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Formation of Pyroglutamic Acid from N-Terminal Glutamic Acid in Immunoglobulin Gamma Antibodies

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The status of the N-terminus of proteins is important for amino acid sequencing by Edman degradation, protein identification by shotgun and top-down techniques, and to uncover biological functions, which may be associated with modifications. In this study, we investigated the pyroglutamic acid formation from N-terminal glutamic acid residues in recombinant monoclonal antibodies. Almost half the antibodies reported in the literature contain a glutamic acid residue at the N-terminus of the light or the heavy chain. Our reversed-phase high-performance liquid chromatography−mass spectrometry method could separate the pyroglutamic acid-containing light chains from the native light chains of reduced and alkylated recombinant monoclonal antibodies. Tryptic peptide mapping and tandem mass spectrometry of the reduced and alkylated proteins was used for the identification of the pyroglutamic acid. We identified the formation of pyroglutamic acid from N-terminal glutamic acid in the heavy chains and light chains of several antibodies, indicating that this nonenzymatic reaction does occur very commonly and can be detected after a few weeks of incubation at 37 and 45 °C. The rate of this reaction was measured in several aqueous buffers with different pH values, showing minimal formation of pyroglutamic acid at pH 6.2 and increased formation of pyroglutamic acid at pH 4 and pH 8. The half-life of the N-terminal glutamic acid was ∼9 months in a pH 4.1 buffer at 45 °C. To our knowledge, we showed for the first time that glutamic acid residues located at the N-terminus of proteins undergo pyroglutamic acid formation in vitro.

Recombinant monoclonal antibodies have become an important modality in protein pharmaceutics due to their predictable properties, controlled functions, and long circulation half-life.

Although antibodies are relatively stable molecules, they are nevertheless subject to a variety of enzymatic and nonenzymatic degradation reactions that can occur during manufacturing, formulation, and storage. A number of molecular modifications for antibodies have been reported in the literature including aggregation, oxidation, proteolytic cleavage, disulfide-bond scrambling, glycosylation, deamidation, and isomerization.[1−4] These modifications have the potential to decrease biological activity, as well as alter pharmacokinetics and antigenicity.

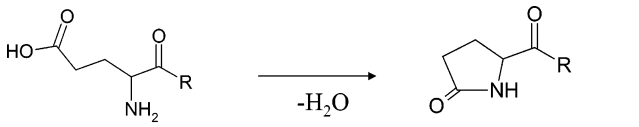

Figure 1. Formation of pyroglutamic acid (pyroE) from glutamic acid.

Pyroglutamic acid (pyrrolidonecarboxylic acid, pyroE) has been identified at the N-terminus of a variety of proteins, including the light and heavy chains of antibodies, fibrinogen, collagen, and kinines, just to name a few.[5] The nonenzymatic formation of pyroglutamic acid from N-terminal glutamine has been known for many years.[6−8] However, much less is known about the formation of pyroglutamic acid from glutamic acid in proteins (see Figure 1). The formation of pyroglutamic acid from glutamic acid has been believed to be an enzymatic reaction.[9,10] Twardzik and Peterkofsky showed that N-terminal pyroglutamic acid can be derived from glutamic acid without prior conversion to glutamine but concluded that N-terminal pyroglutamic acid formation from glutamic acid must be enzymatic rather than spontaneous.[9] More recently, Beck and co-workers identified and characterized the formation of pyroglutamic acid from glutamic acid at the N-terminus of therapeutic peptides.[11] They showed that, in the solid

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state, two peptides, each containing N-terminal glutamate, form pyroglutamate if incubated at 37 °C for a few months and that the formation of pyroglutamate is accelerated in the presence of acetate and trifluoroacetate compared to hydrochloride.[11] A proposed mechanism for the weak acid-catalyzed pyroglutamic acid formation has been presented by Dimarchi et al.[12]

In this report, we have identified and characterized the pyroglutamic acid formation from glutamic acid at the N-terminus of recombinant monoclonal antibodies. N-Terminal glutamic acid residues are very common in immunoglobulin gamma antibodies. Almost a half of the antibodies reported in the literature contain a glutamic acid residue at the N-terminus of the heavy chain and over 6% at the N-terminus of the immunoglobulin light chain κ (over 80% of immunoglobulin light-chain κ contain an aspartic acid residue).[13] Zhang and Henzel reported that 16 out of 270 human proteins contain an N-terminal glutamic acid residue, suggesting that more than 5% of all human proteins could contain glutamic acid residues at their N-terminus.[14] To our knowledge, we showed for the first time that the nonenzymatic cyclization reaction of glutamic acid at the N-terminus of proteins does occur in vitro at mild acid/basic pH conditions typically used during pharmaceutical stability studies.

