using these daughter ions, deamidation at N₃₈₂ was confirmed for both the first and third peaks of Figure 1(A), indicating that they are the isoD₃₈₂ and D₃₈₂ products, respectively. Both the relative peak areas and the elution order provide further support for these assignments, because in rpHPLC, isoD-containing peptides typically elute earlier than their D-containing counterparts.^{19–22} The isoD product is generally favored in unstructured peptides, with a typical isoD:D ratio of 3:1 to 5:1.^{7,23} On this basis, the product peaks in Figure 1(A) are definitively assigned as isoD₃₈₂NN [Fig. 1(A), 36.5 min] and D₃₈₂NN [Fig. 1(A), 39.3 min]. Based on the identification of the two deamidation products at the N₃₈₂ site, the succinimide intermediate [Fig. 1(A), 41 min] is assumed to be associated with this site as well. It should be noted that racemization can also occur via the succinimide to produce the D-forms of asparagine, aspartate, and iso-aspartate containing species. D- and L-forms were not resolved by the UPLC/+ESI-MS assay used here, so the species identified may be mixtures of racemates. At long storage times, loss of parent and/or product peptide species occurred; the appearance of lower mass species suggested that this loss is due to peptide bond hydrolysis (“clipping”). Kinetic studies were truncated if these losses exceeded ~10%.

Deamidation products in the intact protein. Figure 3(A) shows the EIC for the G₃₆₉-K₃₉₀ fragment of intact Fc stressed for 28 h before tryptic digestion and UPLC/+ESI-MS analysis. Five species are detected, eluting at 36 min (Peak 1), 37 min (Peak 2), 37.5 min (Peak 3), 38.5 min (Peak 4), and 39.2 min (Peak 5). The molecular isotope envelopes [Fig. 3(B)] show that: (i) the first, fourth and fifth peaks correspond to singly deamidated products [Fig. 3(B, 1, 4 and B, 5)], (ii) the second peak corresponds to the parent peptide [Fig. 3(B, 2)], and the third peak corresponds to a doubly-deamidated product with +2 amu mass increase relative to the parent [Fig. 3(B, 3)]. By comparison with results for the synthetic model peptide standards and with the products of NNN deamidation, and by high energy MS1 analysis, the first, fourth, and fifth peaks are identified as the isoD₃₈₂NN, ND₃₈₇N, and D₃₈₂NN variants, respectively [see Fig. 4(B)]. By high energy MS1 analysis [Fig. 4(A)] and the molecular isotope envelopes [Fig. 3(B, 3)], the third peak is identified as a doubly deamidated product. The presence of *y*'₄, *y*'₆, and *y*'₈ daughter ions with a mass change of +1 amu and of a *y*'₃ daughter ion with unchanged mass indicate that deamidation occurs at N₃₈₇. A +2 amu mass change for the *y*'₁₀ and *y*'₁₉ daughter ions indicate a second

Figure 2. High energy MS1 spectra of peaks 1 (A), 2 (B) and 3 (C) min of Figure 1. The fragmentation pattern, particularly the y'_3 , y'_4 , and y'_{10} ions, together with the elution and fragmentation patterns of synthetic peptide standards (see text), identifies the peaks as the IsoD₃₈₂NN, NNN (parent) and D₃₈₂NN forms, respectively. See the electronic version of this article for enlarged figures.

deamidation site at N₃₈₂. Thus, the third peak is deamidated at both the N₃₈₂ and N₃₈₇ sites; the identity of the deamidated sites with respect to isoD or D cannot be determined with the present data. The succinimide intermediates (i.e., Su₃₈₂NN, NSu₃₈₇N) were not detected at either site, suggesting that they were not formed or were degraded during tryptic digestion.

