Determination of the Origin of Charge Heterogeneity in a Murine Monoclonal Antibody

Melissa Perkins,[1] Rolf Theiler,[2] Susan Lunte,[1] and Margit Jeschke[2,3]

Received February 10, 2000; accepted June 9, 2000

Purpose. The aim of this study was to elucidate the molecular basis of charge heterogeneity found in a purified monoclonal IgG_1 antibody, MMA383.

Methods. Cation exchange chromatography (CEX) and isoelectric focusing (IEF) were used to monitor charge heterogeneity. CEX in conjunction with carboxypeptidase B digests of the antibody was used to determine the contribution of C-terminal lysines to MMA383 charge heterogeneity. Potential chemical degradation sites were identified by peptide mapping of individual chains, with peptide identification by mass spectrometry (MALDI-TOF MS). Peptide sequencing was used to determine specific deamidation sites. Binding constants of predominant isoforms were compared by surface plasmon resonance (SPR).

Results. Extensive charge heterogeneity of purified MMA383 was detected by CEX and IEF. Removal of C-terminal lysines simplified the IEF pattern to nine predominant isoforms. Quantitation of isoaspartate in each of the isoforms indicated deamidation of MMA383 as a major cause of charge heterogeneity. CEX of the individual isoform chains suggested the presence of one deamidation site on each of the heavy and light chains. The two sites of deamidation were identified using peptide mapping, sequencing and mass spectrometry. SPR results showed no significant difference in the binding parameters among the isoforms.

Conclusions. C-terminal lysine microheterogeneity and deamidation of Asn141 in the heavy chain and Asn161 in the light chain are the major causes of MMA383 charge heterogeneity. Identification of the two deamidation sites will allow replacement of these amino acids in order to create a product less susceptible to degradation.

KEY WORDS: microheterogeneity; monoclonal antibody; deamidation; isoaspartate.

[1] Department of Pharmaceutical Research, The University of Kansas, Lawrence, Kansas.

[2] Biotechnology Development & Production, Novartis Pharma AG, Basel, Switzerland.

[3] To whom correspondence should be addressed at Novartis Pharma AG, Biotechnology Development & Production, WKL 681.5.44, 4057 Basel, Switzerland. (e-mail: margit.jeschke@novartis.pharma.com)

ABBREVIATIONS: AA, amino acid; Asn, asparagine, Asp, aspartic acid; CEX, cation exchange chromatography; EDC, N-ethyl-(N′-dimethylaminopropyl)-carbodiimide; EDTA, ethylenediaminetetraacetic acid; GuHCl, guanidine hydrochloride; isoAsp, isoaspartatic acid; HEPES, N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]; HPLC, high performance liquid chromatography; IEF, isoelectric focusing; Lys, lysine; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MES, 2-morpholinoethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; pI, isoelectric point; Rt, retention time; SEC, size exclusion chromatography; TFA, trifluoroacetic acid; TRIS, Tris(hydroxymethyl)-aminomethane; SDS, Sodium dodecyl sulfate; DTT, 1,4-dithio-DL-threitol

INTRODUCTION

Lewis Y carbohydrate antigen (LeY) is widely expressed on the surface of epithelial cancer cells and is considered a promising antigen for targeted therapy of epithelial cancers in humans (1,2). Since carbohydrate structures are poorly immunogenic, anti-idiotypic antibody vaccines have been employed to induce an active immune response against carbohydrate antigens (reviewed in 3). MMA383 is an anti-idiotypic murine monoclonal antibody (IgG_1) directed against the anti-Lewis Y monoclonal antibody ABL364 (4). To produce a vaccine against LeY positive tumors, the anti-idiotypic MMA383 was manufactured at Novartis to be used as a surrogate antigen for LeY.

Monoclonal antibodies intended for therapeutic use require a comprehensive characterization of their structural integrity, purity, and stability (5). Molecular alterations can take place at every stage of manufacturing: cell culture, purification, formulation, and storage. Since chemical modifications can affect the biological activity of a protein, detection and identification of molecular changes are important analytical issues in biopharmaceutical development. Antibody modifications can occur enzymatically or nonenzymatically, e.g. through unfavorable storage conditions, and may cause charge or size heterogeneity. Common modifications of the primary sequence include N-glycosylation, methionine oxidation, proteolytic fragmentation, and deamidation (6–9).

In this study we have investigated the molecular basis for charge heterogeneity observed in the therapeutic monoclonal antibody MMA383. In our approach, potential modifications were first evaluated using the whole antibody molecule. Upon enzymatic removal of all C-terminal lysines to eliminate this source of heterogeneity, MMA383 was comprised of nine major isoforms when analyzed by CEX which were subsequently purified. Both IEF and CEX analysis of isolated heavy and light chains indicated the presence of one modification site on each chain. Peptide mapping was performed on both chains and revealed a suspect peptide in each chain. Finally, analysis of these peptides by MALDI-TOF MS, CE, and N-terminal sequencing allowed the identification of the modified amino acid residues.

This work presents a new methodical approach that defines the molecular events leading to multiple charge variants present in purified monoclonal antibodies.

MATERIALS AND METHODS

Materials

The murine monoclonal antibody MMA383 (IgG_1) was purified at Novartis Pharma AG during development of the manufacturing process. ABL364 murine monoclonal antibody (IgG3) that specifically recognizes Lewis Y carbohydrate antigen was developed at the Wistar Institute, U.S.A.. Research-grade material was manufactured at Novartis. All chemicals and reagents used were of analytical grade.

Size-Exclusion Chromatography (SEC) of MMA383

MMA383 purity was analyzed by SEC using a HPLC System equipped with a Supelco Progel-TSK $G3000SW_{XL}$ column (7.8 × 300 mm). Chromatography was performed with

0724-8741/00/0900-1110$18.00/0

150 mM sodium phosphate buffer pH 6.5 as mobile phase. Sample absorbance was monitored at 210 nm.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Isoelectric Focusing (IEF)

SDS-PAGE was performed under reducing conditions on 4–20% gradient, polyacrylamide TRIS/glycine gels (Novex). IEF was carried out on CleanGel polyacrylamide gels (Pharmacia Amersham) containing a 8:3:3:3:3 ratio of ampholytes of pI 3-10 (Servalyte), pI 8-10.5 (Pharmalyte), pI 6.5-9 (Pharmalyte), pI 8-10 (Biolyte), and pI 6-8 (Biolyte), respectively, on a Multiphor II electrophoresis system (Pharmacia Amersham) thermostated at 10°C. The ampholytes were prefocused by applying 300Vh at 10W. Samples were focused by applying 500Vh at 5W followed by 6000Vh at 10W. Gels were fixed and silver stained.