EXPERIMENTAL SECTION

Material. The recombinant monoclonal antibodies analyzed in this study were expressed in Chinese hamster cells and purified using standard manufacturing process steps. The antibodies were prepared in 10 mM sodium acetate, 5% sorbitol, pH 5.2, at a concentration of 30 mg/mL and stored at −70 °C. Upon thawing, buffer exchange was performed using Slide-A-Lyzer dialysis cassettes (Pierce, Rockford, IL) according to the manufacturer's protocol. Protein concentration after buffer exchange was adjusted to 10 mg/mL. The following buffers were used in this study: 10 mM succinate (pH 4.1, pH 4.7, pH 5.2, pH 5.7), 10 mM phosphate (pH 6.2, 7.0, 8.0), 10 mM acetate (pH 5.5), and 10 mM histidine (pH 5.5). All reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified.

Reduction and Alkylation. The recombinant human antibodies were diluted to 2 mg/mL with 6 M guanidine hydrochloride, 0.25 M Tris-HCl pH 7.5, 1 mM EDTA to a total volume of 500 μL. The solution was then treated with 5 μL of 0.5 M DTT stock solution (final DTT concentration of 5 mM), and the reaction mixture was placed at 37 °C for 30 min. After this, the sample was cooled to room temperature and treated with a 12-μL aliquot of 0.5 M iodoacetic amide to a final concentration of 12 mM, and the sample was placed at room temperature for 40 min. The reduced and alkylated material was buffer exchanged using a gel filtration NAP-5 column (Amersham BioSciences, Uppsala, Sweden). The column was equilibrated with 10 mL of 0.1 M Tris-HCl pH 7.5 buffer, and a 500-μL volume of sample was loaded on the column. The reduced and alkylated antibody was eluted with a 1-mL aliquot of the 0.1 M Tris-HCl pH 7.5 buffer resulting in a final reduced alkylated protein sample of ∼1 mg/mL. Samples were stored at −70 °C and thawed immediately prior to use.

HPLC Separation of Reduced and Alkylated Antibodies. The reduced and alkylated antibodies were analyzed using an Agilent 1100 HPLC unit equipped with a diode-array detector, autosampler, microflow cell, and temperature-controlled column compartment (Agilent, Palo Alto, CA). The column was heated at 80 °C to enhance separation. Mobile phase A consisted of 0.1% trifluoroacetic acid (TFA; Pierce, Rockford, IL) in water, and mobile phase B consisted of 90% 1-propanol and 0.085% TFA in water. Separation was performed on a Zorbax SB column (150 × 1.0 mm) packed with 3.5-μm particles, 300-Å pore size C8 resin (Agilent). The flow rate was 50 μL/min, and 4 μg of the reduced and alkylated samples were injected. The column was initially equilibrated with 15% solvent B. Three minutes after sample injection, the concentration of mobile phase B was increased to 42% over 27 min. The column was reequilibrated by ramping up mobile phase B to 100% over 1 min, holding for 5 min at 100% B, and dropping down to 15% B over 1 min, followed by 5 min at 15% B. UV absorption was monitored at 214 and 280 nm.

Peak areas were calculated using ChemStation software (Agilent) using the tangent skimming technique for incomplete resolved peaks. Quantitation based on peak areas of incomplete resolved peaks was described in great detail before.[15]

The same HPLC method was used for fraction collection with the following minor modifications: the column was a Zorbax SB column (150 × 4.6 mm), 3.5-μm pore size, 300-Å C8 resin. The flow rate was 1.0 mL/min, and 20 μg of protein was injected. Fractions were collected manually.

Mass Spectrometry of the Reduced and Alkylated Protein. Electrospray ionization time-of-flight (ESI-TOF) mass spectrometry of reduced and alkylated antibodies was performed on a Q-TOF Micro mass spectrometer (Waters, Milford, MA) equipped with an ESI atmosphere−vacuum interface. Mass spectrometry was performed on-line with the reversed-phase chromatography. The ESI-TOF mass spectrometer was set to run in positive ion mode with a capillary voltage of 3400 V, sample cone voltage of 50 V, and m/z range of 1000−5000 with a mass resolution of 5000. The instrument was tuned and calibrated using multiply charged ions of trypsinogen (MW 23981.0, T1143, Sigma). The deconvolution of electrospray ionization mass spectra was performed using a MaxEnt1 algorithm, which is a part of the MassLynx software (Waters).

Tryptic Digestion. The collected fractions of the reduced and alkylated antibody from the reversed-phase column were dried using a Speed Vac concentrator. The samples were reconstituted in 100 μL of 0.1 M Tris-HCl to a final concentration of ∼1 mg/mL of antibody. Tryptic digestion was performed for 4 h at 37 °C using two additions of trypsin (Roche, Indianapolis, IN); at the beginning and after the first 2 h of digestion. The enzyme/protein ratio of each addition was 1:50 (w/w). The samples were analyzed immediately after digestion.

