Ph. D. Thesis

Mass spectrometric analysis of noncovalent complexation and glycosylation of proteins

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I. INTRODUCTION, AIMS

The structural investigation of biomolecules (carbohydrates, peptides, proteins, glycoproteins, nucleic acids) is an important field of current research, where the relationship between the molecular structure and biological function is studied. Information of their altered structure in different pathological conditions may lead to promising biomarkers and a better understanding of pathophysiology.

Improvements in methodology and instrumentation in mass spectrometry resulted in novel ionization techniques (ESI, MALDI) and convenient on-line coupling to chromatography making macromolecules and complex biological mixtures (blood, urine) amenable for mass spectrometric study. These developments opened new fields for application of mass spectrometry, among which proteomics, biochemistry and biomedical applications are in the forefront of interest.

Proteins, peptides and nucleic acids participate in most biological process as weakly bound, noncovalent complexes, including protein interactions with inhibitors, peptides, and other proteins or with other small molecules as ligands. Investigation of noncovalent complexes has been one of the main fields of proteomic research since a few decades. Beside of most frequent analytical techniques like NMR-, IR-, CD-spectroscopy and X-ray diffraction, mass spectrometry became a widespread tool in studying noncovalent complexes, where the most important aspect is that dissociation of the complexes should be avoided during the ionization process. From a simple mass spectrum information of molecular mass of the intact complex and the binding stoichiometry can be derived.

Another important field of proteomics is the study of post-translational modifications where glycosylation has key role. In glycosylation processes regulated attachment of oligosaccharide content to the peptide chain is taking place causing significant changes in the function of proteins. The growing interest for the glycosylation owing to those observations, that changes in glycosylation of plasma proteins in different pathological condition including cancer, rheumatoid arthritis and immune system deficiencies could be detectable. Mass spectrometry combined with liquid chromatography is one of the best suited analytical techniques that can provide information of glycosylation of proteins and oligosaccharide structures.
The main goal of my work was to study the application of different mass spectrometric techniques in the structural investigation of proteins, glycoproteins, to study their microheterogeneity, and to examine the complexation of proteins with small ligand molecules. Due to the limitation of this thesis, the investigation of two proteins, belonging to the lipocalin family, has been discussed. The aim of the study was:

a) to develop a mass spectrometry based technique for the detection of the noncovalent complexation of beta-lactoglobulin (BLG) and a polyunsaturated fatty acid, cis-parinaric acid (cPA). We aimed to characterize their interaction, specificity of the ligand binding properties to which competitive complexation experiments have been performed. Our further plan was to identify the cPA binding site of the BLG by mass spectrometry using limited trypsinolysis of the protein.

b) to develop a novel mass spectrometry based analytical method to study the glycosylation site occupancy of a highly heterogenic glycoprotein, alpha-1-acid glycoprotein (AGP).

c) to study the oligosaccharide composition of AGP isolated from the sera of various individuals suffering from ovarian tumor and lymphoma and compare to that of healthy individuals using PNGase digestion and MALDI-MS analysis to reveal how the glycosylation pattern changes in pathophysiology and to check the potential utility of glycosylation-based biomarkers. The results were evaluated using statistical method (Linear Discriminant Analysis, LDA).

II. METHODS

Mass spectrometric measurements of the complex of BLG-cPA and the intermediate tryptic fragments still having the ligand binding property were performed using a Perkin–Elmer Sciex API-2000 triple quadrupole instrument (Toronto, Canada) equipped with a TurboIonspray Source in positive ionization mode.

The tryptic digest of the glycoprotein in glycosylation site investigation of AGP was analyzed on a Voyager DE-Pro MALDI-TOF mass spectrometer (Applied Biosystems, Framingham, MA) equipped with a 337 nm nitrogen laser, in linear positive mode and on a
Micromass Q-Tof Micro mass spectrometer equipped with a Waters CapLC HPLC system (Manchester, UK), in positive electrospray ionization mode. Glycopeptides were selectively detected and identified by MS/MS experiments in survey scan (Parent Ion Discovery) mode.

Purified human serum AGP samples obtained from healthy individuals (12) and cancer patients (16 ovarian tumors, 15 lymphoma) were hydrolyzed by PNGase F enzyme and were derivatized with anthranilic acid. Mass spectrometric measurements were carried out on a Voyager DE-Pro MALDI-TOF (Applied Biosystems, Framingham, MA) mass spectrometer in linear mode (positive and negative as well). Statistica 7.0 software package (StatSoft Inc., Tulsa, OK, USA) was then applied to perform Linear Discriminant Analysis (LDA) on the obtained MALDI-MS data.