Detection of the NisoD₃₈₇N deamidation product. The NisoD₃₈₇N synthetic peptide was analyzed in an attempt to confirm that this product is not produced by deamidation at the N₃₈₇ site in samples of the intact protein. The NisoD₃₈₇N form is expected to be the major deamidation product at this site but was not detected in stressed samples of Fc-IgG,

Figure 3. Deamidation products in Fc IgG. Representative extracted ion chromatogram (EIC) (**A**) and molecular ion isotope envelopes (**B**) of IsoD₃₈₂NN (1), NNN (2), a doubly deamidated product (3), ND₃₈₇N (4), and D₃₈₂NN (5) peaks in the EIC, for a sample stressed for 28 h at 37°C, pH 7.4 before digestion. The EIC shows the elution order with IsoD₃₈₂NN eluting first followed by NNN, doubly deamidated and D₃₈₂NN peptides. The isotope envelopes show the +1 amu mass increase for the singly deamidated species and +2 amu for the doubly deamidated product.

although the corresponding ND₃₈₇N form is clearly present [see Fig. 3(A)]. A similar result was reported by Chelius *et al.*¹⁸ Interestingly, UPLC/MS analysis demonstrated that the NisoD₃₈₇N synthetic peptide coelutes with the parent NNN peptide in EIC chromatograms, suggesting that this product could go undetected if it is formed. Additional analysis of isotopic distributions was conducted to estimate the amount of NisoD₃₈₇N in the samples. Because the isoD product has greater mass (+1 amu) than the parent NNN peptide, the presence of NisoD₃₈₇N as an impurity in the NNN peak would be expected to skew the relative intensities of the peaks in the molecular ion isotope envelope (see [Fig. 1(B)]). Specifically, in a sample containing both species, the first isotopic peak would be unique to NNN (i.e., the NNN “M” peak), but the second peak would contain contributions from both NNN and NisoD₃₈₇N (i.e., the NNN “M + 1” peak and the NisoD₃₈₇N “M” peak). The amount of NisoD₃₈₇N in the sample was estimated by subtracting the theoretical intensity of the M + 1 peak of NNN from the observed intensity of the second peak. The “theoretical intensity” of the M + 1 peak of NNN was assumed to be identical to that measured for isoD₃₈₂NN, a species that was cleanly resolved in EIC. The percentage contribution of NisoD₃₈₇N to the second peak was esti-

mated for each of three samples analyzed for each of the twelve time points of the study. By this estimation, the average percentage contribution of NisoD₃₈₇N to the second peak for each time point was less than 2% of the NNN intensity, supporting the assertion that the isoD product is not formed at the N₃₈₇ site.

Deamidation kinetics

The deamidation products for the NNN synthetic peptide are consistent with the accepted mechanism for deamidation at a single Asn site. In the discussion below, deamidation kinetics in these samples will be modeled as an irreversible first-order reaction to produce the succinimide (Su₃₈₂NN), followed by parallel irreversible first-order reactions to produce the isoD and D products (isoD₃₈₂NN, D₃₈₂NN; [Fig. 5(A)] as in previous reports.^{7,10,23} In contrast, deamidation in the intact Fc IgG produced singly deamidated products at two sites (i.e., isoD₃₈₂NN and ND₃₈₇N) and a doubly deamidated product. The corresponding succinimide intermediates (i.e., Su₃₈₂NN and NSu₃₈₇N) were not detected. Reaction kinetics for the intact protein are consistent with parallel irreversible first-order deamidation to produce the singly deamidated products, followed by their further degradation to produce the doubly deamidated product [Fig. 5(B)]. In the discussion below, reaction kinetics for the NNN model peptide are presented first followed by results for the intact protein.

Deamidation kinetics in the NNN synthetic peptide. Kinetic profiles for deamidation in the NNN synthetic peptide are shown in Figure 6. The NNN peptide degrades in a pseudo-first order manner with ~40% remaining after 100 h. Loss of the parent peptide is accompanied by transient formation of the succinimide intermediate and monotonic increase in the isoD- and D-containing products at N₃₈₂ (i.e., isoD₃₈₂NN and D₃₈₂NN, respectively), which are formed in the ratio of ~4:1 (see Fig. 6). The percentages were calculated assuming that sum of the N-, succinimide, isoD- and D-containing species is 100%; possible racemization and side reactions (e.g., clipping) are not included in the analysis.

