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Unambiguous Identification of Pyroglutamate in Full-Length Biopharmaceutical Monoclonal Antibodies by NMR Spectroscopy

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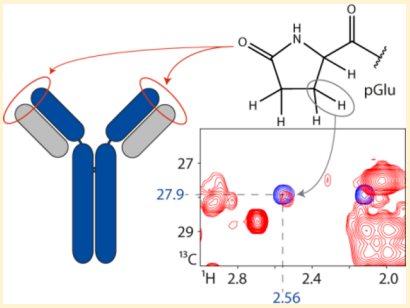
Supporting Information

ABSTRACT: Biotherapeutic proteins are an indispensable class of pharmaceuticals that present a high degree of structural complexity and are prone to chemical modifications during production, processing, and storage, which have to be tightly controlled. Pyroglutamate (pGlu), a cyclization product of N-terminal Gln or Glu residues, is a widespread post-translational modification in proteins, including monoclonal antibodies (mAbs). The unambiguous identification and quantification of this modification in proteins is challenging, since the mass difference of −17 Da or −18 Da, when formed from Gln or Glu, respectively, is not unique. Moreover, deamidation and dehydration occur not only during cyclization to pGlu, but also during other reactions leading to different types of modifications, like succinimide or isopeptide bond moieties due to cross-linking between Asn or Gln and Lys side chains. Here we report the unambiguous identification and quantification of pGlu in intact mAbs with natural isotope distribution by NMR spectroscopy. The assignment of all ^{1}H, ^{13}C and ^{15}N random coil chemical shifts of pGlu in short reference peptides led to the identification of unique chemical shift pairs that are distinct from the random coil chemical shifts of the natural amino-acid residues. These characteristic correlations are suited for the detection of pGlu in denatured proteins. We achieved complete denaturation of mAbs using a straightforward protocol, and could detect and quantify pGlu, in agreement with available mass spectrometric data. The application to the mAbs rituximab and adalimumab illustrates the potential of our approach for the characterization of biotherapeutics containing isotopes at natural abundance.



Pyroglutamate (pGlu) is a posttranslational modification (PTM) of proteins, which is formed by the spontaneous or enzyme-catalyzed conversion of an N-terminal Gln or Glu residue into a pyrrolidinone ring under the loss of ammonia (from Gln) or water (from Glu) (Figure 1a).[1−3] This PTM affects the protein charge, as the positively charged free N-terminus is transformed into a γ-lactam nitrogen, and, in the case of Glu, the γ-carboxylate is neutralized upon amidation.

Highly relevant is the formation of pGlu in biotherapeutics, in particular, monoclonal antibodies (mAbs).[2,4−8] Since it is important to detect and unambiguously identify all possible changes occurring in a biotherapeutic,[2,3,6−8] the content of pGlu needs to be monitored and documented, which is usually done by performing amino-acid sequence analysis of digested samples with LC-MS.[2,4,7,9−11] However, in-source pGlu formation during mass-spectrometric analysis[12] and pGlu formation during protease digestion[9] have been both reported, which implies the risk of overestimating the pGlu-containing variant. In addition, other PTMs that also result in a mass difference of −17 Da or −18 Da, like succinimide (Snn)[12] or isopeptide bond formation upon cross-linking between Asn/Gln and Lys side-chains,[13] could make the identification of pGlu ambiguous, especially when the sequence coverage is incomplete, which can occur during tandem MS of intact proteins.[14−17]

Recently, we have proposed an NMR-based approach for the detection of modifications in denatured proteins.[18,19] Under these conditions, only the typical random coil chemical shift[20] correlations of the 20 natural amino acids are observed, due to the lack of secondary and tertiary structure, which enormously simplifies the analysis of the 2D NMR spectra. However, modified amino acids or additional moieties will generate new

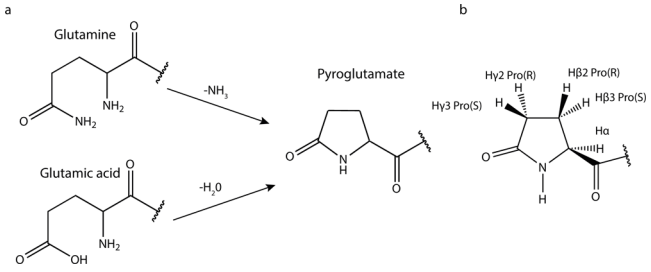


Figure 1. Formation and nomenclature of pyroglutamate. (a) Cyclization of Gln or Glu to pGlu under loss of ammonia or water. (b) Stereochemical nomenclature of pGlu.