Cation-Exchange Chromatography (CEX) of MMA383

MMA383 (approximately 200 μg) was loaded onto a Pharmacia Mono S column (5/5) and eluted with 10 mM sodium phosphate buffer in the presence of 50 mM glycine and 0.25 g/l sodium azide by a pH gradient (A at pH 6.5, B at pH 8.0). The linear gradient was from 30 to 85% B over 50 min. Protein elution in CEX was generally followed by absorbance at 280 nm.

For preparative CEX, approximately 400 mg MMA383, devoid of C-terminal lysines were loaded onto a Pharmacia SP-Sepharose HP packed column in 50 mM sodium succinate, pH 5.1. MMA383 isoforms were eluted with a linear pH gradient from 0 to 100% B. Samples were collected manually. After adjustment to pH 5.1 with succinic acid, the collected fractions were subjected to diafiltration against 10–50 mM sodium succinate, pH 5.1, on a 30K ultrafiltration membrane (Amicon Corporation) and lyophilized until further use.

Carboxypeptidase B Treatment

MMA383 (7 mg/ml in 10 mM sodium succinate, pH 5.1) was diluted to 3.5 mg/ml with 50 mM TRIS-HCl, pH 7.5, and treated with carboxypeptidase B (Boehringer) at an enzyme: substrate ratio of 1:5000 (w/w) at 25°C. A solution of 1 M 2,2'-bipyridyl in 50% ethanol/50% 50 mM TRIS-HCl, pH 7.5, was used as a stop solution. The 1 M 2,2'-bipyridyl solution was added to the digest in a ratio of 1:48, and the pH adjusted to 5.5 with 0.5 M succinic acid.

Preparation and Separation of Carboxymethylated Heavy and Light Chains

The heavy and light chains of individual lyophilized isoforms (20 mg) were separated by SEC on Superose 12 prep grade (Pharmacia) in the presence of 4 M guanidine hydrochloride after reduction of the disulfide bridges with dithiothreitol and alkylation of the Cys residues with iodoacetamide. The purified chains were concentrated by ultrafiltration, desalted against 20% (v/v) aqueous formic acid on a column of Biogel P2 (BioRad), and lyophilized after five-fold dilution with water.

CEX of the Light Chains and the Heavy Chains from Individual MMA383 Isoforms

Light chains (approximately 200 μg) were loaded onto a Pharmacia Mono S column and eluted with 20 mM 2-morpholinoethanesulfonic acid (MES) buffer in the presence of 9 M urea by a pH gradient (A at pH 5.0, B at pH 7.2). The linear pH gradient was from 30 to 65% B over 50 min.

Heavy chains (approximately 200 μg) loaded onto a ProPac™ WX-10 column (4 × 250 mm, Dionex) and eluted with 20 mM disodium tetraborate buffer in the presence of 9 M urea by a pH gradient (A at pH 7.95, B at pH 10.25). The linear pH gradient was from 5 to 100 % B over 45 min. The chromatograms of all chain isoforms were normalized using the total peak area.

Stress Testing of Intact Antibody

Aliquots (1 mg) of parental MMA383 (purified variant I-1) in 10 mM sodium succinate buffer pH 5.1 were diluted with 0.5 mM ethanolamine (pH 9.8) to a final pH of 9.5 and incubated in a 25°C water bath for up to 120 h before termination by the addition of 0.5 M succinic acid to a pH of 5.0. Subsequently, samples were concentrated and diafiltered against 50 mM sodium succinate, pH 5.1, on a 30K ultrafiltration membrane (Amicon Corporation).

Peptide Mapping of Individual Chains

Individual isoform chains were dissolved in 9.5 M urea and diluted with a solution of Endoproteinase Lys-C (Boehringer) in 112 mM HEPES buffer, pH 7.5, to obtain final concentrations of 2 mg/ml of protein, 20 μg/ml endoproteinase Lys-C, 1 M urea, and 100 mM HEPES, pH 7.5. After 24 h at 20°C the reaction was terminated by adding 150 mM succinic acid to approximately pH 4. Peptides were resolved with a linear gradient from 0 to 52% B (A: 0.1% aqueous trifluoroacetic acid (TFA) and B: 0.1% TFA in 90% acetonitrile/10% water) over 100 min at 2.4 ml/min on a Nucleosil 300-5C18 column (1.0 × 25 cm, Alltech).

Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS)

Samples were prepared by mixing 5 μl of the peptide (at approximately 1–10 pmol/μl in acetonitrile and aqueous 0.1% TFA) with 5 μl of matrix solution (α-cyano-4-hydroxycinnamic acid saturated in a 2:1, aqueous 1% TFA:acetonitrile solution). This solution was applied to a stainless steel probe and dried under vacuum. Mass spectrometric analysis was performed on a Biflex Bruker laser desorption time-of-flight mass spectrometer equipped with a nitrogen laser (337 nm, <25 nsec pulse width, LSI Laser Science, Inc.). The instrument was externally mass calibrated in the reflectron mode using a mixture of peptides of known mass. Average error was <0.03% in the reflector mode.

Peptide Sequencing

The peptides were sequenced using standard Edman degradation techniques (10) and was performed on a Perkin-Elmer/Applied Biosystems sequencer model 473A equipped with an on-line HPLC analyzer.

Quantitation of isoAspartate

Isoapartate was measured enzymatically using protein isoaspartyl methyl transferase (PIMT) and a tritium marker. The assay was done using an isoaspartate assay kit according to the instructions provided by the manufacturer ("ISOQUANT™ Deamidation Detection Kit", Promega Corporation).