HPLC Separation of Tryptic Peptides. The HPLC separation method of the tryptic peptides has been described before.[16] In brief, the tryptic peptides were separated on a Polaris C18 ether column (250 × 2.0 mm Varian, Torrance, CA) using a linear gradient from 0 to 65% B over 195 min. Solvent A was 0.1% TFA in water, and solvent B was 0.089% TFA, 90% acetonitrile (Baker,

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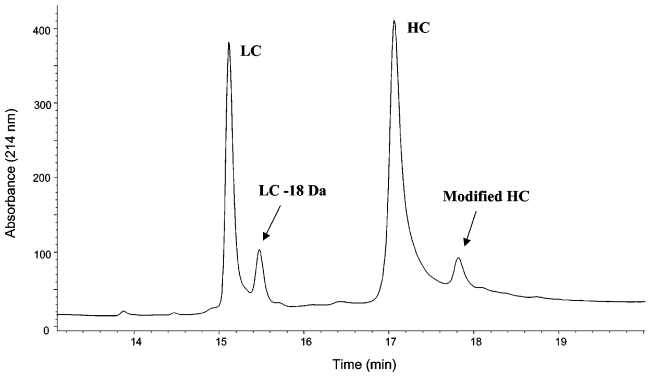

Figure 2. Reversed-phase chromatogram of the reduced and alkylated antibody A exhibiting pyroglutamic acid formation. The light chain (LC) and the heavy chain (HC) are labeled accordingly. The antibody was aged in a pH 4.6 buffer at 45 °C for 3 months. The pyroglutamic formation in the light chain is denoted by the label (LC-18 Da). The modified heavy chain represents a different modification.

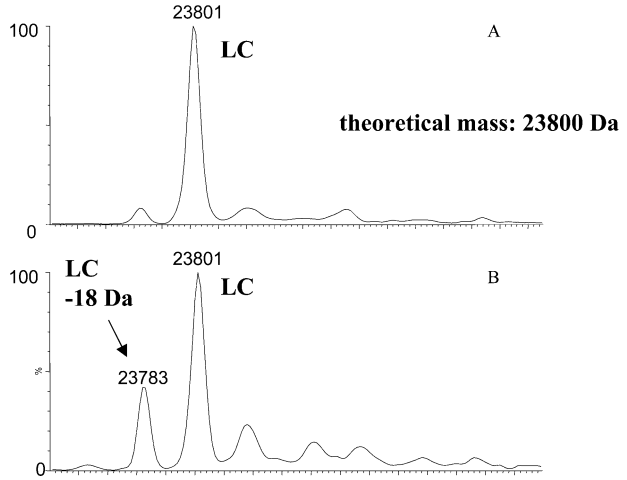

Figure 3. Deconvoluted ESI mass spectra of two light-chain isoforms of antibody A separated on the reversed-phase chromatogram in Figure 2. The theoretical mass was calculated based on the amino acid sequence as 23 800 Da, which matches the observed molecular weight of 23 801. (A) Deconvoluted ESI mass spectrum of the native light-chain main peak eluting at 15.0 min and (B) deconvoluted mass spectrum of the light-chain postpeak eluting at 15.5 min. Both light-chain isoforms were detected in the post light chain peak, since baseline separation of the peaks in Figure 2 was not achieved.

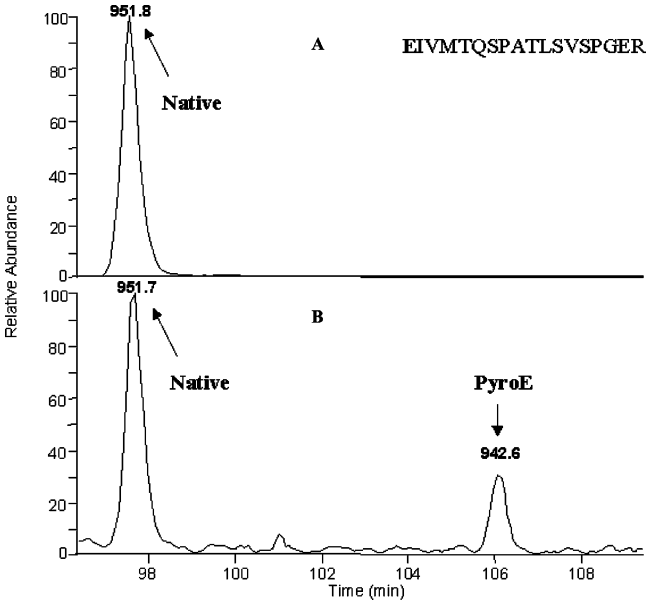

Figure 4. Reconstructed ion chromatograms from the tryptic peptide maps of the two light-chain isoforms of antibody A separated on reversed-phase chromatogram in Figure 2 (A, light-chain main peak; B, light-chain postpeak). Shown are only the selected ions at *m*/*z* 951.5 and 942.7 corresponding to the doubly charged N-terminal tryptic peptides of the light-chain variants with glutamate and pyroglutamate, respectively.

Phillipsburg, NJ) in water. Before sample injection, the column was equilibrated with 0% solvent B. The column temperature was maintained at 50 °C. The flow rate was 0.2 mL/min, and a total of 20 μg of protein digest was injected onto the column for analysis.

