Native mass spectrometry: A powerful tool to study protein-ligand complexes in drug discovery

Author(s): Cubrilovic, Dragana

Publication Date: 2014

Permanent Link: https://doi.org/10.3929/ethz-a-010171697

Rights / License: In Copyright - Non-Commercial Use Permitted
NATIVE MASS SPECTROMETRY: A POWERFUL TOOL TO STUDY PROTEIN-LIGAND COMPLEXES IN DRUG DISCOVERY

A thesis submitted to attain the degree of
DOCTOR OF SCIENCES of ETH ZURICH
(Dr. sc. ETH Zurich)

presented by
DRAGANA CUBRILOVIC

M.Sc. in Chemistry, Goethe University, Germany

born on 16.11.1985
citizen of Germany

accepted on the recommendation of

Prof. Dr. Renato Zenobi, examiner
Prof. Dr. Dario Neri, co-examiner

2014
Dedicated to Dušan
I. DISCLAIMER

I hereby declare that this dissertation represents the result of my own original work, and it has not been submitted to any other institution for another degree or qualification. I also declare that it contains no material written by another person without proper citation in the text.

______________________________
Dragana Cubrilovic

______________________________
Date
II. Acknowledgements

I would like to thank Prof. Dr. Renato Zenobi for giving me the chance to do my doctoral studies in his research group in such a great international environment. I want to thank him for his support and giving me the freedom in the new research projects. I also thank Prof. Dr. Dario Neri for agreeing on being my co-examiner.

Many thanks to Ms. Brigitte Bräm for her great help in all the administrative work.

I was lucky to be involved in very exiting collaborations. I am very thankful to Prof Dr. Gerhard Klebe, Prof. Dr. Torsten Steinmetzer, Prof. Dr. Gerhard Wider, Prof. Dr. Thorsten Berg, Dr. Wolfgang Haap, Daniela Hoffman and Adam Biela. Thanks for your contributions to the jointly published papers.

During my doctoral studies I had the great chance to work at Hoffmann-La Roche in Basel as well as the Oxford University. Thanks to Dr. Gregor Dernick and Dr. Angela Russell for the great supervision and the hospitality in their groups.

I was very lucky to be a part of the Marie Curie Initial Training Network-CHEBANA. I had a great chance not only to share the research experience but also to get to know so many great people from all over the world. Special thanks to Dr. Heike Mader for the organization of all CHEBANA events and her support in all CHEABANA related questions.

Many thanks to the noncovalent ESI-MS team: Dr. Basri Gülbakan and Dr. Konstantin Barylyuk. It was a pleasure to work with you in the same team and to have fruitful discussions during our meetings. Special thanks to Dr. Konstantin Barylyuk for all his advises, his help in maintaining the instruments, assistance in experiments and all the discussions during our coffee breaks. I appreciate your support a lot.

I am very thankful to Dr. Lukas Meier, Dr. Stefan Schmid, Dr. Simon Weidmann and Dr. Alfredo Ibanez. You gave so much helpful advices. Thanks for all helpful discussions. I also want to thank Dr. Martin Badertscher for his great support to evaluate my data. Thanks to all the members of the Zenobi group for the nice atmosphere during my time at ETH Zurich.

Without the support of my wonderful parents, my brother, Dusan, and his lovely parents, I would never be where I am today. Thanks for your love, and being always there for me.
III. Publications


IV. Conference Contributions


V. Oral Presentations

**D. Cubrilovic:** Quantifying Noncovalent Interactions by Native Mass Spectrometry, Novartis, Basel, Switzerland, May, 2011.

**D. Cubrilovic:** Native Mass Spectrometry: Powerful Tool for Determination of Protein-Ligand Binding Constants, Hoffmann-La Roche, Basel, Switzerland, October, 2011.


VI. Table of Contents

1 INTRODUCTION AND BACKGROUND .................................................................................. 4

1.1 INTRODUCTION ............................................................................................................. 3
  1.1.1 COMPETITIVE INHIBITION.................................................................................... 4
  1.1.2 ALLOSTERIC REGULATION IN PROTEINS ......................................................... 5
  1.1.3 PROTEIN–PROTEIN INTERACTIONS INHIBITION ........................................... 7

1.2 NATIVE MS: TOOL FOR DETECTION THE NONCOVALENT COMPLEXES ...................... 8

1.3 NATIVE MS FOR THE STUDY OF PROTEIN–LIGAND COMPLEXES ............................. 11
  1.3.1 DETERMINATION OF PROTEIN–LIGAND BINDING AFFINITIES ....................... 11
  1.3.2 CID EXPERIMENTS TO DETERMINE CE_{50} VALUES ........................................ 14