Biomolecular Interaction Analysis

Binding interactions between individual MMA isoforms (150 nM) and ABL364 antibody (the original antigen of MMA383) were determined using a BIACORE® 3000 biosensor system (Biacore, Inc.). ABL364 (approx. 2.2 ng/mm^2) was bound onto an activated CM5 sensor chip (Biacore, Inc.). HBS-buffer (10 mM HEPES, pH 7.4, 3.4 mM EDTA, 150 mM sodium chloride, 0.005% Tween 20) was used as running buffer. Binding rate determination was done at a flow rate of 20 μl/min with a dissociation time of 150 sec. Regeneration of the chip was accomplished with a pulse of 10 mM glycine/HCl pH 2.5.The resulting sensorgram data were fitted with a 1:1 Langmuir binding model using BIAevaluation 3.0 software (Biacore, Inc.), yielding values for association and dissociation rate constants (k_a and k_d, respectively). Binding affinities were expressed as dissociation constants (K_D (M)), where $K_D = k_d/k_a$.

RESULTS

Purity of MMA383

MMA383 had a purity of > 99% according to SEC (Fig. 1, upper panel). SDS-PAGE under reducing conditions showed the expected two bands of approximately 54 and 25 kDa representing the heavy and light chains, respectively. A number of additional bands were detected by silver staining of the gel but these proteins totaled < 1% by intensity. Although homogeneous by size, marked charge heterogeneity was evident in isoelectric focusing (IEF) and cation exchange chromatography (CEX; Fig. 1, lower panel). IEF showed three major bands and several minor bands ranging from pI 6.6 to 7.4. CEX of MMA383 revealed a very complex elution profile with the main peaks eluting in the more basic portion of the gradient.

Antibodies have been reported to have isoelectric variants due to different factors (reviewed in 8,11). N-terminal sequencing yielded only the light chain sequence, suggesting that neither partial capping of the heavy chain N-terminus nor proteolytic degradation was the cause of isoelectric heterogeneity. Moreover, there was no evidence for the presence of sialic acid, O-linked sugars or unglycosylated antibody (data not shown).

Contribution of C-Terminal Lysine Heterogeneity to MMA383 Charge Heterogeneity

Partial removal of C-terminal lysines from the heavy chain is a common cause of charge heterogeneity in monoclonal antibodies (11). To assess the contribution of this phenomenon, these lysine residues were completely removed by treating the antibody with carboxypeptidase B. The time course of the reaction was followed by CEX analysis which

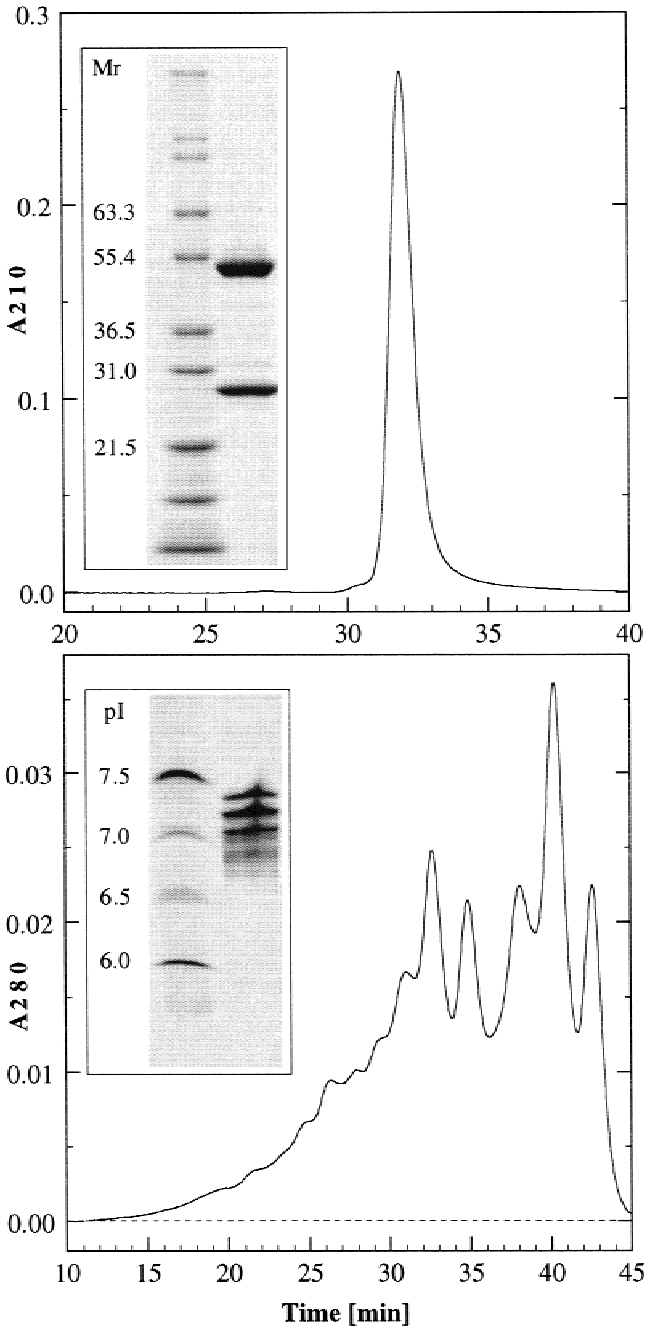

Fig. 1. Size homogeneity and charge heterogeneity of MMA383. Upper panel: SEC analysis of 20 μg MMA383. Upper panel inset: SDS-PAGE separation of 2 μg MMA383 (lane 2) and a wide range molecular mass marker (lane 1). Gels were stained with Coomassie blue. Lower panel: CEX of MMA383 using a Pharmacia Mono S column. Lower panel inset: Isoelectric focusing on a polyacrylamide gel followed by silver staining. MMA383 (lane 2) and IEF protein standards (lane 1).

demonstrated a gradual shift to more acidic pH and a simplification of the overall pattern (Fig. 2). Simplification of the CEX elution profile implied that C-terminal lysine microheterogeneity accounted for part of the charge heterogeneity. Upon removal of C-terminal lysines, CEX analysis showed a pattern of three isoforms which seemed to be repeated three times (Fig. 2, CEX 24 h) indicating an alternative source of this remaining charge variance.