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signals[18,21] and/or influence the chemical shifts of the modified amino acids in such a way that their chemical shifts will be distinct from those of the unmodified amino acids.[19] For the detection of a certain PTM by NMR spectroscopy, it is necessary that at least one of its chemical shift correlations will be unique and well separated from all other signals. Random coil chemical shifts of uncommon amino acids and posttranslationally modified proteinogenic amino acids have rarely been reported so far, for example, for phosphorylated Ser, Thr, and Tyr;[22−25] hydroxylated Asn;[24] succinimide and isoaspartate;[19] or methylated amino acids.[25] So far, only small- to medium sized-proteins and mAb fragments[18,19,21] were studied using this approach, but since protein size should not be a limiting factor for NMR spectroscopy under denaturing conditions, an application to intact therapeutic mAbs is feasible.

Here we report all ^{1}H, ^{13}C and ^{15}N random coil chemical shifts of pGlu and its unique chemical shift correlations that have been used to detect and quantify pGlu in denatured full-length therapeutic mAbs with high accuracy.

EXPERIMENTAL SECTION

Chemicals for Peptide Synthesis and Characterization. All Nα-Fmoc-amino acids, Fmoc-Rink amide MBHA resin, *N*,*N*-dimethylformamide, 1-methyl-2-pyrrolidinone, dichloromethane, diethyl ether, and *N*,*N*-diisopropylethylamine were purchased from Iris Biotech GmbH (Marktredwitz, Germany). Boc-pGlu-OH was purchased from Bachem (Bubendorf, Switzerland). 1-Hydroxybenzotriazole, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, and trifluoroacetic acid (TFA) were obtained from Biosolve (Valkenswaard, The Netherlands). HPLC-grade acetonitrile (ACN), triisopropylsilane (TIS), thioanisole (TIA), 1,2-ethanedithiol (EDT), and piperidine were obtained from Sigma-Aldrich (Vienna, Austria). HPLC-grade TFA was from Alfa-Aesar (Karlsruhe, Germany).

Solid Phase Peptide Synthesis and Characterization. Peptides were synthesized by Fmoc-chemistry using solid-phase peptide synthesis on an automatic peptide synthesizer (Syro I, Biotage, Uppsala, Sweden). The resin-bound peptides were cleaved and deprotected with TFA containing 10% scavengers (H_2O/TIA/EDT/TIS, 1:3:3:3, v/v) at room temperature for 1.5 h. The peptides were then precipitated from cold diethyl ether, recovered by centrifugation at 4 °C, washed three times with cold ether, dried under nitrogen, dissolved in 0.1% aqueous TFA, and lyophilized. Analytical RP-HPLC was performed using a Thermo Scientific Dionex UltiMate 3000 UHPLC system (Thermo Fisher Scientific, Germering, Germany) and a Syncronis C18 column (100 Å, 5 μm, 250 × 4.6 mm, Thermo Fisher Scientific) at 25 °C, with a flow rate of 1.5 mL·min^{-1}. The UV detection was set at 220 and 280 nm. The elution system was (A) 0.06% (v/v) TFA in water, and (B) 0.05% (v/v) TFA in ACN. The products were dissolved in ACN/H_2O (25:75, v/v) containing 0.1% TFA. For assessing the purity of the peptides (90−93%) analytical chromatograms were obtained with the following gradients: 3% B for 8 min, 3% to 60% B in 35 min for pGlu-GGW-NH_2 (Supporting Information (SI) Figure S1); 1% B for 8 min, 1% to 50% B in 35 min for pGlu-PGG-NH_2 and pGlu-GGG-NH_2. Mass spectra were recorded on an Autoflex Speed MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany), by using a saturated solution of α-cyano-4-hydroxycinnamic acid in ACN/MeOH (50:50, v/v) as matrix: pGlu-GGW-NH_2, M_{theor} 428.45 Da, $(M-H)^-_{found}$ 427.3 Da (SI Figure S1); pGlu-PGG-NH_2, M_{theor} 339.35 Da, $(M+H)^+_{found}$ 340.4 Da; pGlu-GGG-NH_2, M_{theor} 299.29 Da, $(M+H)^+_{found}$ 300.3 Da. The concentration of the solution of pGlu-GGW-NH_2 in 7 M urea at pH 2.3, which was used to assess the detection limit, was determined by measuring the UV absorbance at 280 nm using 7 M urea at pH 2.3 as blank and the molar extinction coefficient of 5540 M^{-1} cm^{-1} for Trp.[26]