1.4 CHALLENGES AND LIMITATIONS OF NATIVE MS ..................................................... 16
  1.4.1 SAMPLE PREPARATION ....................................................................................... 16
  1.4.2 SPECIFIC INSTRUMENTAL TUNING FOR NATIVE MS ....................................... 16
  1.4.3 pH AND TEMPERATURE ...................................................................................... 18

1.5 MOTIVATION AND SCOPE OF THE THESIS .............................................................. 20

2 METHODS AND INSTRUMENTATION ................................................................................. 25

2.1 FUNDAMENTALS OF MASS SPECTROMETRY: AN OVERVIEW ..................................... 27

2.2 ELECTROSpray in NATIVE MS ...................................................................................... 28
  2.2.1 IONIZATION TECHNIQUES ................................................................................... 28
  2.2.2 ELECTROSpray IONIZATION .............................................................................. 28
  2.2.3 NANO ELECTROSpray IONIZATION ....................................................................... 31

2.3 MASS ANALYZERS ....................................................................................................... 32
  2.3.1 TIME-OF-FLIGHT MASS ANALYZER ..................................................................... 33
  2.3.2 QUADRUPOLE MASS ANALYZER ..................................................................... 35
  2.3.3 HYBRID QUADRUPOLE TIME-OF-FLIGHT (Q-TOF) INSTRUMENTS .................... 36
  2.3.4 ION MOBILITY ANALYZER .................................................................................. 38

3 QUANTIFYING PROTEIN–LIGAND BINDING CONSTANTS USING ELECTROSpray
   IONIZATION MASS SPECTROMETRY: A SYSTEMATIC BINDING AFFINITY STUDY OF A SERIES
   OF HYDROPHOBICALLY MODIFIED TRYPsin INHIBITORS ................................................. 43

3.1 OVERVIEW .................................................................................................................... 45
3.2 INTRODUCTION .................................................................................................................... 45

3.3 EXPERIMENTAL SECTION.................................................................................................. 50
  3.3.1 MATERIALS AND METHODS ....................................................................................... 50
  3.3.2 MASS SPECTROMETRY ............................................................................................... 50
  3.3.3 DATA PROCESSING ........................................................................................................ 51
  3.3.4 KINETIC INHIBITION ASSAY ..................................................................................... 51

3.4 RESULTS AND DISCUSSION .............................................................................................. 53
  3.4.1 $K_{D}$-DETERMINATION OF THE BENZAMIDINE-BASED INHIBITORS BY THE NANOESI-MS .................................................................................................................. 53
  3.4.2 GAS-PHASE STABILITY VS. BINDING AFFINITY .......................................................... 58
  3.4.3 $K_{D}$-DETERMINATION OF THE CMA SERIES BY NANOESI-MS TITRATION METHOD .................................................................................................................... 60

3.5 CONCLUSIONS .................................................................................................................... 64

4 DETERMINATION OF PROTEIN-LIGAND BINDING CONSTANTS OF A COOPERATIVELY
REGULATED TETRAMERIC ENZYME USING ELECTROSPRAY MASS SPECTROMETRY .......... 67

4.1 OVERVIEW .......................................................................................................................... 69

4.2 INTRODUCTION .................................................................................................................. 69

4.3 EXPERIMENTAL SECTION.................................................................................................. 72
  4.3.1 MATERIALS AND METHODS ....................................................................................... 72
  4.3.2 MASS SPECTROMETRY ............................................................................................... 73
  4.3.3 CRYSTALLOGRAPHIC EXPERIMENTS ........................................................................ 74
  4.3.4 DATA PROCESSING ........................................................................................................ 75

4.4 RESULTS AND DISCUSSION .............................................................................................. 77
  4.4.1 NANOESI-MS ANALYSIS OF FBPASE ......................................................................... 77
  4.4.2 BINDING OF SMALL MOLECULE INHIBITORS TO FBPASE ......................................... 78
  4.4.3 $K_{D}$ AND HILL COEFFICIENT DETERMINATION OF THE FBPASE-INHIBITOR COMPLEXES ............................................................................................................... 80
  4.4.4 MONITORING EFFECT OF SUBSTRATE BINDING TO THE COMPLEX ......................... 84

4.5 CONCLUSIONS .................................................................................................................... 86

5 INFLUENCE OF DIMETHYLSULFOXIDE ON PROTEIN-LIGAND BINDING AFFINITIES ...... 89

5.1 OVERVIEW .......................................................................................................................... 91

