Profiling of soluble and membrane-bound metalloproteinases
Klein, Theo

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2008

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Conclusions and future perspectives
This thesis describes the development of novel methods for activity-based proteomics on zinc-dependent metalloproteases. The functional proteomics field can roughly be divided into two fields. The first approach uses labelling of active enzymes with functionalized probes (usually based on small synthetic inhibitor of the protease of interest) followed by detection or visualization via a fluorescent dye, radioactivity or a biotin moiety on the probe. The second approach focuses on selective enrichment of active proteinases by using immobilized protease inhibitors which can be used to fish out the protease of interest from an aqueous sample followed by detection or identification by Western blot or mass spectrometry. Both approaches were explored within this thesis.

The development and testing of two novel photoactivatable probes for activity-dependent labelling of metalloproteases is described in chapter 3. The structure of the probes was based on optimization of the peptide-like backbone, yielding a novel inhibitor with a high inhibition efficacy even at low nanomolar concentrations (the basic structure for the probes was reversible inhibitor ML5). Addition of a trifluoroazirine photoreactive group for covalent crosslinking of the probe to the active site of the metalloprotease did decrease the affinity towards the tested metalloproteases (as tested by IC\textsubscript{50} determination) but the inhibition constants were still within the nanomolar range. The photocrosslinking approach was chosen since, contrary to for instance serine and cysteine protease the proteolytic cleavage is not mediated by an amino acid residue in the active site, but by an activated water-zinc (II) complex which is coordinated in the active site by three histidine residues. This makes labelling of the metalloproteases more difficult as is the case for the other protease classes, which are readily labelled by probes that attack the intramolecular nucleophilic group that is part of the active site residue (serine or cysteine). The latter approach has yielded several highly successful probes that are now at the point of being used in clinical research (see chapter 2 for references). The photocrosslinking approach is somewhat controversial since the labelling process is not initiated by actual proteolytic activity of the enzyme, but rather by irradiation with UV light. This makes the reaction possibly less efficient and less selective, and some investigators consider the technique not to be truly activity-based. On the other hand, since the labelling of both MMPs and ADAMs using the two novel probes has been demonstrated to be inhibited by TIMPs in a concentration dependent manner gives an indication that the interaction is at least dependent on the availability or accessibility of the catalytic cleft. This may be considered as a measure of activity since the catalytic pocket is not accessible in active metzincins, as it is shielded by the prodomain in the inactive zymogen, and by TIMP in the inhibited form. Preincubation of the enzymes with non-functionalized versions of the probes revealed that even a moderate excess was sufficient to inhibit labelling which is an indication that the labelling is site specific. Since the UV-mediated activation of the trifluoroazirine moiety and subsequent covalent attachment to the protein is by nature non-selective this efficient competition shows that there is at least a structural interaction which positions the probe in a certain part of the enzyme prior to UV irradiation, effectively limiting the possible binding sites. To assess the actual amino acid residue where the probe binds some experiments were performed to determine the labelling site by mass spectrometry.
Recombinant enzymes were labelled with the photoactivatable probe ML22, subsequently digested with trypsin and the tryptic peptides were analysed with LC-MS/MS. One problem that arose during the first attempts was the excess presence of unlabeled peptides, which seemed to indicate a low labelling efficiency. Experiments were further complicated by the primary structure of the proteases themselves, since the tested MMPs and ADAMs have a very low abundance of lysine and arginine residues in the catalytic region the resulting tryptic peptides containing the putative binding site are very large. This makes identification and sequencing by mass spectrometry challenging. The actual binding site has not been identified to date, but experiments are ongoing. Some attempts have been made to create an actual mechanism-based probe for the MMPs using the structure of the N-terminal generic MMP cleavage sequence Pro-Leu-Gly with a crosslinking group that is activated by cleavage and yields a highly reactive quinoline methide group after proteolysis. The first generation inhibitor did unfortunately not show any covalent labelling of MMP-12 (data not shown).