Mass Spectrometry of Tryptic Peptides. The mass spectrometry method for the analysis of the tryptic peptides has also been described before.[16] Briefly, the HPLC was directly coupled to a Finnigan LCQ Deca ion trap mass spectrometer (Thermo Electron, San Jose, CA) equipped with an electrospray ionization source. The spray voltage was 4.5 kV, and the capillary temperature was 250 °C. The fragmentation mass spectra were obtained using ion trap collision energies of 35%. Peptides were identified automatically by two different computer programs. SEQUEST algorithm of BioWorks version 3.1 (Thermo Finnigan, San Jose, CA) was used to correlate the experimental tandem mass spectra against theoretical tandem mass spectra from a database. A software program written in-house was also employed to correlate the experimental tandem mass spectra against theoretical tandem mass spectra generated from the known antibody amino acid sequence for peptide identification.[17,18]

RESULTS AND DISCUSSION

Identification of Pyroglutamic Acid Formation from Glutamic Acid in the Light Chain of a Recombinant Monoclonal Antibody. Reversed-phase LC−MS analysis of several reduced and alkylated recombinant monoclonal antibodies resulted in the appearance of a light-chain postpeak with an apparent molecular mass loss of 18 Da compared with the native, unmodified light chain. Figure 2 shows the reversed-phase chromatogram of the reduced and alkylated antibody A. The deconvoluted mass spectra of the two light-chain isoforms are shown in Figure 3A and B, indicating the presence of a −18-Da modification on the later eluting isoform. The theoretical mass of the light chain of the antibody was calculated to be 23 800 Da, which correlates well with the observed mass of the unmodified light chain of 23 801 Da (Figure 3A). Additionally, a modified heavy chain could be identified (Figure 2), which was not related to the formation of pyroglutamic acid and was not the focus of this study. To identify the cause of the mass difference between the unmodified light chain and the modified light chain (Figure 2), the two light-chain fractions were collected and each fraction was digested with trypsin. The tryptic peptides were separated using reversed-phase HPLC and analyzed by mass spectrometry. The reconstructed ion chromatograms of the doubly charged N-terminal peptides of the light-chain main peak and the light-chain postpeak are shown in Figure 4A and B, respectively. The only difference between the two samples was the formation of pyroglutamic acid at the

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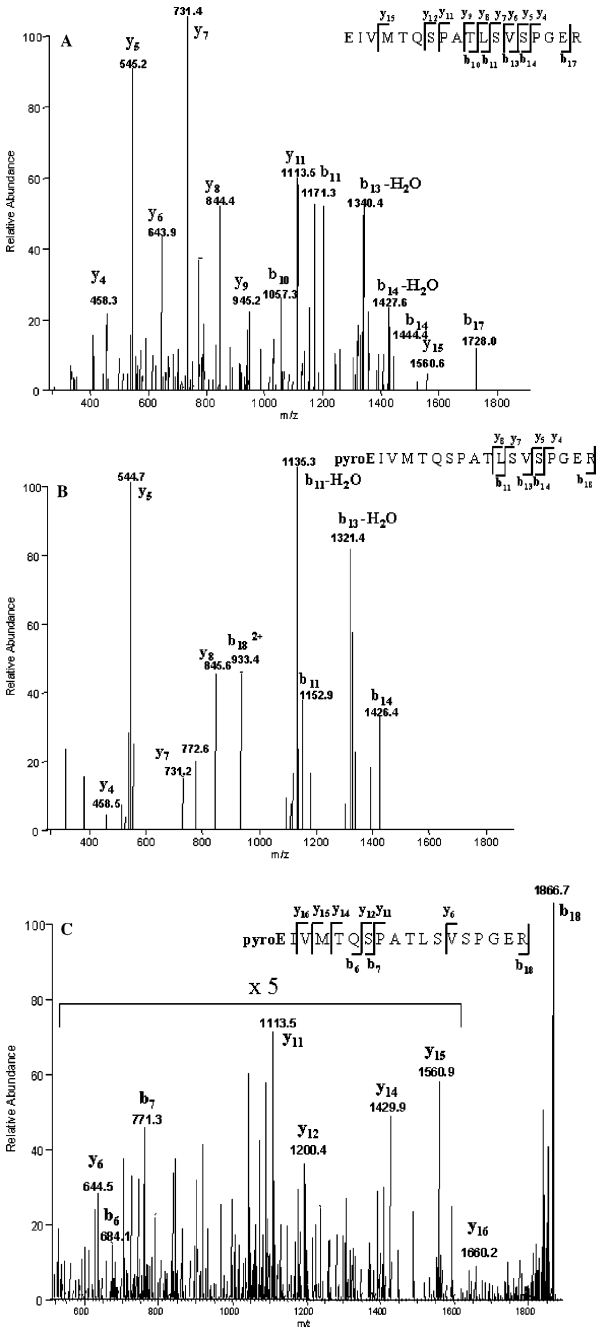

Figure 5. Tandem mass spectra derived by collision induced dissociation of the $(M + 2H)^{2+}$ precursor ions at *m/z* 951.5 (A), *m/z* 942,7 (B), and the $(M + H)^{+}$ precursor ion, *m/z* 1883.7 (C). The peptides were identified as EIVMTQSPATLSVSPGER (A) and its pyroE-containing isoform (B, C).

