Application of Electrospray Mass Spectrometry (ES-MS) for the Analysis of Monoclonal Antibody F\textsubscript{c} Subunits

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INTRODUCTION

Recent developments in the hybridoma technology have opened up new perspectives for the commercialization of monoclonal antibodies (Mabs)\textsuperscript{6} as pharmaceutical products. To determine the physicochemical properties and stability of therapeutic Mabs, the use of a panel of appropriate analytical methods is required (1–4). Recently, ES-MS has been introduced as a powerful technique for the determination of the MW of proteins: it has proven to be a precise and accurate method with high resolution (5–8) when compared to conventional methods, such as gel permeation chromatography and SDS-PAGE. In ES-MS the obligatory ionization is achieved via electrically assisted nebulization, yielding a series of multiply charged molecular ions. The mass spectrometer separates the ions according to the ratio between mass number and number of electrical charges. Since almost no fragmentation occurs, the set of peaks obtained represents multiply protonated molecular ions with successive numbers of electrical charges.

A common feature of IgG class antibodies is the heterogeneity in the N-linked glycosylation in the C\textsubscript{12} domain (9–13). The average molecular mass of entire monoclonal antibodies and conjugates thereof has been determined by laser desorption mass spectrometry (14), but this technique does not resolve the molecular masses of individual species of large proteins that exhibit carbohydrate heterogeneity (14,15). Heterogeneous carbohydrate chains of glycoproteins have been investigated by mass spectrometry, but only of enzymatic or chemical digests with molecular masses up to about 5000 Da (15). The present study shows that mass heterogeneity in relatively large (ca. 25-kDa) glycoproteins, i.e., the F\textsubscript{c} part of two mouse IgG Mabs, MN12 and WT31, can be resolved by ES-MS. In addition, the influence of storage of MN12 under alkaline conditions on the ES-MS spectra of the F\textsubscript{c} subunits has been investigated.

MATERIALS AND METHODS

Materials

Production of the Mabs MN12 (mouse IgG\textsubscript{2a,\textgreek{m}}) and WT31 (mouse IgG\textsubscript{1,\textgreek{m}}) has been described elsewhere (16). Storage of MN12 at pH 10.0 and 37°C for 0, 16, 32, and 64 days was performed as described previously (3).

Papain (2× crystallized and lyophilized powder), DTE, and iodoacetamide were obtained from Sigma (St. Louis, MO). Digoxigenin-labeled lectins, carboxypeptidase Y, transferrin, fetuin, asialofetuin, and alkaline phosphatase-labeled monoclonal antibody against digoxigenin (anti-DIG-AP) were purchased from Boehringer (Mannheim, Germany). Bovine serum albumin (BSA) was obtained from Organon Teknika (Turnhout, Belgium). Purified PIC3 protein from Neisseria gonorrhoeae (17) was kindly provided by Dr. G. F. A. Kersten. All chemicals used were of analytical grade. Spectra/Por molecular porous membrane tubing with a MW cutoff of 12,000–14,000 (Spectrum, Los Angeles, CA) was used for dialysis.

Preparation of F\textsubscript{c} Subunits

Enzymatic Digestion. Solutions of 1 mg/ml purified Mabs MN12, whether previously stored at pH 10 (see above) or not, and WT31 in 0.10 M Tris/HCl (pH 8.0) + 2.0 mM EDTA (buffer A) were prepared. Papain was activated by incubation of a 1.0 mg/ml solution in 0.10 M Tris/HCl (pH 8.0) + 2.0 mM EDTA + 1.0 mM DTE (buffer B) for 20 min at 37°C (activated papain). Enzymatic digestion of the Mabs was carried out by incubating 20 ml of Mab solution with activated papain for 8 hr at 37°C, using a Mab/papain ratio of 100:1 (w/w). Digestion was terminated by addition of 2 ml 0.20 M iodoacetamide and incubation for 1 hr on melting ice.

After storage at pH 10, MN12 had become less susceptible to digestion by papain (see Results and Discussion). Therefore, the pH 4.5 protein A fractions of the papain digests (see below) of the MN12 samples stored at pH 10 (for 0, 16, 32, and 64 days; see above) were extensively dialyzed against buffer B and incubated again with activated papain for 4 hr at 37°C, using a Mab/papain ratio of 100:1 (w/w).
Digestion was terminated by the addition of 0.1 vol 0.20 M iodoacetamide and incubation for 1 hr on melting ice.

The digests were extensively dialyzed at 4°C against binding buffer for protein A affinity chromatography, viz., PBS (MN12) or 1.5 M glycine/NaOH (pH 8.9) + 3.0 M sodium chloride (WT31).