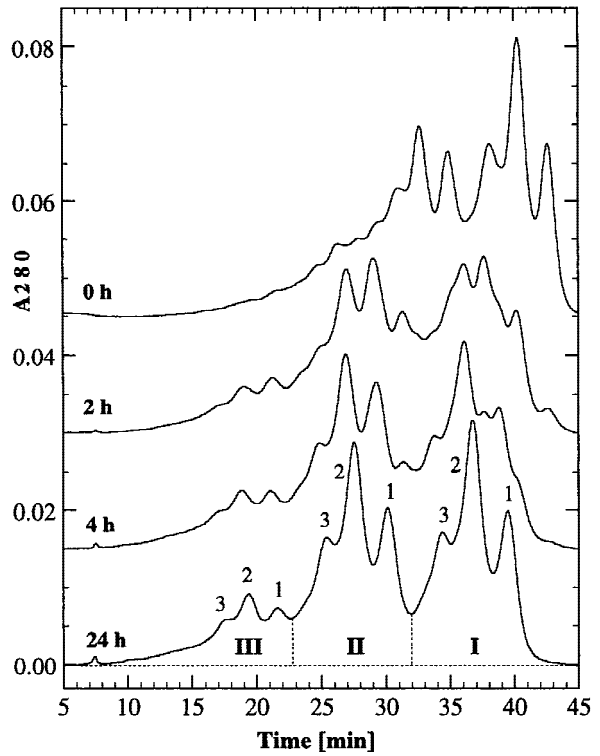

Fig. 2. C-terminal lysine heterogeneity and nomenclature of MMA383 charge variants. C-terminal lysines were removed with carboxypeptidase B. The reaction was stopped at the designated time points and the digest analyzed by CEX on a Pharmacia Mono S column. The isoform nomenclature is shown on the CEX chromatogram of the 24-h sample. Three peak groups were identified in the elution profile at the end of the treatment and given the Roman numerals I, II, and III. Each group contained three dominant peaks which were identified by Arabic numbers 1, 2, and 3.

Characterization of Isolated MMA383 Isoforms Lacking C-Terminal Lysines

The nine isoforms were isolated by preparative CEX and purified by rechromatography for further characterization (Fig. 3A). Because of their low abundance (Fig. 2), the group III isoforms were the least pure. When analyzed by IEF, the pattern of three isoforms was repeated, with decreasing net charge within each group (Fig. 3B). Interestingly, the isoelectric points (pI) of variants II-1 and III-1 were the same as those of variants I-2 and II-2, respectively, and were higher than the pI of the last peak of the preceding group, i.e. variants I-3 and II-3. Therefore, IEF did not reflect the full extent of charge heterogeneity of MMA383; neither was the order of elution in CEX determined by net charge alone.

Deamidation of Asn to Asp and isoAsp leading to an increase in protein acidity is a common degradation mechanism that would explain the observed pI-shifts. Therefore, the content of isoAsp in the purified isoforms was quantified. The results showed a significant increase in isoAsp content from isoform I-1 to III-2/3 (Fig. 3C), indicating that the charge variants of MMA383 present after removal of C-terminal lysines are most likely caused by deamidation. Deamidation of the antibody did not affect sites responsible for antigen binding since the binding affinities toward the original antigen, ABL364, were not significantly different among the isoforms (Fig. 3D).

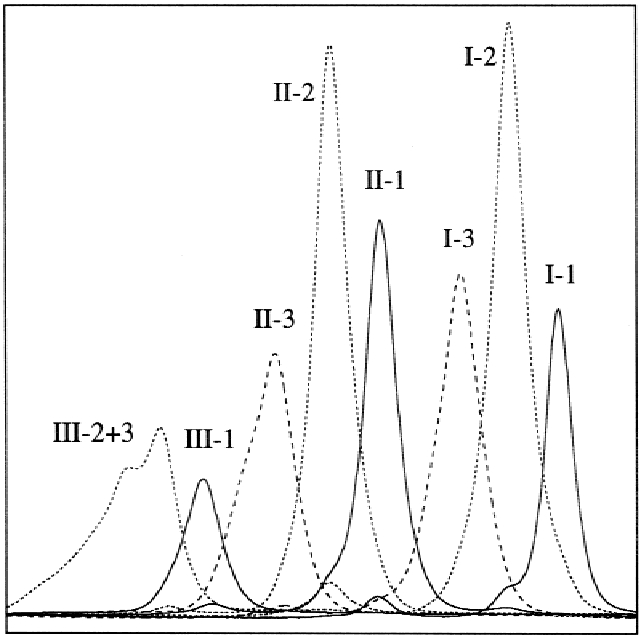

Fig. 3. Properties of charge variants of MMA383. The antibody was digested for 24 h with carboxypeptidase B and individual isoforms were purified as described in the Materials & Method section. A: CEX analysis of purified charge variants performed as in Fig. 2. B: IEF analysis of 5 μg protein. The pI-range of the part of the gel shown extends from 7.5 (top) to 6.5 (bottom). C: IsoAsp content was measured enzymatically using protein isoaspartyl methyl transferase (PIMT) and a tritium marker. D: Binding to ABL364 was determined by surface plasmon resonance spectroscopy using a BIAcore instrument. In B, C, and D the samples appear in the same order as the CEX fractions in A.

Analytical Characterization of Purified Heavy and Light Chains

To further delineate the sites of deamidation, the heavy and light chains of each isoform were purified by SEC after reduction of the disulfide bridges and alkylation of the Cys residues. Each chain could then be studied individually to determine if the charge heterogeneity was due to degradation of the heavy chain, light chain or both. CEX analyses of the light chains of each isoform are shown in Fig. 4A. The chromatograms of group I light chains were virtually identical with