5.2 INTRODUCTION .................................................................................................................. 91

5.3 EXPERIMENTAL SECTION.................................................................................................. 94
  5.3.1 MATERIALS AND METHODS ....................................................................................... 94
  5.3.2 MASS SPECTROMETRY ............................................................................................... 94
5.3.3 DATA PROCESSING .................................................................................................. 95
5.4 RESULTS AND DISCUSSION ...................................................................................... 97
  5.4.1 LYSOZYME-NAG$_3$ BINDING ............................................................................... 97
  5.4.2 TRYPsin-PefABLOC BINDING ........................................................................... 100
  5.4.3 CARBONIC ANHYDRASE-CTA BINDING .............................................................. 101
  5.4.4 INFLUENCE OF DMSO ON THE $K_D$ OF THE INVESTIGATED COMPLEXES .......... 103
5.5 CONCLUSIONS .......................................................................................................... 107

6 DIRECT MONITORING OF PROTEIN-PROTEIN INHIBITION USING NANO ELECTROSPrAY
  IONIZATION MASS SPECTROMETRY .............................................................................. 111
  6.1 OVERVIEW .............................................................................................................. 113
  6.2 INTRODUCTION ....................................................................................................... 113
  6.3 EXPERIMENTAL SECTION....................................................................................... 117
    6.3.1 MATERIALS AND METHODS ............................................................................ 117
    6.3.2 MASS SPECTROMETRY .................................................................................. 118
    6.3.3 DATA PROCESSING ......................................................................................... 119
  6.4 RESULTS AND DISCUSSION ...................................................................................... 121
    6.4.1 NANOESI-MS ANALYSIS OF TNF-$\alpha$ ............................................................ 121
    6.4.2 MONITORING THE DISRUPTION OF THE TNF-$\alpha$ BY NANOESI- AND IM-MS ................... 122
    6.4.3 $K_D$ DETERMINATION OF THE BCL-2-BAK AND BCL-2-BAD COMPLEXES .............. 128
    6.4.4 MONITORING THE BCL-2-BAK AND BCL-2-BAD INHIBITION USING SMALL DISRUPTORS ABT737
        AND ABT263 .................................................................................................. 130
  6.5 CONCLUSIONS .......................................................................................................... 135

7 CONCLUSIONS AND OUTLOOK ..................................................................................... 139

8 REFERENCES ............................................................................................................... 149

A – ABBREVIATIONS ..................................................................................................... 175

B - CURRICULUM VITAE .................................................................................................. 177
VII. Abstract

Noncovalent interactions are critical in maintaining the three-dimensional structure of large biomolecules and play a fundamental role in molecular recognition in all biological processes. The discovery and characterization of protein-ligand interactions (e.g. with other proteins, carbohydrates, lipids, DNA or small molecules) is crucial for understanding biochemical reactions and pathways as well as for a subsequent development of new therapeutics for treatment of different human diseases. The quantitative determination of binding strengths of protein-ligand complexes is of high importance for the design of novel therapeutics in drug discovery processes.

Mass spectrometry (MS) was used for a long time as a tool for the “fingerprint” to identify different compounds. Many researchers believed that detection of noncovalent interactions is impossible, because large intact biomolecule should not survive the transfer into the gas phase. The advent of soft ionization techniques, such as electrospray ionization (ESI) as well as matrix-assisted laser desorption ionization (MALDI) in combination with the availability of modern mass spectrometers achieving sufficient accuracy and sensitivity, revolutionized the field. The evidence in the early 1990ies that ESI could maintain noncovalent interactions preserving their solution structure was even more surprising. Since then native ESI-MS has been developed into a powerful and widely utilized tool in drug discovery, via detection and analysis of the protein-ligand interactions. This method can be used for determining composition, stoichiometry, subunit interactions, and architectural organization of noncovalent complexes.

In the last years more research groups have become active in the field of the ESI-MS based drug discovery for the investigation of protein-ligand interactions and determination of binding affinities. In recent years, binding affinities have been successfully determined by ESI-MS for a variety of noncovalent protein-ligand complexes, and by and large were found to be in agreement with results from other biophysical methods. However in the case of some other investigated complexes no correlations with standard methods have been observed. Even if the complexes are preserved, it is not fully understood to which extent ESI mass spectra represent a
snapshot of solution-phase equilibrium. In particular, is not clear what happens to protein-ligand complexes when weak interactions, e.g., hydrophobic interactions dominate the subunit. Therefore, one of the aims of this thesis was to investigate the protein-ligand complexes driven by hydrophobic interactions and to study how the complex stability is affected in the gas phase. Changes in the length of the hydrophobic side chain should lead to systematic differences in binding affinities accommodating the hydrophobic cavity of the enzyme. The question of this study was whether one could observe the expected trend in the binding affinity when using ESI-MS as a read-out for the solution phase equilibrium. Also we wanted to establish mass spectrometric measurements for drug discovery screening on several relevant and more complex biological systems, important for, both academia and the pharmaceutical industry. Such measurements need to be put on a solid basis to be widely accepted.