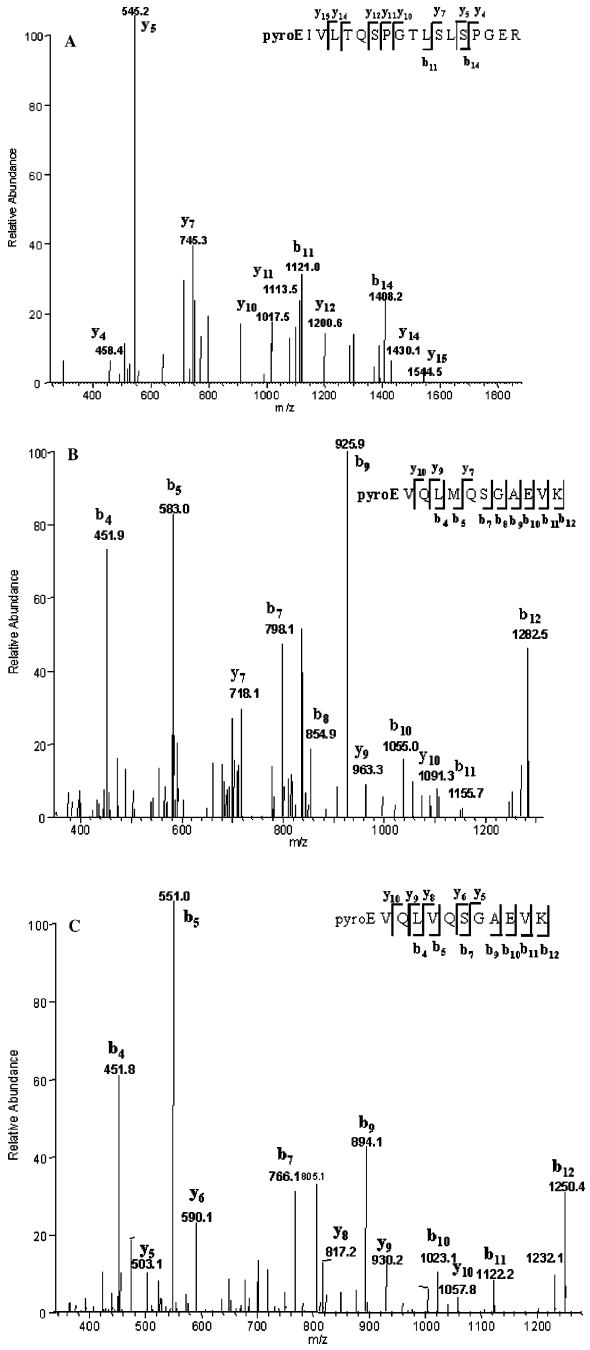

Figure 6. Tandem mass spectra derived by collision-induced dissociation of the $(M + 2H)^{2+}$ precursor ions at *m/z* 933.5 (A), 1300.4 (B), and 1268.5 (C) from tryptic peptide maps of three different human monoclonal antibodies. The peptides were identified as pyroEIVLTQSPGTLSLSPGER (A), pyroEVQLMQSGAEVK (B), and pyroEVQLVQSGAEVK (C).

N-terminal peptide of the light-chain postpeak (Figure 4B). The modified peptide (pyroEIVMTQSPATLSVSPGER) has a later elution time compared to the native peptide (EIVMTQSPATLSVSPGER), which correlates with the elution order of the intact light-chain variants. The tandem mass spectra of the native N-terminal tryptic peptide and its modified isoform are shown in Figure 5. The tandem mass spectra of the modified isoform contained a series of b ions (Figure 5B: b11, b13, b14, and b18. Figure 5C: b6, b7, and b18) all showing the 18-Da mass loss as compared to the native peptide and a series of y ions (Figure 5B: y4, y5, y7, and y8. Figure 5C: y6, y11, y12, y14, y15, and y16) without any mass change. From the tandem mass spectrum shown in Figure 5C, it was clear that the modification of the peptide, with a mass difference of −18 Da, was within the first two amino acid residues from the N-terminus. The ion at *m/z* 1660.2 (Figure 5C) corresponding to the y16 fragment of peptide EIVMTQSPATLSVSPGER (antibody A), left only the first two amino acids at the N-terminus as the possible source for the loss of 18 Da in molecular mass. The only known modification that would result in the loss of 18 Da for those two residues is the formation of pyroglutamic acid at the N-terminus of the peptide. Thus, the light-chain postpeak was unambiguously identified to result from the

Table 1

protein	N-terminal sequence of light chain (LC) or heavy chain (HC)
antibody A	EIVMTQSPATLSVSPGER (LC)
antibody B	EIVLTQSPGTLSLSPGER (LC)
antibody C	EVQLMQSGAEVK (HC)
antibody D	EVQLVQSGAEVK (HC)

formation of pyroglutamic acid from N-terminal glutamic acid in the protein.