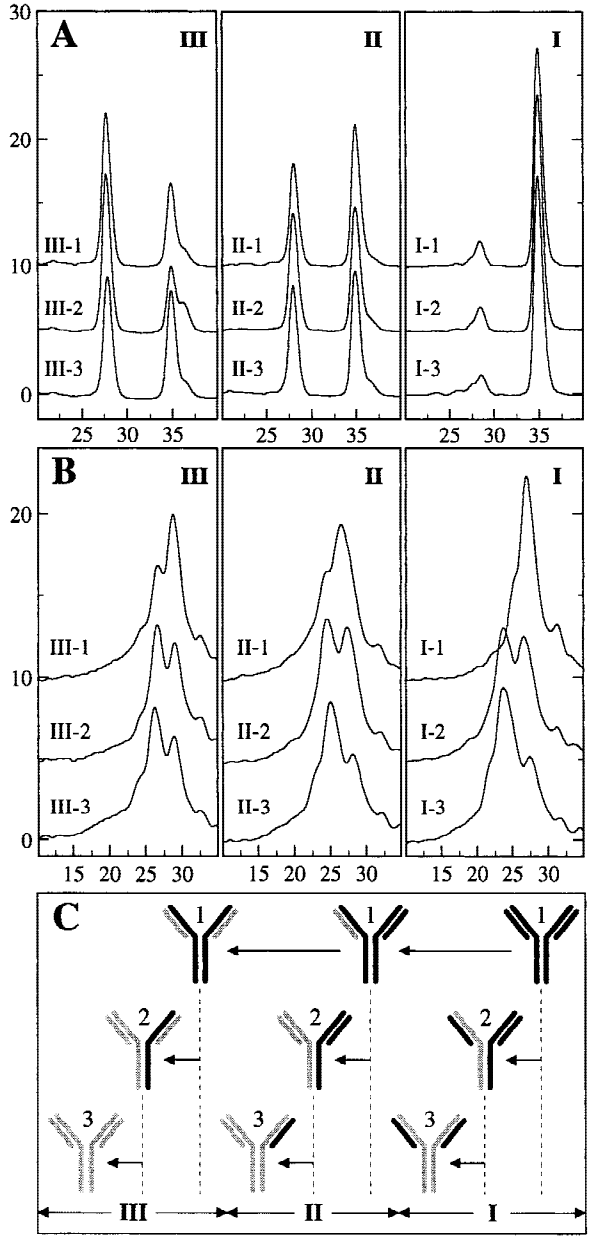

Fig. 4. Assignment of charge variation sites to MMA383 heavy and light chains. A: CEX chromatograms of the light chains of the group I, II, and III isoforms showing the elution profile between 20 and 40 min. B: CEX analyses of the heavy chains of the group I, II, and III isoforms. Chromatograms are shown for retention times of 10 to 35 min. Among the light and heavy chain samples, the chromatograms were normalized using the total area of the peaks. See Methods section for details. C: Schematic presentation of the explanation for the observed shifts. Chains shown in black indicate the native form, while gray is used for chains that underwent deamidation.

a single dominant peak. Group II chromatograms showed the same peak and another more acidic of roughly the same size. Both species were also present in group III but the contribution of the earlier eluting component was larger than in group II. This pattern was consistent with the idea that peak groups II and III are derived from group I through deamidation of one and both light chains, respectively (Fig. 4C).

A similar analysis was performed with the purified heavy chains (Fig. 4B). In contrast to the light chains, the peak patterns within group I heavy chains were very different. Isoform I-1 contained one predominant peak whereas isoform I-2 resolved into two peaks of approximately the same area, one with the retention time of the major peak in I-1 and a more acidic component. The same peaks were seen in isoform I-3 but the acidic component was more predominant. The heavy chains of groups II and III showed essentially the same elution profiles as group I. Apparently, each of the shifts within a group resulted from deamidation of one or both heavy chains (Fig. 4C). Similar results were obtained by IEF analysis of the heavy and light chains (data not shown). One likely explanation why the results of the CEX analyses of heavy chain and light chains did not fit the model as well as expected is that the isolated isoforms were not purified to isoelectric homogeneity (Fig. 3A & 3B) which affected the results obtained for the group III most.

Taken together, these data suggest three charge states of the heavy chain and three charge states of the light chain (Fig. 4C), resulting in nine possible combinations as seen in Fig. 2.

In vitro Degradation of Isoform I-1 at Basic pH

The hypothesis that charge variants are generated by progressive deamidation of the antibody was tested by placing the purified I-1 isoform under conditions that favored deamidation. Isoform I-1 was incubated at pH 9.5 and 25°C for up to five days (Fig. 5). Within three days, the majority of isoform I-1 was converted to isoforms of lower isoelectric points. The pattern was remarkably similar to the original CEX elution profile of MMA383 after removal of the C-terminal Lys residues (Fig. 2; 24 h). This indicates that deamidation is another mechanism responsible for MMA383 charge heterogeneity.

Peptide Mapping of Heavy and Light Chains

In order to determine the specific sites of deamidation, Reversed-Phase HPLC peptide maps of the individual chains were developed. Peptides obtained after digestion with endoproteinase Lys C (Lys C) were identified by MALDI-TOF MS and, when necessary, N-terminal sequencing. Due to the insufficient purity of the isoforms in group III, only chains from groups I and II were mapped.

Comparison of the heavy chain maps revealed substantial differences in the elution window of 90–100 min (Fig. 6A). Two small peaks labeled H9a and H9c eluted before and after the peak labeled H9b and had a mass which was one unit higher than that of H9b, possibly resulting from deamidation of Asn to either Asp and isoAsp. This notion was confirmed by N-terminal sequencing of the peptides (Fig 6). The sequence of peak H9a prematurely terminated after T140, indicating an isoAsp residue in position 141 since isoAsp is known to be incompatible with Edman degradation. An Asp residue was found at position 141 of peak H9c, demonstrating the presence of a deamidation site at this position. The amount of peptides H9a and H9c increased at the expense of the parent fragment H9b in isoforms within a CEX peak group, confirming that the shifts within a peak group were due to deamidation of heavy chain Asn141.