The kinetic data for the parent tryptic fragment and its deamidation products were fitted simultaneously to the reaction scheme in [Fig. 5(A)] to provide values for the rate constants k_{1p} , k_{2p} , and k_{3p} (Table I). The regression lines are in close agreement with the data (see Fig. 6) and the coefficients of variation are relatively small (%CV, Table I), indicating a good fit. The calculated half-life for loss of the parent NNN synthetic peptide is ~81 h (i.e., $0.693 (k_{1p})^{-1} = 3.4$ days), in reasonable agreement with the value of 110 h previously reported by Chelius *et al.* for deamidation in this fragment of an Fc IgG.¹⁸ Because the two studies were conducted in solutions of identical pH, storage temperature, buffer, and buffer concentration, the reasons

Figure 4. High energy MS1 spectra of peaks 3 (**A**) and 4 (**B**) of Figure 3. The peptides were identified using the y' ions as the doubly deamidated and the D_{387} peptide, respectively. Other ions were used to confirm the products. See the electronic version of this article for enlarged figures.

for this difference are unclear, but may involve differences in peptide concentration. Deamidation at N_{387} and N_{388} was not detected during the time course of the studies reported here. Because Tyr is C-terminal to N_{388} , the absence of deamidation products at this site is not surprising as steric hindrance by the bulky Tyr residue typically slows deamidation.⁶ The absence of deamidation products at N_{387} suggests that the presence of a second N, C-terminal to the N of interest, slows deamidation rate. Long-term incubation of the digests at 37°C led to clipping so that the slower deamidation at N_{387} and N_{388} could not be monitored.

Deamidation kinetics in the intact protein. Kinetic profiles for deamidation in the intact Fc IgG are shown in Figure 7. The fully N-containing form of the protein (i.e., NNN) undergoes monotonic loss with

corresponding increases in the singly deamidated (i.e., iso D_{382} NN, ND $_{387}$ N) and doubly deamidated (i.e., iso D_{382} D $_{387}$ N) products. At time zero, the percentage of the fully N-containing form is less than 100 and the percentage of the iso D_{382} NN product is greater than zero, suggesting that partial deamidation of N_{382} occurs during sample preparation or is initially present in the protein. The D_{382} product (i.e., D_{382} NN) was also detected throughout the study at a constant level of ~2–3% (not shown), consistent with its formation during sample preparation.

Kinetic analysis of deamidation in intact Fc IgG was performed according to the reaction scheme shown in Figure 5(B). The D_{382} NN product was treated as an artifact of sample preparation and measured amounts of D_{382} NN (1.5–2%) were reassigned to the parent form (NNN). Kinetic data for the parent

Figure 5. Reaction scheme showing the kinetics of deamidation of **(A)** the NNN synthetic peptide and **(B)** intact Fc IgG.

protein and its degradation products then were fitted simultaneously to provide the microscopic rate constants k_1 and k_2 , assuming that the rate of deamidation at each site is unaffected by deamidation at the second site (i.e., $k_1 = k_4$ and $k_2 = k_3$; [Fig. 5(B)]). Simultaneously, fitting the time-dependent profiles for the four species with a model containing two global fitting parameters produced good agreement between experimental and theoretical values (see Fig. 7).

The calculated half-life for the overall loss of the parent protein is ~ 108 days (i.e., $0.693 (k_1 + k_2)^{-1}$; Table I), ~ 30 -fold slower than the 3.4 day half-life observed for the NNN synthetic peptide. The rate-limiting step in formation of isoD₃₈₂NN in the synthetic

Table I. Kinetic Parameters for Deamidation in Model Peptides and in the Intact Fc Fragment

Parameter ^a	Value (days) ⁻¹	S.E. ^b (days) ⁻¹	CV (%) ^c
Model peptides			
k_{1p}	0.206	0.002	0.9
k_{2p}	0.69	0.05	7.2
k_{3p}	2.8	0.2	7.1
Intact Fc fragment ^d			
k_1	0.0039	0.00006	1.5
k_2	0.0025	0.00006	2.4

^a Values determined by nonlinear regression of data in Figures 6 and 7. See Figure 5 for kinetic schemes and parameter definitions.