Therefore, the allosteric mechanism in the binding of the new inhibitors to a tetrameric enzyme using nanoESI-MS was studied. The system investigated was the homotetrameric enzyme fructose-1,6-bisphosphatase (FBPase), a potential therapeutic target for glucose control in type-2 diabetes. The nanoESI based $K_\text{d}$ values determined were in good agreement with validation measurements. In addition to the determination of $K_\text{d}$ values of the cooperatively regulated enzyme, our results also allowed for a better understanding of the mechanism of enzymatic cooperativity and for getting information about stoichiometry, mechanism of binding and Hill coefficient. Recently, we also investigated the possible effects of DMSO on protein-ligand complexes during the screening process, which have received little attention in the literature. As a part of this thesis, we studied the inhibition of protein-protein interactions (PPI). A large number of PPIs are involved in signalling pathways related to cancer and many other human diseases. NanoESI-MS was applied to monitor the extent of the protein-protein inhibition as well as the mechanism of binding.

Considering that native ESI-MS is beginning to be recognized as a viable tool for early drug discovery, we expect that in the near future the usage of this analytical technique will be further accelerated.
VIII. Zusammenfassung


Bindungsmechanismus zu studieren. Nachdem ESI-MS als nützliches Instrument für die frühe Wirkstoffforschung erkannt wurde, erwarten wir eine beschleunigte Verwendung dieser analytischen Verfahrensweise in naher Zukunft.
Chapter 1

1 Introduction and Background

This chapter contains a general introduction of native mass spectrometry analysis of protein-ligand interactions, its potential for drug discovery, as well as its current challenges and advantages. The chapter closes with the objective of this thesis.
1.1 Introduction

Drug research and discovery are fields that have evolved into a distinct branch of science, and are also the main driving force behind medical progress in the last century. This is very multidisciplinary research that includes disciplines as diverse as chemistry, multiple branches of biology, biophysics, computer sciences, mathematics and engineering. [1-3]

Protein function depends on molecular conformation that allows in turn the modulation of intermolecular interactions. Information about protein-ligand interactions is the fundamental basis of medicinal chemistry. Drug development attempts to identify small molecules that bind specifically to target proteins and either induce a conformational change or ‘outcompete’ the active site for its natural ligand. Discovery and characterization of protein–ligand complexes are important steps to complete description of biochemical reactions and pathways. Better understanding of the molecular basis of protein recognition in combination with the relationship between structure and binding selectivity and affinity enables the design of novel therapeutics that may be used to treat a variety of human diseases. [4, 5]

Among the most important steps in developing a new drug are target identification as well as the validation. Some of the important standard techniques in drug discovery research are: surface plasmon resonance (SPR) spectroscopy, isothermal titration calorimetry (ITC), enzyme-linked immunosorbent assay as well as nuclear magnetic resonance spectroscopy (NMR). Beside these conventional methods for characterization of protein-ligand interactions, native ESI-MS is gaining more popularity. [6]

In the following three subsections a brief overview of the different mechanisms of small molecule binding to the protein studied in this thesis with native nanoESI-MS is given.
1.1.1 Competitive Inhibition
Enzymes catalyze virtually all cellular processes. Therefore enzyme inhibitors represent one of the most important agents and are of major interest in pharmaceutical research. Enzyme inhibitors are molecules that interfere with catalysis and are able to slow or halt enzymatic reactions. Two different broad classes of enzyme inhibitors exist: reversible and irreversible. One common type of reversible inhibition is called competitive. Competitive inhibitors binding to the serine protease trypsin were investigated in this thesis, so that this type of inhibition will be described in brief. In this type of inhibition, another molecule competes with the substrate for the active site of an enzyme. In many cases the competitive inhibitor has a similar structure as the substrate, but does not contribute to the catalysis. A schematic illustration of competitive inhibition is shown in Figure 1.1. [7, 8]

![Diagram of competitive inhibition](image)