Sample Preparation. For the biopharmaceuticals adalimumab (Humira, AbbVie; exp. year: 2016, 2 mL, 10 mg·mL^{-1}) and rituximab (Mabthera, Roche; exp. year: 2013, 2 mL, 10 mg·mL^{-1}) the formulation buffer was changed to ddH_2O with Amicon Ultra-15 Centrifugal Filter Units (Cutoff: 30 kDa, Merck). Alternatively, buffer exchange to 175 mM ammonium acetate-d_3 (acetic acid-d_3, ARMAR Chemicals) at pH 7 gave comparable results (data not shown). After lyophilization the proteins were dissolved in 500 μL of a 7 M urea-d_4 (98 atom %D, ARMAR Chemicals) solution in D_2O (100 atom %D, ARMAR Chemicals) resulting in a concentration of 30−40 mg·mL^{-1}. The pH was adjusted to 2.3 or 7.4 by adding DCl or NaOD (ARMAR Chemicals). To reduce disulfide bonds, 11 mmol·L^{-1} tris(2-carboxyethyl)-phosphine hydrochloride (TCEP) (Sigma-Aldrich) was added, and the samples were incubated for 15 min at 60 °C. The reference peptides were dissolved in the same conditions without TCEP. The presence of TCEP did not change their chemical shifts (data not shown). To measure the chemical shifts of exchangeable protons and Nε of pGlu, the peptides were dissolved in 7 M urea (Merck), ddH_2O + 10% D_2O. After the NMR measurements, 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) (ARMAR Chemicals) was added to each sample and a 1D ^{1}H spectrum was recorded for referencing the proton chemical shifts. The carbon and nitrogen chemical shifts were adjusted regarding to the IUPAC-IUB recommended chemical shifts referencing ratios[27] of 0.251449530 (^{13}C) and 0.101329118 (^{15}N).

NMR Spectroscopy. Unless specified otherwise, spectra were recorded with a 600 MHz Bruker Avance III HD spectrometer equipped with a $^{1}H/^{13}C/^{15}N/^{31}P$ quadruple-resonance probe at 298 K. Standard 5 mm NMR tubes (ARMAR, Type 5TA) with a sample volume of 500 μL were used. To assign the HSQC fingerprint spectra of the reference peptides, the following 2D experiments were recorded: $^{1}H-^{13}C$ HSQC, $^{1}H-^{13}C$ HMBC (hmbcgpndqf), $^{1}H-^{1}H$ TOCSY, $^{1}H-^{1}H$ COSY (cosygpppqf), $^{1}H-^{13}C$ HMQC−COSY,[18,28] $^{1}H-^{1}H$ ROESY, $^{1}H-^{15}N$ HSQC, and $^{1}H-^{13}C$ HCO.[19] For the stereochemical assignment of Hβ2 and Hβ3 of pGlu, a 1D steady-state NOE difference spectrum (zgf2pr) with 32 scans and a recycle delay of 6 s was performed, with irradiation at either 4.38 ppm (pGlu Hα) or −1.7 ppm. Data was processed with Topspin 3.5 (Bruker) and analyzed with Sparky 3.114 (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco, CA). Integration and signal-to-noise (S/N) determination were performed with Sparky by using standard protocols (https://www.cgl.ucsf.edu/home/sparky/manual/views.html).

Detection Limit. To estimate the detection limit, denatured adalimumab with a concentration of 220 μM in D_2O (100 atom %D, ARMAR Chemicals, Germany), as described in the sample preparation section, was doped with a reference peptide (pGlu-Gly-Gly-Trp-NH_2), using mAb:peptide molar ratios of 1:1, 1:0.5, and 1:0.25. The latter would correspond to a sample in which one out of four mAb

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molecules contain pGlu at one of the four N-termini. $^{1}H-^{13}C$ HSQC spectra of the doped samples (1:1, 1:0.5, and 1:0.25) were measured with a 600 MHz Bruker Avance III HD spectrometer equipped with a $^{1}H/^{13}C/^{15}N/^{31}P$ quadruple-resonance RT probe. Several experiments with different numbers of scans were tested. In addition, the 1:0.25 sample was measured on two other NMR spectrometers: 600 MHz Bruker Avance III HD spectrometer equipped with cryogenic $^{1}H/^{13}C/^{15}N/^{31}P$ QCI quadruple resonance probe, and 900 MHz Bruker Avance III HD equipped with a cryogenic $^{1}H/^{13}C/^{15}N$ TCI triple-resonance probe.