^b S.E. = standard error of regression.

^c CV (%) = percent coefficient of variation, $100 \times (\text{S.E.})/\text{value}$.

^d Regression assumes $k_1 = k_4$ and $k_2 = k_3$; see Figure 5.

peptide is formation of the succinimide rather than its hydrolysis (i.e., $k_{1p} < k_{3p}$, Table I), as expected for deamidation in unstructured peptides. The rate of formation of isoD₃₈₂NN in the synthetic NNN peptide is then roughly equal to the rate of succinimide formation (k_{1p}), a rate that is ~ 50 -fold greater than the rate of isoD₃₈₂NN formation in the intact protein (k_1 vs. k_{1p} , Table I). This suggests that slowing of deamidation at N₃₈₂ is a dominant contribution to the overall reduction in degradation rate. The rates of formation of the two singly deamidated products, isoD₃₈₂NN and ND₃₈₇N, are comparable in the intact protein (k_1 vs. k_2 , Table I).

Molecular dynamics simulation

Using the PDB crystal structure for human Fc-IgG1 (1h3u), SYBYL MDS showed that N₃₈₂ is located in a

Figure 6. Kinetic profile for deamidation of the NNN synthetic peptide stored at 37°C, pH 7.4. The symbols are experimental data points; the lines represent the fit obtained with the kinetic model described in the text. $n = 3 \pm \text{S.D.}$

Figure 7. Kinetic profile for deamidation in Fc IgG stored at 37°C, pH 7.4. The symbols are experimental data points; the lines represent the fit obtained with the kinetic model described in the text. $n = 3 \pm \text{S.D.}$

Table II. Solvent Accessibility and Interatomic Distances by SYBYL MDS^a

Asparagine residue	Solvent accessibility (%)	C=O to (N + 1)NH distance, (Å)
N382	71.04	3.31
N387	66.82	4.36
N388	40.48	4.13

^a Values determined by SYBYL molecular dynamics simulation (MDS) of PDB entry 1h3u.

loop between two β -sheet regions, whereas the N₃₈₇ and N₃₈₈ residues are located in a relatively unstructured loop (see Supporting Information, Appendix II, for visualization). Solvent accessibility values and interatomic distances were calculated for all three N residues (Table II). Previous reports suggest that deamidation is favored by an interatomic distance between the attacking backbone nitrogen and the N-side chain carbonyl (i.e., C=O to (N + 1)NH, Table II) of 1.89 Å or less, while distances greater than 4.9 Å are unfavorable. Here, the calculated interatomic distances are greater than 1.89 Å and less than 4.9 Å for all three N residues (Table II), suggesting some susceptibility to deamidation based on structure. The smallest simulated interatomic distance corresponds to N₃₈₂, experimentally observed to deamidate most readily (Table II). Calculated solvent accessibility values decreased in the order N₃₈₂ > N₃₈₇ > N₃₈₈, again corresponding to the measured deamidation rates (Table II).

The MDS results also help to explain the somewhat unusual distribution of deamidation products in the intact protein, in which: (a) only the isoD product is observed at N₃₈₂ (i.e., isoD₃₈₂NN but not D₃₈₂NN), (b) only the D product is observed at N₃₈₇ (i.e., ND₃₈₇N but not NisoD₃₈₇N), and (c) no deamidation is observed at N₃₈₈. The formation of isoD₃₈₂NN to the exclusion of D₃₈₂NN suggests that hydrolysis of the succinimide occurs preferentially at the carbonyl contributed by the backbone amide and not at the carbonyl contributed by the N side chain. Such preferential hydrolysis could be related to the location of N₃₈₂ between two β -sheet regions, which may hinder the attack of water from the side-chain side. In contrast, hindered hydrolysis of the succinimide is an unlikely explanation for the preferential formation of D at N₃₈₇, because the site is relatively solvent exposed. The absence of deamidation products at N₃₈₈ probably reflects the combined effects of primary sequence, with deamidation hindered by the N₃₈₈ + 1 Tyr residue, and the limited solvent exposure of this site (Table II).