Formation of N-Terminal Pyroglutamic Acid from Glutamic Acid in Other Antibodies. The formation of the N-terminal pyroglutamic acid from glutamate was also observed in three other recombinant monoclonal antibodies. Standard tryptic peptide mapping procedures were used for the identification of pyroE in those antibodies.[16] The N-terminal sequences of the tryptic peptides of the four different antibodies are shown in Table 1. Antibody A and antibody B share a high degree of sequence homology at the N-terminus of the light chains (Table 1). Antibodies C and D have similar N-terminal sequences of heavy chains (Table 1). Reversed-phase separation of the reduced and alkylated antibody B resulted in a light-chain postpeak similar to antibody A (Figure 2). The other two antibodies showed the formation of pyroglutamic acid from glutamic acid at the heavy chains. We could not achieve quantifiable separation of the native heavy chain from the pyroE-containing heavy chains using our reversed-phase chromatography method of reduced and alkylated antibodies C and D. Instead, tandem mass spectra of completely separated peptides generated by tryptic mapping revealed the formation of pyroglutamic acid in the heavy chains of these molecules. Panels A−C in Figure 6 show the tandem mass spectra of the three modified peptides of antibody B, antibody C, and antibody D, respectively. The ion at m/z 1544.5 (Figure 6A) corresponding to the y15 fragment of peptide EIVLTQSPGTLSLSPGER (antibody B), left only the first three amino acids at the N-terminus as the possible source for the loss of 18 Da in molecular mass. For antibody C, the ion at m/z 1091.3 (Figure 6B), corresponding to the y10 fragment of peptide EVQLMQSGAEVK, left only the first two amino acids at the N-terminus as the source of the 18-Da loss. And finally, for antibody D, the ion at m/z 1057.8 (Figure 6C), corresponding to the y10 fragment of peptide EVQLVQSGAEVK, left only the first two amino acids at the N-terminus as the possible source for the loss of 18 Da in molecular mass. Since there is no modification of isoleucine or valine that would result in a molecular mass loss of 18 Da, we concluded that the −18 Da modifications were a result of formation of pyroglutamic acid from glutamic acid at the N-terminus for all those peptides.

Temperature, pH, and Time Dependence of Pyroglutamic Acid Formation. After identifying this unusual modification, the formation of pyroglutamic acid from glutamate was further characterized, under different conditions for antibody A. The amount of pyroglutamic acid was measured based on the peak area of the light-chain main peak and the light-chain postpeak in the reversed-phase chromatograms of reduced and alkylated antibody samples (Figure 2). The effect of temperature on the formation of pyroglutamic acid is shown in Figure 7. The amount of pyroglutamic acid increased over time at 37 and 45 °C, and the rate of pyroglutamic acid formation increased with temperature. The half-life ($t_{1/2}$) of the N-terminal glutamate was calculated

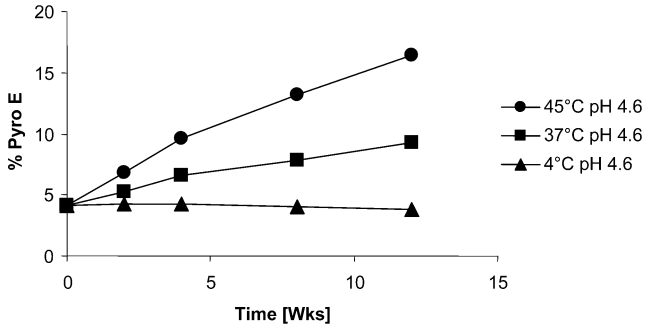

Figure 7. Effect of temperature on pyroglutamic acid formation. Antibody A was incubated at pH 4.6 at different temperatures for various periods of time. The percentage of pyroglutamic acid was calculated based on the peak areas of the light chain and the light-chain postpeak from the reversed-phase chromatogram (see Figure 2).

(SigmaPlot software), assuming a first-order reaction as 2.7 years at 37 °C (pH 4.6) and 1.1 year at 45 °C (pH 4.6). At 4 °C and below, there was very little if any condensation of glutamic acid during a 12-week incubation period.

To investigate the effect of pH on the rate of the formation of pyroglutamic acid, the recombinant antibody A was incubated at 37 and 45 °C at pH 4.1, 5.2, 5.7, 6.2, 7.0, and 8.0. Aliquots were taken at different time points (Figure 8) and abundance of the light-chain post peak (% PyroE) was measured using the reversed-phase HPLC method after reduction and alkylation as described under the Experimental Section. The results are summarized in Figure 8A and B. The half-life of the N-terminal glutamate at 37 °C was calculated as 2.1 (pH 4.1), 14.9 (pH 6.2), and 2.1 years (pH 8.0). As expected, the half-life decreased at 45 °C as compared with 37 °C to 0.7 (pH 4.1), 4.2 (pH 6.2), and 0.9 years (pH 8.0). Interestingly, the formation of pyroglutamic acid in antibody A at both temperatures appeared to be minimal at a pH around 6.2 (Figure 9).

The unusual pH dependence observed for the formation of pyroglutamate in antibody A was also observed for the condensation reaction of free glutamic acid by Wilson and Cannan.[19] They reported that, at neutral pH, the equilibrium of the conversion from free glutamic acid to pyroglutamic acid favors almost complete conversion to pyroglutamic acid.[19] The rate of change was slow, and only 1% conversion occurred in 2−3 h at 100 °C. At pH 4 and pH 10, the reaction from glutamic acid to pyroglutamic acid proceeded to ∼98% completion in somewhat less than 50 h at 100 °C.[19] The reported difference in reaction rates between the neutral pH and pH 4 or pH 10 was similar to our case of N-terminal glutamic acids. We believe that the reaction of free glutamic acid is very similar to the reaction of N-terminal glutamic acid as long as the conformation of the protein or peptide does not constrain the reaction.