Database Evaluation. The PDB search for pGlu or pyrrolidone carboxylic acid (PCA) residues in three-dimensional protein structures was performed via the OCA webtool (access date: 22 April 2019).[29] UniProt search[30] was performed with the keyword "KW-0873" standing for pGlu/PCA (access date: 8 May 2019).

RESULTS

Random Coil Chemical Shifts of pGlu. Complete chemical shift assignment of pGlu was obtained for two short pGlu-containing peptides in 7 M urea at two different pH values (2.3 and 7.4). Acidic conditions are sometimes required for complete denaturation; moreover, the random coil chemical shifts of the 20 common amino acids were reported at pH 2.3.[31] The second condition at the physiological pH of 7.4 was chosen, because in some cases it may be necessary to preserve acid-sensitive modifications like sialylated moieties. Although random coil chemical shifts have not been reported for pH 7.4, the comparison between random coil chemical shifts reported at different pH values[20,32,33] reveal that only the resonances of amino acids with ionizable side chains are sensitive to pH, as expected. Complete resonance assignments for pGlu (Table 1) were obtained using $^{1}H-^{13}C$ HSQC, $^{1}H-^{1}H$ TOCSY, $^{1}H-^{1}H$ COSY, $^{1}H-^{13}C$ HMBC, and $^{1}H-^{13}C$ HMQC–COSY spectra measured with the peptides pGlu-$(Gly)_3$-NH_2 (Figure 2a) and pGlu-Pro-$(Gly)_2$-NH_2 (Figure 2b), which were prepared by Fmoc-based solid phase peptide synthesis. The latter sequence was used to evaluate the effect of an adjacent Pro residue on the chemical shifts of pGlu. A comparison with random coil chemical shifts of the 20 common natural amino acids revealed one unique and well isolated chemical shift correlation in the 2D $^{1}H-^{13}C$ HSQC spectrum at 27.9 ppm for the ^{13}C and 2.56 ppm for the ^{1}H shift (Figure 2c). This characteristic cross-peak corresponding to a correlation between the Cβ and the downfield Hβ resonance is suited to detect pGlu in denatured proteins. This signal was stereospecifically assigned to Hβ3 (Figure 1b) using a 1D steady-state NOE difference spectrum (SI Figure S2). The presence of a neighboring trans- or cis-configured Pro residue slightly changes the chemical shifts of the unique C–H correlation, which are, however, still sufficiently different from all other random coil chemical shifts (Figure 2c,d).

Table 1. Chemical Shifts of pGlu in the Reference Peptides with the Sequence pGlu-$(Gly)_3$-NH_2 and pGlu-Pro-$(Gly)_2$-NH_2 under Denaturing Conditions Containing 7 M urea-d_4 in D_2O (Except N and HN Shifts That Were Obtained in H_2O) at pH 2.3 and 7.4

			pGlu-Pro-$(Gly)_2$-NH_2 pH 2.3		pGlu-Pro-$(Gly)_2$-NH_2 pH 7.4	
	pGlu-$(Gly)_3$-NH_2 pH 2.3	pGlu-$(Gly)_3$-NH_2 pH 7.4	pGlu (trans Pro)	pGlu (cis Pro)	pGlu (trans Pro)	pGlu (cis Pro)
C	178.4	178.4	175.6	nd[a]	175.6	nd[a]
Cα	59.7	59.7	58.1	58.2	58.1	58.2
Cβ	27.9	27.9	26.8	26.9	26.8	27.0
Cγ	32.0	32.0	31.9	31.9	31.9	31.9
Cδ	184.8	184.8	184.5	184.6	184.6	nd[a]
N	125.2	nd[a]	126.2	126.2	nd[a]	nd[a]
HN	7.93	nd[a]	7.88	7.81	nd[a]	nd[a]
Hα	4.39	4.38	4.66	4.37	4.67	4.37
Hβ3[b]	2.56	2.56	2.61	2.56	2.61	2.55
Hβ2[b]	2.12	2.12	2.08	2.04	2.08	2.04
Hγ	2.45	2.44	2.42	2.44	2.42	nd[a]

[a]not determined; [b]Hβ2 and Hβ3 were stereochemically assigned (Figure 1b).