In case of the light chain maps, no significant differences were found within a CEX peak group but small peaks labeled L9b and L9*b were increased in the maps of group II com-

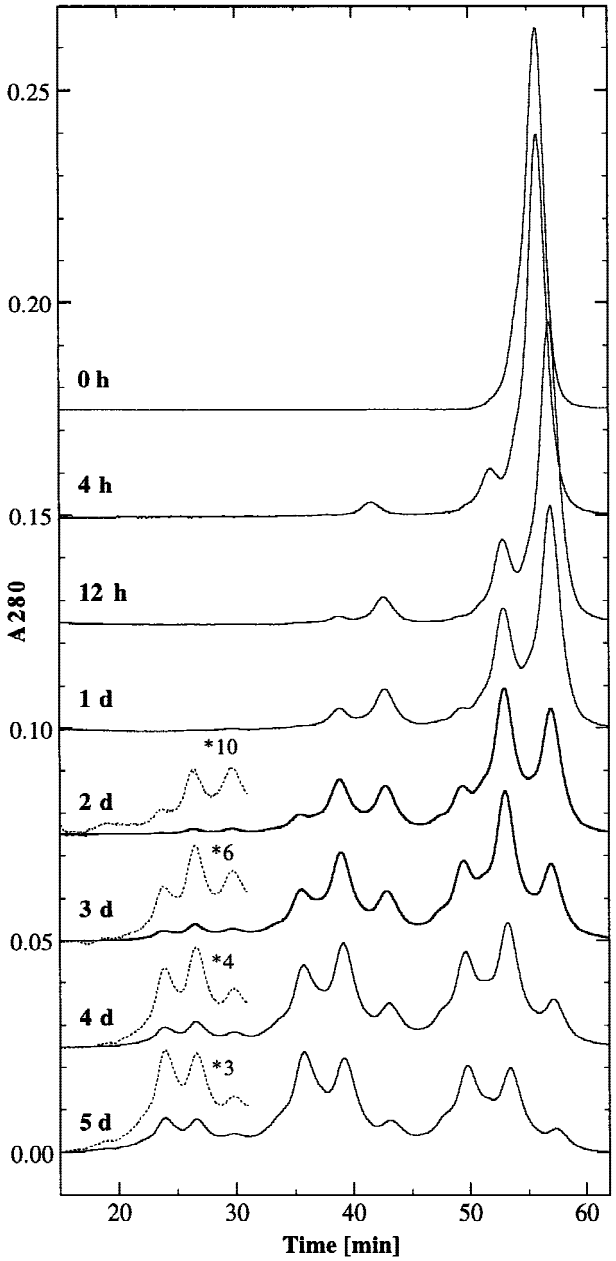

Fig. 5. Alterations of the charge properties of isoform I-1 during incubation at pH 9.5 and 25°C. Isoform I-1 (1 mg/ml) was incubated for up to 5 days at 25°C in 50 mM ethanolamine pH 9.5. After termination of the stress, aliquots of 100 µl (=200 µg of concentrated and diafiltered protein) were analyzed by CEX on a Pharmacia Mono S column after the designated time points. For the last four incubation times, the region of the peak group III has been amplified by the indicated factors (broken lines).

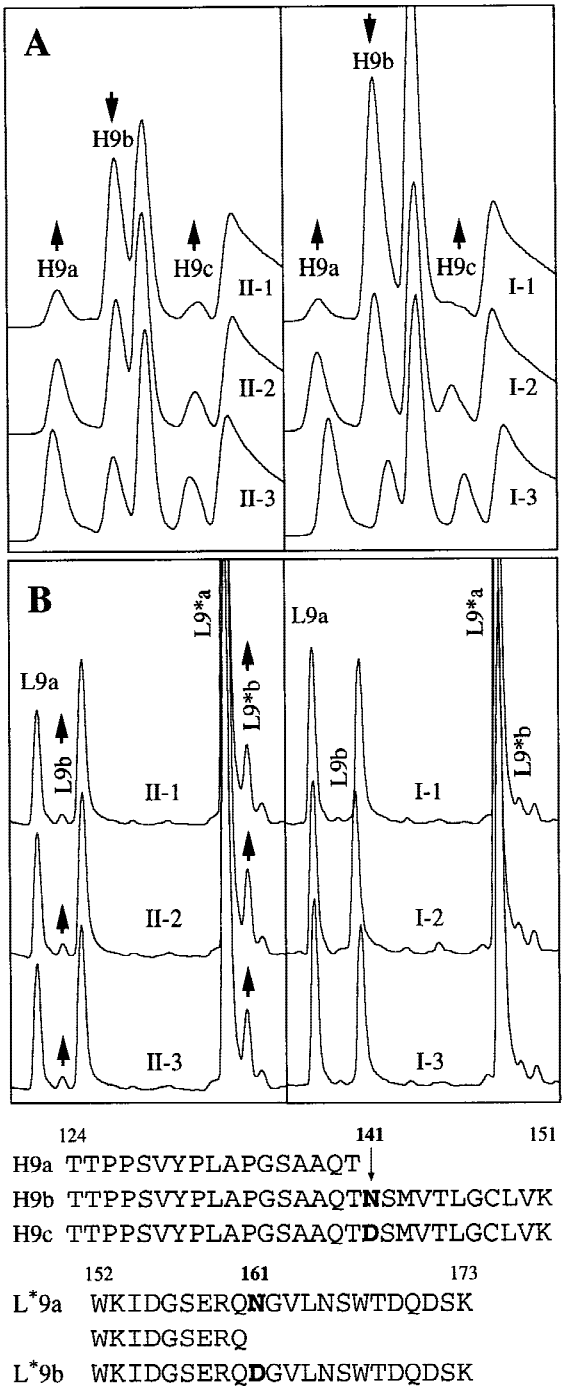

Fig. 6. Peptide mapping of MMA383 heavy and light chains. Endoproteinase Lys-C digested individual chains from isoform group I and II were analyzed using a Nucleosil 300-5C18 column. Peptide elution was monitored by absorbance at 280 nm. Upper panels: Details of peptide maps of heavy chains showing the region of Rt 90-100 min. Peaks H9a, H9b, and H9c contain peptide H9. Note the differences in the relative amounts of these peaks in e.g. I-1, I-2 and I-3. Lower panels: Sections of peptide maps of light chains showing region Rt 70-85 min where fragments L9 and L9* elute. Changes between groups are indicated by arrows (e.g. compare I-1 with II-1). Amino acid sequences obtained from N-terminal sequencing of indicated peaks containing heavy chain peptide H9 (in A) and light chain peptide L9* (in B) is shown below. Superscripted numbers identify the amino acid number in the primary sequence.

pared to group I (Fig. 6B). Peptides L9* resulted from a partial cleavage at Lys153. MALDI-TOF MS revealed that peptides L9b and L9*b were deamidated since their masses were one unit higher than L9a and L9*a, respectively. The exact location of deamidation was identified by sequencing peak L9*a of group I along with peaks L9*a and L9*b of group II (Fig 6). Peak L9*a from group I showed the predicted native amino acid sequence which was also found for 50% of peak L9*a from group II. For the remaining 50% of peak L9*a the sequence broke off prematurely after Q160,

consistent with the presence of an isoAsp residue as position 161. In L9*b of group II, position 161 was Asp. MALDI-TOF MS analysis and capillary electrophoresis confirmed that the Asn and isoAsp forms were co-eluting at the position of L9*a (data not shown). Thus, the shifts from CEX peak group to peak group were due to deamidation of light chain Asn161.