For pyroglutamic acid formation from glutamic acid, the amino group needs to react with the carboxyl group of the side chain. At basic conditions, the amino group is a strong nucleophile, which would account for the increased rate at pH 8. The counterbalance would be that the carboxyl carbon atom is less electrophilic at higher pH and O^- of the deprotonated carboxyl side chain is not a good leaving group. Under acidic conditions, the carboxyl side chain forms a good leaving group (OH) and the carboxyl carbon atom becomes more electrophilic as compared with basic conditions. However, the amino group would be protonated and thus a poor nucleophile. The minimum at pH 6, therefore, might be due

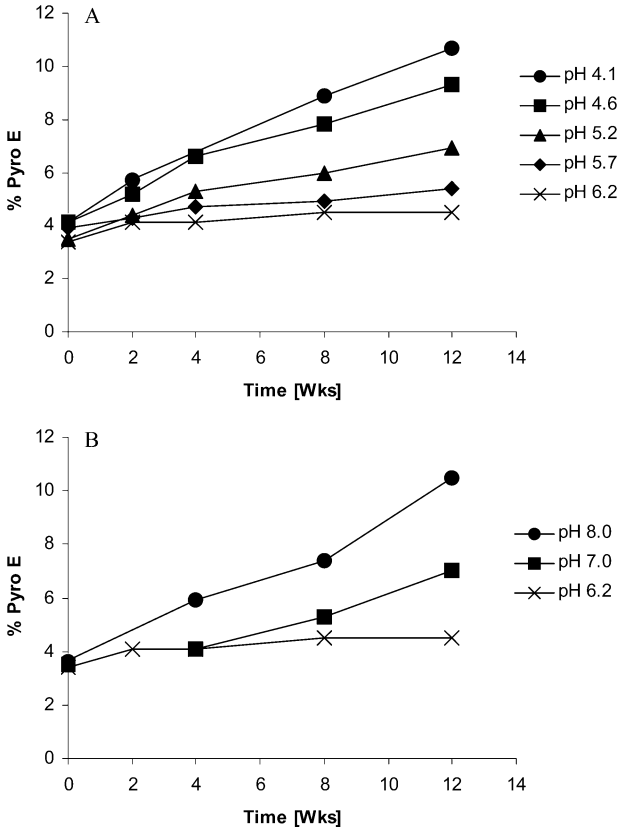

Figure 8. Effect of pH and time on pyroglutamic acid formation. Antibody A was incubated in aqueous buffers at pH 4.1, 4.6, 5.2, 5.7, and 6.2 (A) and pH 6.2, 7.0, and 8.0 (B) at 37 °C for various periods of time. The percentage of pyroglutamic acid was calculated based on the peak areas of the light chain and the post light chain from the reversed-phase chromatogram (see Figure 2). Each sample was analyzed three times. The standard deviations of peak area measurements were less than 1%.

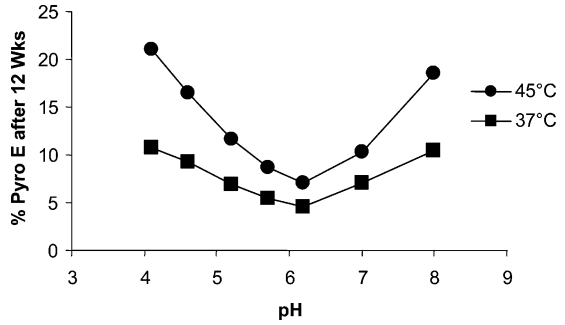

Figure 9. Pyroglutamic acid formation at a minimum around pH 6. Antibody A was incubated at pH 4.1, 4.6, 5.2, 5.7, 6.2, 7.0, and 8.0 at 37 and 45 °C for various periods of time. The percentage of pyroglutamic acid was calculated based on the peak areas of the light chain and the post light chain from the reversed-phase chromatogram (see Figure 2). Each sample was analyzed three times.

to the relative inability of both the amino group to act as a nucleophile, as well as the lack of formation of a good leaving group.

Condensation of N-terminal glutamic acid to pyroglutamic acid in peptides has been reported previously, but only in the solid state.[11] Beck et al. reported that the condensation reaction of N-terminal glutamic acid was accelerated if the peptide was produced as the acetate or trifluoroacetate salt, as compared to the hydrochloride salt.[11] Dimarchi et al. proposed a mechanism