Detection of pGlu in mAbs. The mAbs rituximab and adalimumab were chosen as model systems for biotherapeutics. Rituximab has been previously reported to contain 4 pGlu per mAb,[34] which are readily formed from initial N-terminal Gln residues. In contrast, adalimumab has not been reported to contain pGlu.[35] Its heavy chains start with N-terminal Glu, whose tendency to form pGlu is orders of magnitude smaller than that of N-terminal Gln.[1] An overlap of 2D $^{1}H-^{13}C$ HSQC spectra of denatured and reduced rituximab and the reference peptide pGlu-$(Gly)_3$-NH_2 under the same conditions (pH 2.3) is shown in Figure 3. The characteristic Cβ-Hβ3 signal of pGlu is visible in the spectrum of rituximab and perfectly matches with the Cβ-Hβ3 correlation of pGlu in the reference peptide. For quantification, the volume of the signal was compared to a reference signal, which originated from a group with the same multiplicity and a similar $^{1}J_{CH}$ value. The $^{1}J_{CH}$ couplings were determined using $^{1}H-^{13}C$ HSQC spectra recorded without ^{13}C decoupling in F2 and $^{1}H-^{13}C$ HMBC spectra. $^{1}J_{CBHB3}$ of pGlu was determined to be approximately 140 Hz. With three independent samples of the same batch of rituximab, the Cβ-Hβ3 correlation signal was integrated and normalized to the integrals of Arg Cδ-Hδ (28× in rituximab, $^{1}J_{CH} \approx 139$ Hz) and Lys Cε-Hε (98× in rituximab, $^{1}J_{CH} \approx 142$ Hz), which resulted in 4.23 ± 0.32 pGlu residues per rituximab molecule with reference to Arg Cδ-Hδ (SI Table S1), in accordance with previous MS data.[34] In the case of adalimumab, no signal for pGlu was detected, which, again, confirms the low propensity of N-terminal Glu to cyclize.[1]

Sensitivity and Detection Limit. To estimate the detection limit, we doped samples of 220 μM adalimumab with different amounts of a pGlu-containing peptide that also contained a Trp residue for UV determination of the concentration (pGlu-Gly-Gly-Trp-NH_2). By performing a 189 h measurement at a 600 MHz spectrometer without cryogenic probe it was possible to observe the characteristic signal using a sample with a mAb:peptide molar ratio of 1:0.25 corresponding to 0.25 pGlu residues per mAb (S/N of the pGlu β3 signal in the $^{1}H-^{13}C$ HSQC spectrum was 8, as calculated by Sparky). By using a 600 MHz spectrometer with cryogenic probe the measurement time could be reduced to 48 h (S/N: 10) (SI Table S2, Figure S3). With a 900 MHz spectrometer and cryogenic probe a measurement of only 25 h led to similar results (S/N: 12) (SI Table S2, Figure S3). To demonstrate the robustness of the approach, four independent samples were measured and compared (SI Table S2).

Although $^{1}H-^{13}C$ correlations are well dispersed and suitable for the quantification of isolated signals, they rely on

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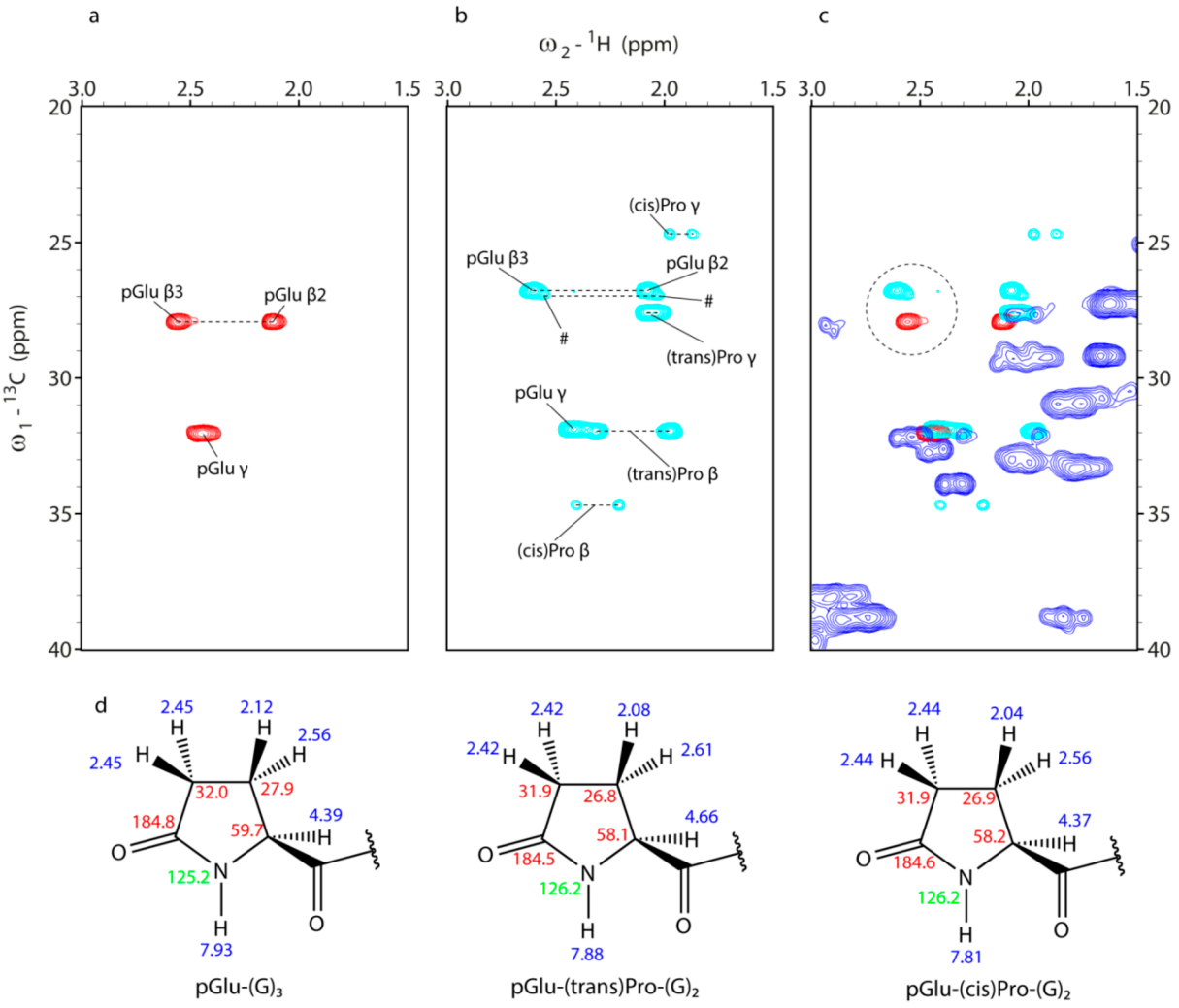