Separation of $F_a$ and $F_c$ Fractions. The $F_a$ and $F_c$ fractions were separated by protein A affinity chromatography, using an HR 10/10 column containing 8 ml protein A-Sepharose CL4B Fast Flow (supplied on special request by Pharmacia, Uppsala, Sweden), as described previously (16,18). Dialyzed digest was put on the column using the above-mentioned buffers to bind the $F_c$ fraction to the column. Bound material was eluted with 0.1 M sodium citrate, pH 4.5, neutralized with 1 M Tris, and extensively dialyzed against PBS at 4°C.

Reduction and Concentration of $F_c$ Fractions. Reduction of $F_c$ was carried out by incubating 1 vol of dialyzed $F_c$ fraction with 0.1 vol of 0.10 M DTE for 2 hr at 37°C. The reaction was terminated by the addition of 0.1 vol of 0.20 M iodoacetamide and incubation for 1 hr on melting ice. The reduced and alkylated samples were extensively dialyzed at 4°C against 50 mM ammonium carbonate (pH 7.7) + 0.01% (w/v) sodium azide. Next the samples were concentrated by ultrafiltration to a protein concentration of 3 to 5 mg/ml, using an 8MC ultrafiltration cell (Amicon, Lexington, MA) and a Diaflow PM10 ultrafiltration membrane with a MW cut-off of 10,000 (Amicon). Finally, the samples were filtered through a Minisart NML 165 34 K cellulose acetate filter with 0.2-μm pores (Sartorius, Göttingen, Germany) and kept at 4°C until used for ES-MS analysis.

**Immunodot Blots**

Protein solutions (1 μl, 1 mg/ml) were applied onto nitrocellulose (BA85, 0.45 μm, Schleicher Schuell, Dassel, Germany). The blots were left to dry for 30 min at room temperature and then boiled in PBS for 15 min. Blocking of the blots was performed in PBS containing 0.05% (w/v) Tween 20 and 1% (w/v) BSA (PBS-T-BSA) for 1 hr at room temperature. Next the blots were washed four times for 10 min in TBS and then once in PBS containing 1 mM MgCl₂, 1 mM CaCl₂, and 1 mM MnCl₂. Then the blots were incubated with the lectins [10 ml of AAA (1:1000),DSA (1:500), GNA (1:500), PNA (1:500), or SNA (1:100) in PBS-T-BSA] for 2.5 hr at room temperature, followed by five washes with TBS. Next the blots were incubated with anti-DIG-AP (1:1000 in TBS) for 2 hr at room temperature. After five washes with TBS, the blots were developed with 10 ml substrate solution containing 0.4 mg/ml 4-nitro blue tetrazolium chloride + 0.2 mg/ml 2-chloro-4-nitrophenyl phosphate + 0.8 % (v/v) dimethylformamide in 50 mM Tris/HCl (pH 9.5) + 50 mM MgCl₂ + 0.1 M Na Cl and after color development washed with distilled water and dried thoroughly.

**SDS-PAGE**

SDS-PAGE was carried out on a PhastSystem (Pharmacia), using PhastGel 12.14 combs and low molecular weight standards (Cat. No. 161-0304, Bio-Rad, Richmond, CA). Electrophoresis and subsequent silver staining of the gels were performed following the manufacturer's instructions.

**RESULTS AND DISCUSSION**

**Analysis of $F_c$ Subunits of MN12 and WT31**

The ES-MS spectra of the reduced and alkylated $F_c$ subunits of MN12 and WT31 are shown in Fig. 1. The calculated average molecular masses are listed in Table I. The spectra of both MN12 and WT31 display several multiply protonated components. In particular, the spectrum of MN12$F_c$ is complex. Two main components with molecular masses of 25,713 and 25,854 Da are observed (peaks K and L in Fig. 1a, Table I). In addition, at least 10 other components, with molecular masses ranging from 24,835 to 25,602 Da (peaks A–J in Fig. 1a), can be discerned. The WT31$F_c$ spectrum shows four main components, with molecular masses ranging from 25,726 to 26,017 Da (peaks W–Z in Fig. 1b, Table I).

There was a discrepancy between the apparent molecular mass of 31 kDa in SDS-PAGE (not shown) and the molecular masses observed by ES-MS (ranging from 24.8 to 25.9 kDa). This is probably due to aberrant migration of $F_c$ in SDS-PAGE. Inefficient binding of SDS to glycoproteins has been documented (19) and may account for an overestimation of the molecular mass in SDS-PAGE. Clearly, the resolution of ES-MS is much higher than that of SDS-PAGE. Besides, the precision in the mass determinations of the individual components is high: using Eqs. (1) and (2), intraassay standard deviations were at most 3 Da per component. This is in good agreement with the coefficients of variation of 0.01–0.05% reported for ES-MS of proteins (6,8,15). Similar