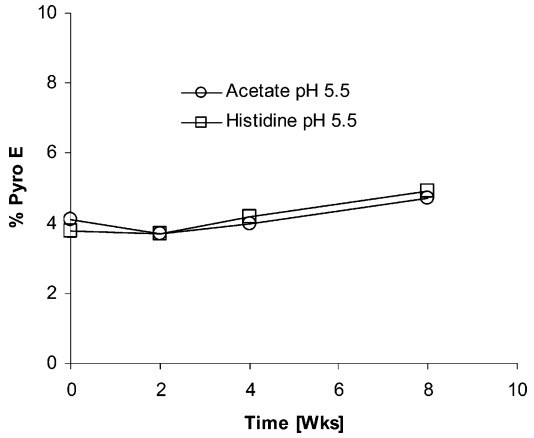

Figure 10. Effect of acetate buffer (weak acid) compared to histidine buffer on pyroglutamic acid formation. Antibody A was incubated at pH 5.5 and 45 °C in acetate buffer or histidine buffer for various periods of time. The percentage of pyroglutamic acid was calculated based on the peak areas of the light chain and the post light chain from the reversed-phase chromatogram (see Figure 2).

for the weak acid-catalyzed pyroglutamic acid formation in the solid-state.[12] Theoretically, acetate could also accelerate the formation of pyroglutamic acid in liquid formulations. To investigate whether weak acids could catalyze the formulation of pyroglutamic acid from glutamic acid as suggested in the literature, we compared the condensation rate at pH 5.5 in histidine buffer with the condensation rate in sodium acetate (weak acid) buffer (Figure 10). Our data did not suggest that the rate of formation of pyroglutamic acid was accelerated by weak acid.

Discussion. Our reversed-phase liquid chromatography method of the reduced and alkylated antibody separated the pyroglutamic acid containing light-chain isoform from its native counterpart and was used to monitor this degradation reaction. The method reported herein is not only interesting for people investigating heavy and light chains of antibodies containing glutamic acid residues but also glutamine-containing antibodies.[8] The identification of pyroglutamic acid in antibodies has been described in the literature, typically using tryptic peptide maps.[20−23] However, the tryptic digestion conditions of 37 °C overnight favor the complete conversion of glutamine to pyroglutamic acid, so that only the pyroglutamic acid variant could be detected. Ion exchange chromatography is also a useful tool for the identification of pyroglutamic acid isomers.[24,25] Other techniques for the separation of pyroglutamic acid from glutamine are hydrophobic interaction chromatography and isoelectric focusing, but they face similar challenges in distinguishing glutamic versus pyroglutamic acid

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protein variants.[26,27] The previous studies showed that glutamine was completely converted to pyroglutamic acid, suggesting that the previous methods were not accurate enough to show the presence of native glutamine in antibodies.[20−26]

In principle, there could be two mechanisms of pyroglutamate formation, enzyme versus nonenzyme catalyzed. For the experiments performed in this study, no enzymes were added, and they were carried out with just protein and buffer at different pHs and temperatures. Further, from the various analytical techniques, such as reversed-phase chromatography and size exclusion chromatography, as well as mass spectrometric analysis of the intact antibody and tryptic peptides of the antibody, there were no nonantibody peaks detected. Collectively, this argues for a nonenzymatic conversion of N-terminal glutamic acid to pyroglutamic acid. In the starting material, ∼4% of the material contained the pyroglutamic acid modification. It is possible that the two-week fermentation process at neutral pH and 37 °C, followed by a one-week purification procedures may have led to this conversion.

The biological effect from the formation of pyroglutamic acid has not been investigated in great detail in this study. However, we could not detect any loss of activity in samples that had converted by more than 30% to pyroglutamic acid. On the contrary, it has been suggested that the formation of pyroglutamic acid would in fact stabilize the protein by making it more resistant to chemical or enzymatic degradation.[28−30] In this work, the nonenzymatic formation of pyroglutamic acid from glutamic acid was identified both in the light chain (antibody A and B) and in the heavy chain (antibody C and D) of the molecules studied. It is reasonable to believe that most proteins starting with a glutamic acid residue at the N-terminus can undergo this modification, but further studies are needed to confirm our findings for other polypeptides.

We also believe that pyroglutamic acid formation in therapeutic antibodies should not cause a pharmacological problem, since pyroglutamic acid is formed from both N-terminal glutamic acid and glutamine residues in vivo. However, it will be important to monitor the degradation of N-terminal glutamic acid residues until it is proven that, indeed, the formation of pyroglutamic acid does not affect the safety or the efficacy of a therapeutic monoclonal antibody.

CONCLUSION

We identified and characterized the chemical degradation of glutamic acid at the N-terminus of the light and heavy chains of recombinant monoclonal antibodies to pyroglutamic acid in vitro after aging the proteins in different buffers. Our reversed-phase liquid chromatography method of the reduced and alkylated antibody separated the pyroglutamic acid-containing light-chain isoform from its native counterpart and was used to monitor this degradation reaction. We identified the pyroglutamic acid from tandem mass spectra of N-terminal peptides produced after tryptic digestion of the collected pyroglutamic acid light-chain isoforms and separation of the tryptic peptides using a reversed-phase liquid chromatography mass spectrometry system. Based on the kinetic information presented in this study, it is reasonable to believe that, in recombinant antibodies, pyroglutamic acid is formed nonenzymatically during the fermentation and purification procedures. It also continues during storage at elevated temperatures. The lowest rate of conversion of N-terminal glutamate to pyroglutamate was found in pH 6.2 solutions.

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