DISCUSSION

Charge heterogeneity is a common phenomenon in antibodies and recombinant proteins. Manufacturers of pharmaceutical proteins are expected to define and characterize the pattern of heterogeneity and assure batch-to-batch consistency for approval by regulatory authorities (5). The aim of this study was to identify specific sites and chemical mechanisms responsible for the extensive charge heterogeneity detected in the therapeutic antibody MMA383. The experimental approach outlined in the introduction allowed the identification of three sources of heterogeneity in MMA383: partial removal of Lys445 of the heavy chain by an endogenous carboxypeptidase activity, deamidation of heavy chain Asn141, and deamidation of light chain Asn161.

Charge variation arising from enzymatic removal of a C-terminal lysine from either one or both heavy chains is frequently observed in monoclonal antibodies (11) with up to three charge variants resulting from limited removal of the terminal lysine. In MMA383, the relative percentage of each C-terminal variant could not be quantified accurately due to substantial additional heterogeneity. The extent of C-terminal Lys removal is likely to be related to the endogenous carboxypeptidase activity of the host cell line used for the expression of the antibody (11) and might therefore be reduced by choosing alternative expression systems or the use of specific carboxypeptidase inhibitors. Although this post-translational modification generally does not impair the functional properties of the antibody, the C-terminal heterogeneity is usually monitored for reasons of product consistency.

Deamidation has been shown to be one of the major chemical degradation mechanisms in protein pharmaceuticals during production and storage (8). In a study with Orthoclone OKT3, two susceptible deamidation sites were identified. One of these was found in the constant region of the light chain at the same position as in the MMA383 light chain (12). This indicates that the kappa light chain is particularly susceptible to deamidation at this site. To our knowledge, deamidation of Asn 141 of a γ1 heavy chain, the other susceptible site in MMA383, has not been reported in the literature.

Deamidation of Asn at neutral to alkaline pH is facilitated if the side-chain of the subsequent residue is small or absent, like in Ser or Gly (13,14). The two deamidation sites identified in MMA383 are no exception: Asn161 in the light chain is followed by a glycine residue, Asn141 in the heavy chain by a Ser. The antibody contains additional Asn-Gly and Asn-Ser sequences for which no evidence of deamidation could be found. In a molecular model created by grafting the sequence of MMA383 onto the crystal structure of a mouse Fab fragment (15), the two susceptible Asn residues were in highly exposed turn regions and not involved in interactions with neighboring side chains (unpublished data). Thus, additional factors, such as exposure to solvent and the three-dimensional structure of the protein influence the rate deamidation (16–18).

At neutral and alkaline pH, the succinimide intermediate spontaneously hydrolyzes to isoAsp or Asp in an approximate ratio of 3:1 (19,20). The isoAsp can distort the conformation of a protein and has been linked to loss of biological activity (21,22), enhanced susceptibility to proteolysis (for review see 23), and altered immunogenicity (24). In MMA383, proteolysis does not appear to be facilitated by deamidation at either site. Prevalent proteolytic antibody fragments present after long-term stress at 40°C are not generated by cleavage of the polypeptide chains near these sites (data not shown).

Analyses of purified chains of individual charge variants by CEX served to identify the chain(s) carrying the modification(s). Although both chains needed to be mapped in the present case, much time and effort could have been saved if the changes had been confined to just one chain. Finally, these CEX analyses offered a simple explanation of the puzzling repetitive pattern observed in CEX of the antibody after digestion with carboxypeptidase B.

The comparison of the MMA383 charge variants with IEF and CEX showed that CEX could resolve variants that comigrated in IEF. The shift in retention time of the individual isoforms in CEX was dependent on whether the heavy or light chain was affected. Charge differences in the light chains resulted in more pronounced shifts, thus creating a repeated triplet-pattern. The finding that CEX could discriminate between variants with identical net charge but different modification sites was remarkable considering the size of an antibody molecule and the fact that deamidation affects the binding to a cation exchanger indirectly since the number of positively charged groups mediating the binding remains the same. The separation was achieved by pH-gradient elution at very low ionic strength suggesting that long-range electrostatic interactions are involved which are readily suppressed by too many screening ions.

Our study on MMA383 exemplifies the complexity generated by three sources of charge heterogeneity in an antibody molecule. Since deamidation leads to either Asp or isoAsp, which can occur in one or both chains of the same type and in all four chains in various combinations, the total number of chemical entities amounts to 45 if structural isomers are counted. The C-terminal Lys heterogeneity raises this number to 135 even if structural isomers are excluded from this last calculation. Thus, it is easy to see why the CEX analysis of MMA383 exhibits an almost continuous pattern of overlapping peaks. Although different batches of the drug substance most likely contain the same charge variants, their relative amounts will depend on the exact history of the batch. This, in turn, gives credence to the notion that batch-to-batch consistency with respect to the isoform pattern will be impossible to achieve with this type of product.

Deamidation of MMA383 occurred in the constant regions of the antibody Fab part and did not affect antigen binding. Apparently, the sequences connecting the constant and variable parts were sufficiently flexible to accommodate any distortions due to the conformation introduced by deamidation. Although deamidation did not cause detectable changes in biological activity, the generation of isoAsp in pharmaceutical proteins is a regulatory concern. In instances where deamidation results in loss of biological activity, the charge heterogeneity and concomitant lack of batch-to-batch consistency would pose a serious problem. Stress testing implied that substantial degradation already takes place during

cell cultivation and cannot be avoided. In principle, it would be possible to design a purification process delivering only the parent antibody molecule but the yield would be low. Thus, the only practical way to eliminate deamidation would be the replacement of the unstable amino acids by introducing point mutations.