III. RESULTS

1. A stable, specific complex formation between bovine BLG and cis-parinaric acid, a polyunsaturated fatty acid in a molar ratio of 1:1 has been detected by electrospray ionization mass spectrometry. Competitive complexation experiments were performed using saturated and unsaturated fatty acid standards with different chain lengths and number of double bonds to study the specificity of the interaction. On the basis of these experiments it can be concluded that only the BLG-cPA complex was detected in all cases. The remarkable high complexation ability of cPA to BLG may depends on different factors like the appropriate size, the carboxyl group of fatty acid, and the degree of unsaturation of the molecule. (Paper I.)

2. An intermediate BLG fragment was identified based on limited trypsinolysis combined with mass spectrometry, which has the same ability to bind cis-parinaric acid as the intact protein. The structure of this residual protein core was successfully identified, which was a disulfide bonded residue [41-70]S-S[149-162] that showed ligand binding properties similar to those of the intact BLG. (Paper I.)
3. A new methodology has been developed for studying site specific glycosylation pattern of a highly complex glycoprotein, human AGP. Tryptic digestion was performed using a new anionic surfactant (RapiGest SF), which was recommended to mostly digestion-resistant membrane proteins before, and resulted simple, efficient proteolysis in the case of heavily glycosylated protein, like AGP as well. (Paper II.)

4. Following digestion, the complex glycopeptides-peptide mixture was directly injected onto a capillary HPLC-ESI-MS instrument, and the glycopeptides were selectively identified based on their characteristic sugar oxonium ions whereby N-linked glycosylation pattern of AGP was explored. Using this method all glycosylation sites of AGP have been characterized and altogether 80 different glycopeptides were identified by mass matching and MS/MS experiments, about three times more than known previously. From these results it can be established that glycosylation shows a markedly different pattern for the various glycosylation sites. At sites I and II tri-antennary, however, at sites III, IV and V tetra-antennary complex-type oligosaccharides predominate. The presence of additional N-acetyl lactosamine (Gal-GlcNAc) units (even higher degree of branching and/or longer antennae) at sites IV and V were detected. (Paper II.)

5. Investigation of glycosylation pattern of AGP by MALDI-TOF MS was achieved on the basis of its PNGase F digested and derivatized glycan structures. With this method AGP samples from 43 individuals (healthy individuals and patients with lymphoma and with ovarian tumor) have been analyzed and 34 N-glycan structures were determined. Mass spectra of oligosaccharides derived from healthy volunteers and cancerous patients showed peaks with higher intensities for high branching tetra-antennary and fucose containing compounds in case of cancer samples. (Paper III.)

6. From the glycosylation pattern determined by mass spectrometry fucosylation and branching indices have also been calculated. These parameters (mainly fucosylation index) showed small discrimination between the patients groups studied, however, these differences are not sufficiently large to use as a potential biomarker. The average values of fucose indexes in case of ovariantumor and lymphoma patient groups were 45 and 60 % higher, respectively, compared to the healthy one. (Paper III.)
7. Linear discriminant analysis of the MALDI MS results showed a very good separation among the three groups (with a classification of 88%). Cross validation indicates that the method has predictive power; identifying cancerous vs. healthy individuals shows 96% selectivity and 93% specificity; identification of lymphoma vs. the mixed group of healthy and ovarian tumor cases is also promising (72% selectivity and 84% specificity). (Paper III.)

IV. IMPORTANCE AND APPLICABILITY OF RESULTS

The structural determination of proteins and protein complexes play an important role in the fundamental understanding of biochemical pathways. The observation of non-covalent complexes formed between proteins and drug molecules can be used typically as a drug target screening method. cPA, a polyunsaturated fatty acid studied in this work has been shown to be toxic in vitro to tumor cells. While cPA is only slightly soluble in water, its non-covalent protein binding property to BLG, a potential carrier protein can increase its bio-availability.

Results presented in this work demonstrated that mass spectrometry gives detailed information on AGP glycosylation and changes in the glycosylation pattern in diseases, which may lead to promising biomarker and better understanding of pathophysiology. The present glycosylation study has to be considered preliminary, as only three patient groups with 10-20 individuals in each have been studied, but shows good promise as a completely new type of biomarker.
IV. PUBLICATION LIST

Publications based on this thesis have been written:


Other publications related to the thesis:


**Selected publications not directly related to the thesis:**


**Oral presentations:**


**Poster presentations:**