Figure 2. Fingerprint region of the pyroglutamate side chain in $^{1}H-^{13}C$ HSQC spectra under denaturing conditions (7 M urea-d_4, pH 2.3). (a) $^{1}H-^{13}C$ HSQC spectrum of pGlu-$(Gly)_3$-NH_2. (b) $^{1}H-^{13}C$ HSQC spectrum of pGlu-Pro$(Gly)_3$-NH_2. The smaller signals labeled with # correspond to pGlu beta with a cis-proline on i+1 position. (c) Overlay of the $^{1}H-^{13}C$ HSQC spectra of the two reference peptides (a, b) with denatured lysozyme displaying the random coil chemical shifts of the common amino acids. The dashed circle indicates the region of the unique pGlu β3 shifts that are not overlapping with random coil shifts of common amino acids. (d) Chemical shift assignments of pGlu in the reference peptides under denaturing conditions at pH 2.3.

the low natural abundance of the ^{13}C isotope of 1.1%. A much higher sensitivity could be, in principle, obtained with the use of 2D $^{1}H-^{1}H$ correlations. Therefore, we decided to explore the potential of $^{1}H-^{1}H$ COSY spectra for the detection of pGlu. A unique and isolated correlation between Hα and Hβ3 of pGlu was visible in the 2D $^{1}H-^{1}H$ COSY spectrum of rituximab (Figure 4). This signal is indicative for the presence of pGlu in this mAb. However, COSY signals can be used only for semiquantitative analysis, because their intensities and volumes depend on $^{3}J_{HH}$ scalar coupling constants, which are not uniform. Moreover, the signals display coupling patterns in both dimensions, which makes it difficult, to integrate and quantify them. In order to test the higher sensitivity of 2D $^{1}H-^{1}H$ COSY spectra, adalimumab samples doped with the above-mentioned pGlu peptide were measured. pGlu signals could be detected at a 600 MHz spectrometer without cryogenic probe overnight with the mAb:peptide molar ratio of 1:0.25, reducing the measurement time from 189 h ($^{1}H-^{13}C$ HSQC) to 8 h ($^{1}H-^{1}H$ COSY). A further reduction of measurement time can be achieved by using a 900 MHz spectrometer with a cryogenic probe leading to comparable results (SI Table S2, Figure S4).

pGlu Content in Data Banks. A search for pGlu (three letter code: PCA) in the protein data bank (PDB)[36] yielded 631 entries including proteins of viruses, bacteria, fungi, plants and vertebrates out of about 130000 considered entries. Using the UniProt search engine,[30] we found 1454 out of approximately 550000 protein entries in the Swiss-Prot data.