**Fig. 1.1** Competitive inhibitor (I) binds to the active site of the enzyme and compete with substrate (S).

The inhibitor occupies the active site and thereby prevents the binding of the substrate to the inhibitor. Knowing the molecular geometry of inhibitors one can conclude which parts of the substrate bind to the enzyme. The quantification of competitive inhibition can be done by steady-state kinetics, using the Michaelis-Menten equation [9]:

\[
V_0 = \frac{v_{\text{max}}[S]}{\alpha K_m + [S]} \quad (1.1)
\]

Where:

\[
\alpha = 1 + \frac{[I]}{K_i} \quad \text{and} \quad K_i = \frac{[E][I]}{[EI]} \quad (1.2)
\]
Where: $V_0 = \text{initial reaction velocity}$, $V_{\text{max}} = \text{maximal velocity}$ and $K_m = \text{Michaelis constant}$.

The variable $\alpha K_m$ also called apparent $K_m$ can be experimentally determined and represents the $K_m$ observed in the presence of the inhibitor. Since the inhibition is reversible, it is possible to favour substrate binding by adding more substrate, i.e.; to bias the competition. If the substrate concentration exceeds the inhibitor concentration the reaction shows a normal $V_{\text{max}}$ since the chance of inhibitor binding to the enzyme is lowered. The substrate concentration for which $V_0 = 1/2V_{\text{max}}$ (apparent $K_m$) increases in the presence of an inhibitor by a factor $\alpha$. This effect on $\alpha K_m$ in combination with the absence of an effect on $V_{\text{max}}$ is an indication of competitive inhibition and is characterized by a double-reciprocal plot. The results are plotted as $1/V_0$ vs. $1/[S]$. [7]

**1.1.2 Allosteric Regulation in Proteins**

Allosteric regulation of protein function is one of many controls on activity and plays an important role in many cellular processes. [10, 11] Understanding allosteric mechanisms may offer a possibility of targeting a protein by small molecule inhibitors for drug discovery. Such regulation is often achieved by the binding of small-molecule effectors or inhibitors to multimeric proteins. [12] The subsequent conformational changes of the protein may give rise to cooperativity in protein function. Two models developed in the 1960s describe cooperativity in ligand binding. Both models explain the oligomeric protein assemblies of identical protein molecules or subunits, wherein each subunit is either in a relaxed state (R) or in a tense state (T). The R state should be more receptive to inhibitor binding compared to the T state. These models are the Monod-Wyman-Changeux (MWC) model [13] and the Koshland-Némethy-Filmer (KNF) [14] model. Both models describe cooperativity due to the binding-induced changes of the protein subunit conformation. In the fist case, it can be concerted (MWC), meaning that subunits in each protein assembly must either all be in the R state or all in the T state (Figure 1.2). The conformational change in one subunit influences an equivalent change in
all others. The small-molecule binding to either state increases the equilibrium to the R state and enables the ensemble to be more receptive to further ligand binding.

**Fig. 1.2** Schematic representation of the two-state concerted model (MWC). The conformational transition between two states of a dimer, R and T occurs in the absence of a ligand. A (activator), I (inhibitor), and S (substrate) can bind to the enzyme and differentially stabilize the R and T states. Adapted from [15] with kind permission of Nature Publishing Group, Copyright© 2013.

The second sequential (KNF) model describes that the subunits of each assembly do not all have to be in the same state. The binding of one ligand to one subunit changes its conformation from the T to the R state. In contrast to the MWC model, this change affects the structures of adjacent subunit and makes it more receptive to ligand binding, with no need to convert them all to the R state (**Figure 1.3**).

**Fig. 1.3** Schematic illustration of the KNF model. The unbound subunits are round and all the ligand-bound subunits are square. No unbound square subunit exists. The conformational change does not take place in the absence of ligand but is specifically ‘induced’ by the ligand, S. Adapted from [15] with kind permission of Nature Publishing Group, Copyright© 2013.

Also a combination of both models is possible. [16] Allosteric triggers are able to decrease (negative cooperativity) or increase protein (positive cooperativity) activity.
1.1.3 Protein-Protein Interactions Inhibition

Protein-Protein interactions (PPI) are of central importance to many biological processes, from intercellular communication to programmed cell death. They represent a large and important class of targets against human diseases. The difficulty of PPI inhibition compared to active site targeting is the more exposed and less defined binding site. Moreover, X-ray structures of protein-protein pairs often do not exhibit small and deep cavities suitable for small-molecule binding sites. PPI interfaces are large, up to thousand of square angstroms, which in terms of competitive inhibition would mean that a small molecule must cover 800-1100 Å of a protein surface. Since natural small molecule known to bind at protein-protein interfaces are rare, experimental development of PPI inhibitors is very challenging. [17-19] Figure 1.4 illustrates the protein-protein interaction inhibition.