Although the first probe ML22 performed well in labelling ADAM proteases, and was actually the first ABP described in literature capable of labelling ADAMs, labelling of MMPs was considerably less efficient. Since an ideal probe for profiling purposes has a broad selectivity attempts were made to improve the labelling of MMPs by transfer of the photocrosslinking moiety from the P'2 site to the P'1 site. The resulting inhibitor PPG3 demonstrated a family-wide selectivity in labelling both MMPs and ADAMs and labelling of the latter was even more efficient than for ML22. Attempt to identify the labelling site with mass spectrometry using the novel inhibitor probe are ongoing. Since in both probes the labelling of ADAM-10 was exceptionally strong and active ADAM-10 is known to be present in A549 cellular lysate (see figure 1 where the activity-based extraction method was used, followed by Western blot detection of ADAM-10) labelling of endogenous ADAM-10 followed by pull down of biotinylated (endogenous or labelled) proteins with immobilized streptavidin beads was performed. Western blot analysis of the resulting extract showed that in the labelled fraction of the lysate a small extracted band was visible, corresponding to the molecular size of active ADAM-10, while in the non-incubated control lysate no extraction was observed. As observed in the results of ADAM-10 extraction by inhibitor ligand pull down the extraction of the mature form of ADAM-10 from cellular lysates is never complete (compare figure 1, considering the enrichment effect in the elution fractions by a factor of~8). This may imply that a considerable amount of ADAM-10 is present in an inactive, presumably TIMP-inactivated form. This finding is in line with the anticipated role of ADAM-10 in constitutive shedding events leading to a continuous turn-over of the protease. The low labelling efficiency in the lysate by the photoactivatable probe may be explained by this.
Chapter 6

As demonstrated in chapter 4, the activity-based enrichment technique can be used for extraction of active ADAM-17 from a complex proteome (lung carcinoma cell lysate). This report was the first example of application of this technique to ADAM proteases, with earlier being demonstrated for MMPs (recombinant MMP-12 and endogenous gelatinases in synovial fluid). The synthetic work on the first generation reversible inhibitors in Leiden resulted in the availability of an enantiomerically pure building block and the possibility to generate a large library of reversible hydroxamate-based inhibitors with variable amino acid residues in the peptide backbone. The amino acid residue on both the P’2 and P’3 position were demonstrated to have a substantial effect on the inhibition potential of the tested MMPs (MMP-9 and -12) and ADAM-17, with many inhibitors having a strong inhibitory effect on MMP-12, while some inhibitors had a preference for the gelatinase MMP-9 and one was found to exhibit the strongest inhibition towards ADAM-17. The introduction of a primary amine group attached to a spacer or linker enables the immobilization to a solid support by simple chemistry and the use of the novel material for activity-based solid phase extraction of metalloproteases from aqueous samples. The sample chosen for proof of principle was the lung carcinoma cell line A549, and inhibitor FF was selected since optimization experiments demonstrated that the extraction yield of ADAM-17 was still high even at lower concentrations of enzyme. By applying this screening technique, suitable inhibitors for immobilization may be selected and tested prior to application to biological samples, and may improve the extraction results (screening procedure is discussed in chapter 2).

The SPE approach coupled to immunochemical detection by Western blot has proven to be applicable to analysis of endogenous metalloproteases, and attempts have been made to apply the technique to analysis of clinical samples. Sputum samples from an incidental

Figure 1:
Western blot of cartridge-extraction of an A549 lysate (2.5x10⁶ cells) on an immobilized ML5 cartridge FT: flowthrough; W: wash fraction; E1-3: 30 µL elution fractions 10 µM ML05; E4-5: 30 µL elution fractions with 10 mM EDTA; SDS: final wash with SDS-PAGE sample buffer; 8% SDS-PAGE, immunoblotted with anti-ADAM10 ectodomain antibody (R&D systems).
Conclusion and perspectives

cigarette smoke exposure study (courtesy of Dr. N. ten Hacken, University Medical Center Groningen) were analyzed for the presence of active MMP-12. Although the sputum of some volunteers contained considerable amount of MMP-12, and the putative active 22 kDa isoform was detected on Western blot, no detectable extracted amount was observed in EDTA elution fractions. This may indicate that the majority of the MMP-12 is in fact complex with one of the endogenous inhibitors (TIMPs) or that prolonged storage of the samples has deteriorated the active protease. Analysis of the samples for MMP-9 revealed a strong presence of both the zymogen as the mature isoform of this protease in the original sample, but only a very minor extracted amount, which was unexpected since the original study revealed an excess of MMP-9 over TIMP-1 in some of the selected samples.