DISCUSSION

The presented approach allows for the detection and quantification of pGlu in proteins by NMR spectroscopy, which we have applied to two therapeutic mAbs. However, our method is applicable to proteins in general. The Protein Data Bank (PDB) counts 631 entries for pGlu from viruses, bacteria, fungi, plants and vertebrates, and the Swiss-prot part of the UniProt database contains 1454 entries for pGlu. These numbers are certainly an underestimation of the pGlu occurrence in native proteins, as in the PDB the N-termini of the entries are often engineered and/or flexible and, thus,

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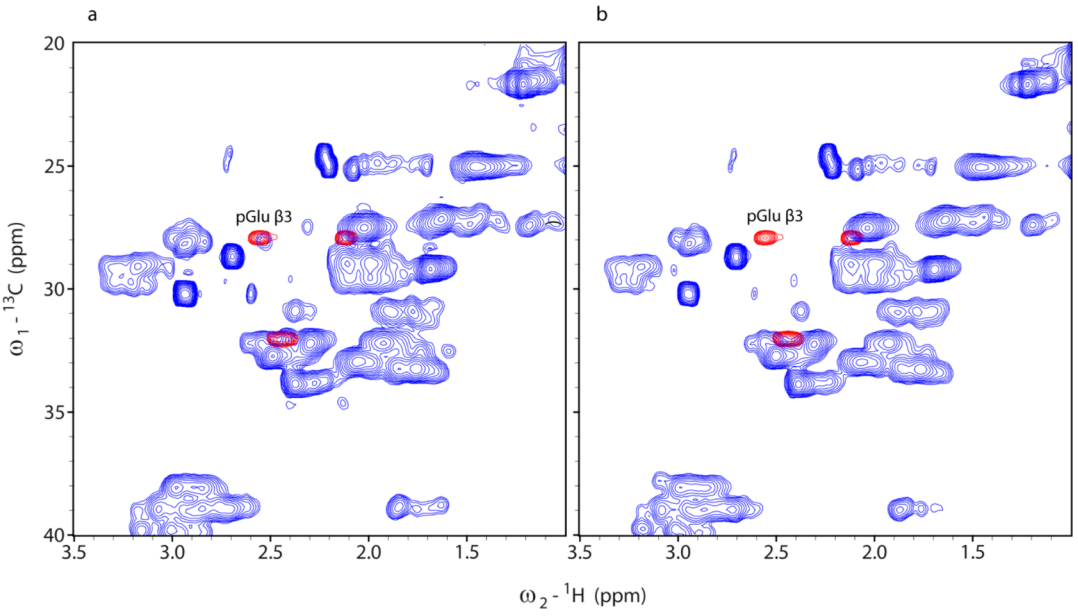

Figure 3. $^{1}H-^{13}C$ HSQC spectra of denatured and reduced mAbs rituximab (220 μM, panel a), adalimumab (220 μM, panel b), respectively superimposed with the spectrum of the reference peptide pGlu-$(Gly)_3$-NH_2 in D_2O containing 7 M urea-d_4 at pH 2.3. The measurement times of the mAb samples were 21 h using 140 scans.

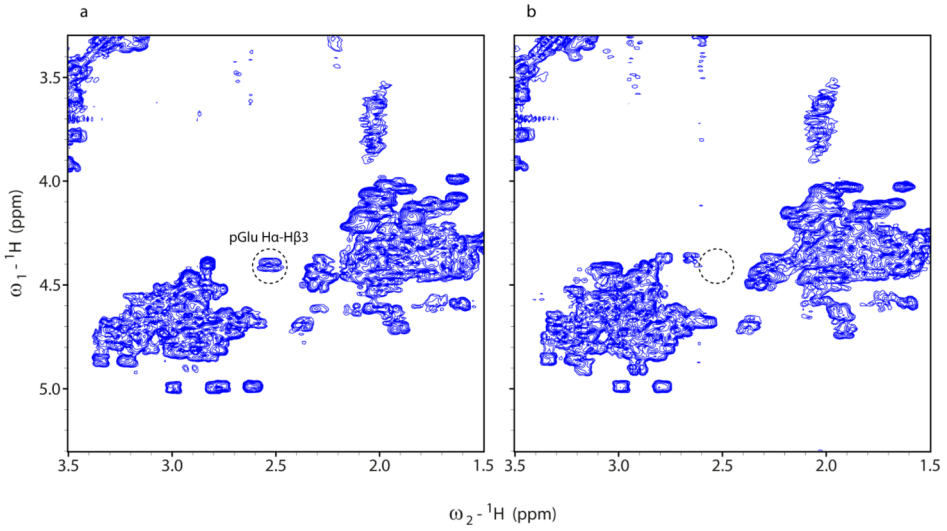

Figure 4. Characteristic pGlu signals in $^{1}H-^{1}H$ COSY spectra. a) The $^{1}H-^{1}H$ COSY spectrum of denatured and reduced rituximab (220 μM) shows one isolated peak corresponding to Hα-Hβ of pGlu. b) The $^{1}H-^{1}H$ COSY spectrum of denatured and reduced adalimumab (220 μM). Characteristic pGlu signals are absent. The measurement times of the samples were 18 h using 56 scans.