![Diagram of protein-protein interaction inhibition](image)

**Fig. 1.4** Schematic illustration of the protein-protein interaction inhibition.

Usually structural methods approaches such as NMR spectroscopy or X-ray crystallography, in combination with biophysical techniques such as surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC) are used for PPI investigation. [18] As part of this thesis native mass spectrometry was introduced as a valuable and complementary tool to study PPI inhibition.
1.2 Native MS: Tool for Detection the Noncovalent Complexes

For more than five decades mass spectrometry (MS) has been a well-established technique in analytical chemistry, mainly because of instrumental breakthroughs in terms of scanning speed, sensitivity of mass analyzers, acquisition and data treatment. The importance of the new ionization techniques was underscored by the 2002 Nobel Prize in Chemistry for John B. Fenn and Koichi Tanaka for the development of electrospray ionization mass spectrometry (ESI-MS) and matrix-assisted laser desorption (MALDI), respectively. [20-25] In the pharmaceutical industry MS is applied for the characterization and the evaluation of the purity of recombinant proteins,[26] the characterization of monoclonal antibodies [27]; in the proteomic field, for the identification of proteins at femtomolar levels via the detection of their related peptides. [28]

It was not too long ago that the detection and the characterization of intact noncovalent complexes, such as protein-ligand systems using MS, was met with scepticism in the biophysical community. A technique like MS that detects species in the gas phase and analyzes complexes maintained by weak interactions (such as electrostatic and van der Waals interactions, H-bonds, hydrophobic effects) looked inappropriate to the community because of the intrinsic fragility of these complexes. It should be mentioned at this point without getting into details that the MALDI technique has also been used as a tool for the detection of noncovalent complexes under very specific conditions. However, ESI is the preferred ionization method for noncovalent MS analysis. [29-32] For the first time mass spectrometry of noncovalent complexes also called “noncovalent”, “non-denaturing” or “native” MS, was reported in the early 1990s by two American groups who detected the intact protein-ligand complexes in the gas phase on an ESI mass spectrometer. [33-35] In the following decade, several laboratories worldwide performed experiments to improve and establish the experimental conditions to study noncovalent complexes. [35-38] One of the major breakthroughs was the modification in the internal energy of the ions generated, also referred to as collisional cooling. [37-39]

For the detection of noncovalent complexes, ESI-MS appears to be the best ionization method for the following reasons: (i) it is a very soft ionization technique,
which allows noncovalent interactions to survive the ionization process; (ii) it works with liquid samples; and (iii) samples are gently transferred from the solution into the gas phase maintaining the noncovalent complexes while sufficient desolvation can be achieved. [20] **Figure 1.5** shows the difference in spectra between native mass spectrometry analysis and denatured conditions for the protein *H. pylori* urease.

![Figure 1.5](image)

**Fig. 1.5** Denatured and native mass spectra of *H. pylori* urease. a.) To generate denatured spectrum the protein was sprayed from the water:acetonitrile (50:50, vol/vol) solution containing 0.1 % (vol/vol) formic acid. Charge distribution stem from the multiply charged monomeric subunits α (26.6 kDa, orange) and β (61.7 kDa, magenta) of urease. b.) A mass spectrum of native urease electrospayed from an aqueous ammonium acetate solution. The narrow charge state distribution represents the intact α12β12 urease machinery with a measured mass of 1,063.4 ± 1.0 kDa. The cartoons are adapted from the X-ray structure of the intact α12β12 urease. Adapted from [40], with kind permission of Nature Publishing Group, Copyright© 2008.

The number of publications related to the native ESI-MS analysis of different complexes like protein-protein [41-43], proteins and small molecules [44-47], nucleic acids [48], proteins and nucleic acids [49], as well as nucleic acids and small molecules [50-52], is growing from year to year. [4, 35, 53] In addition, native ESI-MS
is used for structural and functional characterization of intact protein oligomers [54] and complex cell machineries. [43, 55] Also, large assemblies like exosomes and proteasomes were studied from endogenously expressed proteins [43, 56, 57], and the analysis of several megadalton complexes was recently described. [53, 58-60]