Discussion

The rapid, high-resolution UPLC method was able to separate the N-containing 22-amino acid tryptic frag-

ments and their deamidated variants. The parent NNN peptide was well resolved from its deamidated counterparts [Figs. 1(A) and 3(A)], thus emphasizing the utility of UPLC as a rapid, high resolution technique. The synthetic NNN peptide deamidated ~ 30 -times faster than the intact protein, suggesting that the rate of deamidation is slowed by protein structure. Protein structure also affected the product profiles. In the NNN synthetic peptide, the N₃₈₂ site deamidated to form the expected isoD₃₈₂ and D₃₈₂ products in the typical ratios of 4:1; in the intact protein, only the isoD₃₈₂ is observed without detectable D₃₈₂. Molecular dynamics simulations supported the formation of isoD₃₈₂ because the N₃₈₂ residue was shown to be located in a structurally constrained orientation. In the NNN synthetic peptide, deamidation was not observed at the N₃₈₇ site, because peptide bond hydrolysis ("clipping") near the N- and C-termini prevented observation of the slower N₃₈₇ deamidation reaction. In the intact protein, only the D₃₈₇ product was observed at this site with no appearance of the corresponding isoD₃₈₇ form. Together, these results indicate a significant role of the local secondary structure in both deamidation rate and product profiles in this region of Fc IgG.

The product profile is similar to that reported previously by Chelius *et al.* for Fc IgG.¹⁸ That group also observed deamidation at the N₃₈₂ and N₃₈₇ sites with exclusive formation of the isoD₃₈₂ and D₃₈₇ products. However, the doubly deamidated product was not detected by Chelius *et al.* for the intact protein, while the succinimide intermediate at N₃₈₂ was observed. Their detection of the succinimide at N₃₈₂ supports the hypothesis that the exclusive formation of the isoD product at this site involves the preferential hydrolysis of backbone side of the succinimide. As noted above, our failure to detect the succinimide in the intact Fc IgG samples may be the result of its hydrolysis during tryptic digestion. Recently, Ren *et al.* reported the degradation products in an Fc fusion protein, and detected the N(isoD)₃₈₇N deamidation product, albeit at levels considerably lower than the corresponding ND₃₈₇N product.²⁴ The group also detected succinimide intermediates at both the N₃₈₂ and N₃₈₇ sites.²⁴ These results further support the role of structure-dependent hydrolysis of the succinimide in altering the D:isoD product ratio.

In the work by Chelius *et al.*, the half-life for loss of a G₃₆₉-K₃₉₀ tryptic fragment (110 h) is comparable to that for our NNN synthetic peptide (81 h; $0.693 (k_1)^{-1}$, Table I). In contrast, that group reported a half-life for loss of the intact Fc IgG protein of 38 days, while we observed a half-life of 108 days ($0.693 (k_1 + k_2)^{-1}$, Table I), a difference of nearly threefold. The 38-day half-life was estimated based on concentrations of the parent G₃₆₉-K₃₉₀ tryptic fragment measured at three time points; the 108-day half-life reported here is the result of simultaneous nonlinear

regression of multiple products at more than a dozen time points, and is therefore supported by considerably more kinetic data. Minor differences in experimental conditions for the two groups may also have influenced the results.