With the present study, we were able to understand the origin of and the mechanisms generating charge heterogeneity in MMA383. Accumulating information about hot spots for degradation in monoclonal antibodies will facilitate faster identification of degradation sites in the future. Both the knowledge of the degradation sites and the methodology for their elucidation will be supportive in the development of other antibodies of the same class.

ACKNOWLEDGMENTS

We are very grateful to Marjory Barnes for peptide sequencing and Sabine Dambacher for MALDI-MS analysis of numerous peptides. Michael Gröbke, Daniela Meier, Dr. Daniela Gabriel, and Jean Matscher are acknowledged for their skillful technical assistance. Special thanks to Dr. Wilhelm Stark for 3D-modelling of MMA383. We would like to thank Drs. Patrick Dennis, Bernhard Helk, Roland Günther, and Nancy Harmony for critical reading of the manuscript and fruitful discussions.

REFERENCES

1. G. Ragupathi. Carbohydrate antigens as targets for active specific immunotherapy. *Cancer Immunol. Immunother.* **43**:152–157 (1996).
2. L. H. Pai, R. Wittes, A. Setser, M. C. Willingham, and I. Pastan. Treatment of advanced solid tumors with immunotoxin LMB-1: An antibody linked to Pseudomonas exotoxin. *Nat. Med.* **2**:350–353 (1996).
3. P. B. Chapman. Anti-idiotypic monoclonal antibody cancer vaccines. *Sem. Cancer Biol.* **6**:367–374 (1995).
4. H. Loibner, H. Jaksche, and E. Janzek. A monoclonal antiidiotypic antibody that mimicks the Lewis Y carbohydrate antigen—Immunization of Rhesus monkeys leads to a strong immune response specific for Lewis Y positive cancer. *Proc. Soc. Biol. Therapy* **8**:66 (1995).
5. Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use. US Department of Health and Human Services, Food and Drug Administration, Center for Biologics Evaluation and Research, February 28, 1997.
6. R. Jefferies, J. Lund, H. Mizutani, H. Nakagawa, Y. Kawazoe, Y. Arata, and N. Takahashi. A comparative study of the N-linked oligosaccharide structures of human IgG subclass proteins. *Biochem. J.* **268**:529–537 (1990).
7. R. Jefferis and J. Lund. Glycosylation of antibody molecules: Structural and functional significance. *Chem. Immunol.* **65**:111–128 (1997).
8. M. Manning, K. Patel, and R. Borchardt. Stability of protein pharmaceuticals. *Pharm. Res.* **6**:903–918 (1989).
9. D. B. Volkin, H. Mach, and C. R. Middaugh. Degradative covalent reactions important to protein stability. **8**:105–22 (1997).
10. G. Allen. *Sequencing of Proteins and Peptides,* North-Holland, Amsterdam, 1981.
11. R. J. Harris. Processing of C-terminal lysine and arginine residues of proteins isolated from mammalian cell culture. *J. Chromatogr. A.* **705**:129–134 (1995).
12. D. Kroon, A. Baldwin-Ferro, and P. Lalan. Identification of sites of degradation in a therapeutic monoclonal antibody by peptide mapping. *Pharm. Res.* **9**:1386–1393 (1992).
13. P. Bornstein and G. Balian. Cleavage at Asn-Gly bonds with hydroxylamine. In C. H. W. Hirs and Serge N. Timasheff (eds.), *Methods Enzymol. Vol. XLVII, Enzyme Structure,* (Part E), Academic Press, New York, 1977.
14. R. Stephenson and S. Clarke. Succinimide formation from aspartyl and asparaginyl peptides as a model for the spontaneous degradation of proteins. *J. Biol. Chem.* **264**:6164–6170 (1989).
15. B. C. Branden, H. Souchon, J.-L. Eisele, G. A. Bentley, T. N. Bhat, J. Navaza, and R.J. Poljak. Three-dimensional structures of the free and the antigen-complexed Fab from monoclonal antilysozyme antibody D44.1. *J. Mol. Biol.* **243**:767–781 (1994).
16. S. Clarke. Propensity for spontaneous succinimide formation from aspartyl and asparaginyl residues in cellular proteins. *Int. J. Pept. Protein Res.* **30**:808–821 (1987).
17. A. A. Kossiakoff. Tertiary structure is a principal determinant to protein deamidation. *Science* **240**:191–194 (1988).
18. R. Tyler-Cross and V. Schirch. Effects of amino acid sequence, buffers, and ionic strength on the rate and mechanism of deamidation of asparagine residues in small peptides. *J. Biol. Chem.* **266**:22549–22556 (1991).
19. S. Clarke, R. Stephenson, and J. Lowenson. Lability of asparagine and aspartic acid residues in proteins and peptides, spontaneous deamidation and isomerization reactions. In T. Ahern and M. Manning (eds.) *Stability of Protein Pharmaceuticals, Part A, Chemical and Physical Pathways of Protein Degradation,* Plenum Press, New York, 1992, pp. 1–29.
20. E. Haley, B. Corcoran, F. Dorer and D. Buchanan. β-Aspartyl Peptides in Enzymatic Hydrolysates of Protein. *Biochemistry* **5**: 3229–3235 (1966).
21. T. J. Ahern and A. M. Klibanov. The mechanisms of irreversible enzyme inactivation at 100C. *Science* **228**:1280–1284 (1985).
22. J. Cacia, R. Keck, L.G. Presta, and J. Frenz. Isomerization of an aspartic acid residue in the complementarity-determining regions of a recombinant antibody to human IgE: Identification and effect on binding affinity. *Biochemistry* **35**:1897–903 (1996).
23. H. T. Wright. Sequence and structure determinants of the nonenzymic deamidation of asparagine and glutamine residues in proteins. *Protein Eng.* **4**:283–294 (1991).
24 F. C. Westall. Released myelin basic protein: The immunogenic factor? *Immunochemistry* **11**:513–515 (1974).