Conventional methods for characterization of noncovalent complexes in vitro are surface plasmon resonance (SPR) spectroscopy, isothermal titration calorimetry (ITC), frontal affinity chromatography combined with mass spectrometry detection, enzyme-linked immunosorbent assay as well as nuclear magnetic resonance spectroscopy (NMR). [5, 61-65] All these commonly used techniques have particular strengths and weaknesses. Native MS has become complementary to these classical biophysical methods. Moreover, the nanoESI-MS assay has advantages in terms of simplicity (label-free measurements), selectivity (possibility of using additional stages of MS combined with ion activation methods or ion mobility spectrometry), sensitivity (low sample consumption), speed (mass spectra can be acquired in less than a minute) and specificity (gain information about binding stoichiometry, analyze mixtures and measure multiple equilibria simultaneously). [35] The fact that there is no need for labelling or immobilization of the sample is important, since ligand or target modifications can have deleterious effects on binding properties. Another advantage is the recent automatization of the sample loading, which allows “medium”-throughput screening of compound libraries. [66-68]

In the next subchapters more details how ESI-MS investigation of protein-ligand complexes can be integrated in pharmaceutical drug discovery will be given, and the advantages and limitations of this technique will be described.
1.3 Native MS for the Study of Protein-Ligand Complexes

The discovery and characterization of protein-ligand interactions (e.g. with other proteins, carbohydrates, lipids, DNA or small molecules) is crucial for understanding biochemical reactions and pathways as well as for a subsequent design of new therapeutics for treatment of different human diseases. [5] In case of protein-ligand interactions several key questions important for drug discovery can be solved using nanoESI-MS: (i) confirmation of the existence of a noncovalent interaction between the target protein and small molecule; (ii) determination of the ligand binding stoichiometry, i.e. how many ligand molecules interact with the target protein; ESI-MS allows the direct visualisation of ligand binding stoichiometry through peak shift without any mathematical modelling compared to fluorescence techniques; (iii) distinction between specific or non-specific binding of the molecules, i.e., is the ligand binding site-specific or does it bind non-specifically elsewhere at the surface of the protein, (iv) the determination of solution phase binding affinities. [20] The discovery and characterization of these interactions are key steps in order to understand biochemical reactions and pathways. Furthermore, due to the high sensitivity of MS, low sample consumption can be afforded: 100 pmol of protein sample per ligand binding are enough for the measurement, so that microgram quantities are sufficient to complete MS screening. Reduced amounts of biological material also mean lower screening costs.

1.3.1 Determination of Protein-Ligand Binding Affinities

Information about solution-phase binding affinities is one of the key steps in the drug discovery process for the design of novel therapeutic agents against a particular target that may be used to treat a variety of diseases and infections. In the last years more research groups have become active in the field of ESI-MS based drug discovery for the investigation of protein-ligand interactions and determination of binding affinities. [69, 70] Native MS is not conventional a method for determination of binding affinities, but it is attracting more interest in the last years. The dissociation constants (KDs) can be calculated from carefully controlled ESI-MS experiments. In recent years, binding affinities have been successfully determined by
ESI-MS for a variety of noncovalent protein-ligand complexes. In many cases, they were found to agree with $K_D$ values obtained with more established techniques. [4, 54, 71-74] On the other hand, it does not work for some other complexes. Moreover different instruments may lead to a difference in binding affinities for the same interaction. However, up to date a lot of effort has been made to improve the methodology in order to get better reliability and applicability. [75-83]

Another very important question that should be addressed is whether during the ESI process the solution-phase equilibrium is distorted or whether relative concentrations of interactions partners are preserved. Some studies reported that during the ESI process the chemical equilibrium is not shifted despite the fact that that the solution concentration is increased. [84-91] During the spray process three processes occur in a nanoESI plume, which contribute to retaining the solution phase association-dissociation chemical equilibria. First, the kinetics of the reaction is slowed down, because of the cooling due to the solvent evaporation. Second, the transition from a nanoESI capillary to the MS sampling orifice happens on the ms timescale, which is comparable with the binding kinetics of noncovalent complexes. Third the explosion of larger and charged droplets to the small daughter droplets via Coulomb explosion (Figure 1.6) is very fast, on the $\mu$s timescale.

Despite the increase in solute concentration due to solvent evaporation, the electrospray timescale is too short and the diffusion coefficients of noncovalent complexes are too low to form specific association. Nonspecific complexes may be bound to weakly to survive the process from the ion transfer to the mass spectrometers vacuum. [91, 92] However the exact lifetime of electrospray droplets is not fully understood yet and is reported to be in the range from ns to ms.