Similar results have also been reported for isomerization of a D-residue located in the CDR1 region of an antibody,^{25,26} a reaction that also proceeds via a succinimide intermediate. In that work, succinimide hydrolysis in the denatured protein yielded isoD to D in a ratio of 3.5:1, but in the native antibody only D was formed under neutral and basic conditions. Unpublished work on deamidation in the CDR1 region by Vlasak *et al.* has shown that the ratio of isoD to D changes over time, presumably due to isomerization of isoD to D as the process tends toward equilibrium. Wearne *et al.*²⁷ studied deamidation in RNase A and observed that the structured protein deamidates 30-fold slower at N₆₇ than in the reduced and denatured form, a difference attributed to the local β -turn. Capasso *et al.* later observed that the isoD:D ratio for this protein was initially as high as in unstructured peptides (~3:1) but decreased to 1:2 as the reaction approached equilibrium.⁹ A study by Harris *et al.* of Herceptin®, a commercial monoclonal antibody drug product, showed that N₃₀ in the light chain deamidates to form only the D product.²¹ Atypical isoD:D ratios have also been observed for crystalline.^{10,28} Capasso *et al.*^{29–31} proposed that the D product will dominate over its isoD counterpart wherever the D configuration has a lower energy, which in turn depends on the protein three-dimensional structure, and noted that equilibration between isoD and D via the succinimide occurs more slowly than deamidation. A similar rationale may hold for deamidation at the N₃₈₇ of the Fc IgG studied here.

An alternative deamidation mechanism has been proposed by Clarke *et al.*,³² in which the backbone carbonyl oxygen rather than the (N + 1) nitrogen attacks the side chain carbonyl to form an isonimide ring with release of water. Subsequent hydrolysis of the symmetric isonimide ring from either side yields only the D-containing product, unlike hydrolysis of the succinimide which produces both isoD- and D-containing products. This mechanism has been promoted in several studies in which the formation of the D-containing product was observed to the exclusion of the isoD product, with the absence of the succinimide intermediate.^{33,34} In this study, the formation of the D₃₈₇ to the exclusion of isoD₃₈₇ is consistent with an isonimide mechanism at that site. However, because the isonimide intermediate was not detected here, this explanation must be regarded as speculative.

Methods

Materials

Fc-IgG was prepared at Merck Research Laboratories from a humanized monoclonal antibody expressed in

NSO cells, a murine myeloma cell line. The human IgG1 Fc fragment was prepared by digestion with immobilized papain (Pierce, Rockford IL), followed by protein A purification. Impurities were further removed by cation-exchange high performance liquid chromatography (HPLC) using a ProPac WCX-10 9 × 250 mm column (Dionex, Sunnyvale, CA) and gradient elution (mobile phase A: 10 mM sodium phosphate pH 6.5; mobile phase B: 10 mM sodium phosphate, 0.5M NaCl pH 6.5; gradient: 3–26% B in 20 min at a flow rate of 2 mL/min).

Synthetic model peptides corresponding to the tryptic fragment G₃₆₉-K₃₉₀ (i.e., GFYPDSIAVEWESNGQPENNYK) and its deamidated variants were purchased from several sources. The parent peptide GFYPDSIAVEWESNGQPENNYK (abbreviated “NNN”) and the D₃₈₇-containing deamidated variant, GFYPDSIAVEWESNGQPEDNYK (abbreviated “ND₃₈₇N”), were synthesized by the Biochemical Research Services Laboratory of the University of Kansas. The isoD-containing variants, GFYPDSIAVEWES(isoD)GQPENNYK (abbreviated “isoD₃₈₂NN”) and GFYPDSIAVEWESNGQPE(isoD)NYK (abbreviated “NisoD₃₈₇N”), were purchased from American Peptide Company (Sunnyvale, CA). All other chemicals and reagents were of the highest commercial grade and used without further purification. Milli-Q water was used to prepare all solutions.

Sample preparation and accelerated stability studies

Accelerated stability studies were conducted for two types of samples at 37°C and pH 7.5: (i) the synthetic model peptide, NNN, and (ii) the fully-folded, intact Fc IgG (~50 kD) (“intact protein”). For the NNN peptide (i), samples contained 50 μ L of 0.5 mg/mL of peptide in 100 mM Tris-HCl buffer (pH 7.5). Samples were stored in capped microcentrifuge tubes at 37°C for up to 5 days. Triplicate samples were withdrawn at designated time intervals and stored at –20°C before LC-MS analysis.