The issue of sample storage on MMP activity has just begun to be explored. First reports indicate that even storage at -80ºC under normal circumstances can have a detrimental effect after time. This implies that for preservation of (dilute) samples containing metalloprotease activity care has to be taken. Addition of immobilized inhibitor beads immediately after sample acquisition may help to trap and enrich the active proteases for further storage. This approach may also overcome the problem of autoproteolytic degradation of MMPs as observed for e.g. MMP-9. Experimental work on this procedure is underway at present.

The online analysis system described in chapter 5 is still a work in progress. Although the system has performed rather well in term of reproducibility and sensitivity for MMP-12 the identification of endogenous active MMPs has proven difficult. Several issues appear to have to be dissolved. Firstly the level of non-specific interaction that, although low when considering the high protein load on the extraction cartridge, is still at a level that makes identification and detection of very diluted active proteases difficult. Secondly sample handling has to be strictly regulated to conserve MMP activity prior to analysis. At present a study is underway where rat urine samples were incubated with the immobilized inhibitor beads immediately after sampling. A further project is planned focusing at use of alternative solid supports for the inhibitor. Preliminary results show that non-specific binding of proteins (e.g. albumin) in a novel functionalized solid support (ResQ) is comparable to Sepharose, but the pressure stability and solvent tolerance may lead to an improved washing procedure.

When ADAM activity is to be analyzed some additional problems have to be faced. Since the majority of ADAMs is membrane-anchored in their mature, active form they require solubilisation prior to analysis by, for instance activity-based SPE. The usual method to achieve this is the use of (non-denaturing) surfactants in the lysis buffer, an approach that has been used throughout total proteome preparation within this thesis. Although many surfactants are reported to be non-denaturing, they may have an effect on protease activity as demonstrated by a decreased proteolytic activity of recombinant ADAM-17 in the presence of deoxycholate and Brij-35 (data not shown). Negative effects of surfactant in analysis are also described in chapter 3, where the photocrosslinking efficiency of probe ML22 in the presence of the commonly used detergent Triton X-100 was found to be
dramatically reduced. Using CHAPS in stead of Triton was demonstrated to be sufficient to overcome this problem.

When preparing total cell lysates, besides the obvious precautions like storing the samples on ice, addition of a protease inhibitor cocktail is advisable to avoid proteolytic degradation of the proteome by intracellular endopeptidases. Commercially available inhibitor cocktails usually contain inhibitors of all major subclasses of mammalian endopeptidases (serine, cysteine and aspartic proteases, aminopeptidases and depending on the cocktail a broad-range metalloprotease inhibitor such as EDTA). The cocktail we used in early development of the activity-based extraction method in preparation of A549 lysate was from Merck, and when used at the recommended dilution contained the inhibitors in the concentration described in table 1. It was observed that addition of this cocktail had a negative effect on enrichment of active ADAM-17 from A549 lysate, and some experiments were carried out to assess the effect of the individual protease inhibitors on. One observation that is immediately clear from table 1 is that the concentration of the individual inhibitors at the recommended dilution is far higher than the minimal effective concentration, possibly giving rise to non-specific inhibition effects. As shown in an assay evaluating the effect of the used concentration of each individual inhibitor on the activity of recombinant ADAM-17 and the total proTNFα convertase activity in A549 lysate (measured by conversion of the standard “ADAM-17”-fluorogenic substrate from R&D systems) most protease inhibitors have a small effect of proteolytic activity of the recombinant enzyme, but the cleavage of the substrate is almost completely abolished in lysate containing the recommended concentration of the serine protease inhibitor AEBSF (see figure 2).