lack electron density information on these regions. The application of our method will contribute to the fast detection of pGlu in proteins, which will be particularly important for secreted recombinant human proteins, as they preferentially contain an N-terminal Gln.[12]

The detection limit of the quantitative $^{1}H-^{13}C$ HSQC spectrum relies on the natural abundance of ^{13}C, which is only 1.1%, and the performance of the NMR instrument, like the field strength and the sensitivity of the probe head. To estimate the sensitivity of our experimental setup, a two-day measurement of a sample of 220 μM adalimumab doped with a pGlu-containing peptide at the concentration of 55 μM yielded on a 600 MHz instrument with a cryogenic probe a $^{1}H-^{13}C$ HSQC spectrum, in which the signal of interest was clearly visible. This corresponds to an absolute amount of approximately 27 nmol pGlu using a sample volume of 500 μL. Measurements with a 900 MHz spectrometer with cryogenic probe lowered the acquisition time further to 25 h with comparable results. Alternatively, the higher sensitivity of such high-end instruments can be used to detect even lower quantities. Peng et al.[21] recently reported a quantification limit of about 10% for galactose detection in mAb fragments (using a 850 MHz spectrometer with a cryogenic probe for a measurement time of 11 h). As the relative detection limit of pGlu residues per protein molecule also correlates with the concentration in the sample, it would be helpful to use higher-concentrated solutions. However, one limiting factor is the solubility of the protein. In the case of adalimumab, we only reached approximately 220 μM mAb (approximately 31 mg·mL^{-1}).

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To show the precision and accuracy of our method, we performed triplicate measurements of 220 μM denatured and reduced rituximab at 600 MHz for 20 h measurement time using a QXI probe, which yielded 4.23 ± 0.32 residues of pGlu per native mAb, corresponding to an approximate quantification error of 35 nmol pGlu in a 500 μL sample.

Due to the high natural abundance of ^{1}H (99.98%) compared to that of ^{13}C (1.1%), a significant sensitivity gain can be obtained from 2D ^{1}H−^{1}H COSY spectra compared to ^{1}H−^{13}C HSQC spectra. The described isolated cross-peak for Hα-Hβ3 of pGlu in ^{1}H−^{1}H COSY spectra could be detected even at a much lower content. Although ^{1}H -^{1}H COSY spectra are not well suitable for quantification, they can be used for semiquantitative purposes, for example, for the screening of PTMs in target proteins. The combination of ^{1}H−^{1}H COSY and ^{1}H−^{13}C HSQC spectra makes our approach even more powerful.

CONCLUSION

With this work, we provide a comprehensive NMR fingerprint of pGlu and demonstrate its usefulness for the unambiguous detection and quantification of this PTM in biotherapeutical mAbs. ^{1}H−^{13}C correlation spectra can be used for the reliable quantification of pGlu with a detection limit of approximately 27 nmol in 500 μL of a mAb sample within 1 or 2 days using moderate to high-field spectrometers with cryogenic probes. In addition, semiquantitative ^{1}H−^{1}H COSY spectra, which are significantly more sensitive than ^{1}H−^{13}C correlation spectra, are suitable to detect the presence of pGlu. A combination of both spectra increases the reliability of our method due to cross-validation. This NMR-based approach provides the unambiguous identification of pGlu, especially when a comprehensive sequence analysis of proteins by MS techniques is difficult.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.9b02513.

1D (^{1}H) and 2D (^{1}H−^{1}H and ^{1}H−^{13}C) NMR spectra, MALDI-TOF-MS spectra. (PDF)

Electronic NMR data of the pGlu peptide are provided as reference (Bruker format) (GZ)

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M.S., C.C., and A.H. designed the experiments. V.S. synthesized the reference peptides. A.H. and M.S. performed the NMR experiments, assigned the NMR spectra and performed database analysis. M.S., C.C., A.H., and V.S. wrote the manuscript.

Notes

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