Stability studies for the intact protein (ii) were conducted similarly, except that samples were subjected to tryptic digestion prior to analysis. Samples contained 50 μ L of 0.5 mg/mL Fc IgG in 100 mM Tris-HCl buffer (pH 7.5), and were stored in capped microcentrifuge tubes at 37°C for up to 70 days. At each time point, triplicate samples were withdrawn, stored at –20°C, and then digested with trypsin before LC-MS analysis. Tryptic digestion followed the protocol reported by Chelius *et al.*¹⁸ Briefly, an aliquot of intact Fc was first exchanged to 6M guanidine-HCl, 0.2M Tris-HCl, 1 mM EDTA (pH 7.5) such that 100 μ L of 0.5 mg/mL Fc was obtained. Reduction was performed by addition of 2 μ L of 0.5M dithiothreitol (DTT) at 37°C for 40 min followed by alkylation by 4 μ L of 0.5M iodoacetamide at room temperature for 40 min. The reduced, alkylated sample was then buffer

exchanged with a 10,000 MW cut off membrane (Microcon centrifugal filter, Millipore, Billerica, MA) to 100 mM Tris buffer (pH 7.5) such that the Fc concentration was maintained at 0.5 mg/mL. Trypsin (Worthington, MA) was then added in a trypsin:protein ratio of 1:50 (w/w) and incubated for 2 h at 37°C. A second equal amount of trypsin was then added with incubation for 2 more hours, bringing the final trypsin:protein ratio to 1:25 (w/w). Samples were withdrawn and the reaction quenched by addition of 20% formic acid to a final concentration of 2%.

Mass spectrometric analysis (UPLC/+ESI-MS) of Fc-IgG and fragments

The analytical strategy for monitoring deamidation in Fc-IgG involved tryptic digestion of intact protein, ultraperformance liquid chromatography with tandem electrospray ionization mass spectrometry (UPLC/+ESI-MS) analysis of the fragments, and quantitation of peptides and their deamidated variants using extracted ion chromatograms (EIC). UPLC is a recent innovation in high performance liquid chromatography (HPLC), in which unique small particles and very low column volumes allow for greater throughput and rapid analysis. In the G₃₆₉-K₃₉₀ sequence, deamidation can occur at each of the N residues (N₃₈₂, N₃₈₇, N₃₈₈) to produce the succinimide (Su) intermediate, as well as the isoD and D variants. Because each of the three sites can therefore exist in four forms (i.e., N, isoD, D, Su), a total of 64 (i.e., 4³) variants is possible. Identification and quantitation of the product mixture thus requires analytical methods capable of resolving similar peptides in complex mixtures. UPLC is well-suited to the analysis of such mixtures, and was selected as the separation method to minimize possible coelution of isobaric products.

UPLC was performed using a Waters Acquity UltraPerformance liquid chromatography system (Waters, Milford, MA) with a BEH C18 column (1 mm × 150 mm, 1.7 μm particle size) at a flow rate of 100 μL/mL/min and a column temperature of 35°C. Approximately 10 μg of protein or peptide was injected for each analysis (10 μL/injection). Gradient elution was used, (Solvent A: 99% H₂O, 1% methanol, 0.08% formic acid; Solvent B: 99% acetonitrile, 1% H₂O, 0.06% formic acid), with the profile: 0–1 min, solvent B at 1% (v/v); 1–2 min, solvent B increased to 10% (v/v); 2–19 min, solvent B increased to 18% (v/v); 19–45 min, solvent B increased to 22% (v/v); followed by washing and re-equilibration. Mobile phase solvent compositions were: Solvent A = 99% H₂O, 1% methanol, 0.08% formic acid; Solvent B = 99% acetonitrile, 1% H₂O, 0.06% formic acid.