**Figure 2:**
*Residual proteolytic activity of rhADAM17 and endogenous metalloprotease activity in an A549 lysate in the presence of individual components of a standard protease inhibitor cocktail for mammalian cells (Merck). Concentration protease inhibitors: AEBSF 4 mM, Aprotinin 3.2 μM, E-64 50 μM, Leupeptin 80 μM, Pepstatin 60 μM, Bestatin 160 μM. *: corrected for inhibition caused by solvent methanol.*
The inhibitory effect of AEBSF on total ADAM activity was reduced to around 20% at 0.5 mM, and to evaluate whether this decreased activity in cell lysate is caused by inhibition of post-lysis activation of ADAM zymogens by proprotein convertases like furin two proprotein convertase inhibitors were tested. The irreversible proprotein convertase inhibitor decanoyl-RVKR-chloromethylketone and the competitive furin inhibitor hexa-D-arginine were shown to both inhibit the activity of recombinant TACE directly, but had a stronger effect on the proteolytic activity in cell lysate. These results illustrate the complexity of the biological system regulating ADAMs that has to be considered, and effort may be required to further investigate if the contribution of post-lysis zymogen conversion is existent and has a significant effect on levels of active metalloproteases.

The experiments described in this thesis have demonstrated that activity-based profiling of ADAM proteases is possible, but practical limitations are obvious. Since in-situ ADAM activity is likely to have a better correlation with disease development and progression several groups have attempted to develop probes for in vivo imaging using near infrared fluorescence technology (recently reviewed in\(^3\)). We evaluated the base inhibitor structure of ML5 as a potential probe for in vivo imaging by linking of 99m-technetium as a radiolabel, resulting in probe ML23. The freshly prepared \(^{99m}\)Tc-inhibitor structure was evaluated in a binding assay to the human bronchial epithelial cell line 16HBE 14o\(^{-}\) (a kind gift from Dr. D.C. Gruenert, University of California, San Francisco) and the effect of a competitive inhibitor (ML5) on the binding of the probe. 16HBE cells release TNF\(\alpha\) upon stimulation which is an indication for ADAM activity on the cell surface, and this release can be (partially) blocked by 10 µM inhibitor ML5, indicating this inhibitor is suitable for competition regarding ADAM binding on living cells (figure 3). Figure 4 shows that the binding of the radioactively labelled ML23 to the cells is reversible by ML5, indicating that the interaction is selective. Further work on this probe is of course needed to demonstrate suitability for in vivo imaging (like pharmacokinetic profiling in animals) but these
preliminary results show this approach may be worthwhile pursuing, as is being planned in future work.

**Figure 3:**
TNFα production by 16HBE after combined stimulation with PMA and LPS and the inhibitory effect of metalloprotease inhibitor ML5. TNFα is measured in the supernatant of the cells by ELISA (Sanquin).

**Figure 4:**
Radioactivity on 16HBE cells after incubation with ⁹⁹ᵐTc-ML23
On interesting question that was attempted to be tackled by using activity-based SPE was whether ADAM-33, a protein that is likely involved in asthma pathology, is present in its active form in lung epithelia cells. Cartridge extraction of A549 total cell lysates on an immobilized TAPI-2 cartridge followed by Western blot detection revealed a staining pattern similar to that observed by Powell in fibroblasts, with selective enrichment of some of the lower molecular size bands on the TAPI-2 cartridge as opposed to the ethanolamine control cartridge. The reproducibility of these results with other columns and immobilized inhibitors was poor, leading to an impossibility of drawing distinct conclusion, but these findings may be worth further investigation, especially since the biological role of ADAM-33 involvement in asthma and bronchial hyperresponsiveness may involve disturbed cellular adhesion by mutations in the disintegrin- or cysteine-rich domains but also dysfunctional activity of the metalloprotease domain.

In conclusion the work described in this thesis shows that functional proteomics on zinc-dependent proteases is a challenging field, an observation that is corroborated by the very limited amount of literature that has been published on this subject to date. The activity-based SPE method seems to have crystallized to a point that it may be applied to the first clinical studies, but care has to be taken with respect to sample handling and storage. Gaining actual functional insight in metalloprotease activity in diseases may be essential in evaluating the role of these enzymes in development and progression of disease, and profiling results may aid pharmacological intervention by identifying (novel) targets and follow up of the effects of therapy.

References