The UPLC eluate was coupled to a Micromass[®] Q-ToF II mass spectrometer (Waters, Milford, MA) operated in the positive ion mode (+ESI) with a cone voltage of 35 V and a collision voltage of 30 eV. The

relatively high collision voltage produces partial fragmentation of the parent ions and was adopted as an alternative to MS/MS analysis because it produced a sufficient number of daughter ions to allow unambiguous identification of deamidation sites. The G₃₆₉-K₃₉₀ fragment and the corresponding synthetic peptide carry a charge of +2, a theoretical mass of 2544.67 Da and thus an expected *m/z* value of 1272.57. Data were analyzed using MassLynx[®] software (Waters). Quantitation of native peptides and their deamidated variants was based on the isotopic peak intensities of each form. EIC scans were conducted for the *m/z* range 1264–1277 to allow identification and quantitation of the isoD- and D-deamidation products (+1 amu, +0.5 *m/z*) and the Su intermediate (−17 amu, −8.5 *m/z*). Analyte elution times typically ranged from 30 to 45 min; to facilitate comparison of EIC scans, retention times were normalized by setting the retention time of the NNN peptide peak to 37 min [Figs. 1(A) and 3(A)]. The theoretical isotope distribution for the parent peptide was used to separate the contribution of a doubly deamidated product, which eluted as a shoulder with the parent in studies of the undigested protein. The ionization efficiency of the parent peptide was assumed to be equivalent to that of its deamidated forms.

Kinetic modeling

The time-varying concentrations of the N-containing parent species and their deamidated variants were subjected to kinetic analysis to determine the rate constants for deamidation. The native species and the deamidation products form an ensemble that evolves in time based on the microscopic rates of interconversion between species. Assuming that the deamidation process can be well represented as a unimolecular, first-order reaction, the time-dependencies of the population of “*n*” species can be described by a system of linear differential equations of the form:

$$\frac{d[A_i]}{dt} = \left(\sum_{j \neq i} k_{ji}[A_j] \right) - [A_i] \times \sum_{j \neq i} k_{ij} \quad (1)$$

where *A_i*, *A_j*, represent molar fractions of any two species in the ensemble, and *k_{ji}* is the microscopic rate constant describing the conversion of species *A_j* into species *A_i*:

The system of linear differential equations was solved using classical procedures³⁵ to determine the eigenvalues of the system λ_i (*i* = 1, *n*). The eigenvalues λ_i represent experimental rate constants and can be expressed as linear combinations of the microscopic rate constants, *k_{ji}*. For a system comprising “*n*”

distinct species there are at most “ $n - 1$ ” values for λ_i , because one solution is always zero, corresponding to the equilibrium value. The solutions of the system, $[A_i](t)$, were expressed as a sum of exponentials of the form:

$$[A_i](t) = \sum_j \alpha_{ij} \exp(-\lambda_i t) \quad (3)$$

where α_{ij} represent coefficients that are specific to each species, “ i ”. The coefficients α_{ij} are determined from the system of linear equations and from initial ($t = 0$) and equilibrium ($t = \infty$) conditions. Because the system is relatively uncomplicated, the eigenvalues and eigenvectors were calculated “by hand”, that is, no particular application was used to solve the system of equations describing the time-dependence of each species. The explicit form of the system of differential equations, with their corresponding eigenvalues and solutions for the molar fractions of the species, is available as Supporting Information (online).

For a particular data set (i.e., describing either the deamidation of a model peptide or the intact Fc), the microscopic rate constants were determined by performing a global fit, in which the fitting of the experimental points was performed simultaneously for all kinetic traces using the program SigmaPlot (ver. 10.0, Systat Software, San Jose, CA). Statistical information regarding the fitting results, including standard and relative errors of regression coefficients, was provided by the SigmaPlot routines.

Molecular dynamics simulations (MDS)

The Fc IgG1 structure was visualized using SYBYL (Version 8.0, Tripos, St. Louis, MO) which enabled direct measurement of key interatomic distances as represented in the 1h3u RCSB Protein Data Bank (PDB) crystal structure.³⁶ The relative solvent accessibility of specific residues was determined in SYBYL by generating Connolly surfaces for those amino acids and evaluating the total area of each resulting surface.

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