Structural rearrangements of bimolecular noncovalent complexes such as ligand exchange or protein unfolding happen on longer time scale than the time between the generation of the ion source and the detection, which happens on $\mu$s time scale. [7, 93] Therefore the noncovalent complexes do not have to be thermodynamically stable in the gas-phase if they are kinetically stable. [94-97] This is one of the arguments used to explain that nanoESI based $K_D$s values provide a “snapshot” of the solution-phase equilibrium.
Fig. 1.6 History of charged water droplets produced by nanospray. The first droplet shown is one of the droplets produced at spray needle. These droplets undergo several evaporation and fission events. The first generation droplets are shown, as well as the fission of one of the progeny droplets that leads to second-generation progeny droplets (based on the data from [91]). Adapted from [98] with kind permission of Springer, Copyright © 2004, American Society for Mass Spectrometry.

One of the possibilities to determine binding affinities of protein-ligand interactions in solution is the titration method. [99] This method was used to generate the results described in this thesis. Titration experiments can be monitored by native ESI-MS using a constant protein \([P]_0\) and an increasing ligand concentration, until full complexation is reached. This assay is based on the detection and quantification of free \([P]\) and ligand-bound protein \([PL]\) ions by ESI-MS. The ratio \((R)\) of the total abundance of ligand-bound \([PL]\) and free protein \([P]\) is plotted against the added ligand \([L]_0\) concentration.

\[
K_D = \frac{[P]_0[PL]}{[P][L]} \quad (1.3)
\]

\[
[P]_0 = [P] + [P \cdot L] \quad (1.4)
\]

\[
[L]_0 = [L] + [P \cdot L] \quad (1.5)
\]

The equations 1.3-1.5 can be solved for the \(K_D\) for known values \([P]_0\) and \([L]_0\) to yield
equation 1.6:

$$
\frac{I (P-L)}{I (P)} = \frac{1}{2} \left( -1 - \frac{[P]_0}{K_D} + \frac{[L]_0}{K_D} + \sqrt{4 \left( \frac{[L]_0}{K_D} - \frac{[P]_0}{K_D} - 1 \right)^2} \right) \tag{1.6}
$$

From curve fitting, absolute affinity/dissociation constants ($K_a/K_D$) for a given protein–ligand interaction can be determined. Since there is only one concentration in solution, the abundance of all detected charge states of the free protein and complex ions should be taken into account for data processing. It is assumed that (i) ionization efficiency of the bare protein and the complex is equal, and (ii) no dissociation during the transmission through mass spectrometer takes place.

The range of the determined $K_D$ values depends on the protein-ligand complex concentration used for the assay, which is in the low µM range. $K_D$s from the mid-µM to the high nM range may be evaluated. [100, 101] Even lower $K_D$ values may be difficult to obtain since the saturation curves become indistinguishable; on the other hand above $10^{-3}$ M the proportion of complex formed is to low to be accurately detected in an ESI mass spectrum. [102]

### 1.3.2 CID Experiments to Determine CE<sub>50</sub> Values

With the MS titration method, dissociation constants can be determined. Performing collision-induced dissociation (CID) experiments, noncovalent complexes can be dissociated in the phase. The gas-phase stability of the complexes can be evaluated by calculating the dissociation energies of the gas-phase complex ions. Dissociation of the protein-ligand system can be monitored in a stepwise fashion by increasing the collision energy. Complex gas-phase stability can be assessed by the determination of the CE<sub>50</sub> values, the collision energy voltage resulting in 50 % dissociation. [4] However, solution $K_D$s do not necessarily correlate with CID measurements. On the one hand, good correlation was reported for some examples. [45, 103] On the another hand, no correlation was observed for other cases. [104] In the gas phase, ionic, ion-dipole, and dipole-dipole interacting will become stronger in the absence of solvent. Some interactions such as hydrophobic are weakened in the gas-phase. [105] Although some studies document successful preservation of
noncovalent complexes bound hydrophobically in solution. [99] A more detailed discussion on this topic is given in Chapter 3 of this thesis.
1.4 Challenges and Limitations of Native MS

1.4.1 Sample Preparation

To preserve the structural integrity of the noncovalent complexes in solution is a very important step for the detection in the gas-phase of the mass spectrometer. Buffers usually used in biochemistry for purification, solubilisation and stabilisation of the protein (phosphate buffers, Tris, HEPES, etc.), detergents (SDS, TritonX, CHAPS, etc.), reducing agents (TCEP, DTT, etc.) or polymers (e.g. PEG). These buffers can not be used for ESI-MS analysis, since they are not volatile and therefore not compatible with ESI-MS. An additional purification step is required prior the native MS analysis, a procedure also called “desalting”. For the investigation of noncovalent complexes by nanoESI-MS, aqueous ammonium acetate or ammonium bicarbonate solutions, with limited amounts of nonvolatile salts or detergents, are usually used. These buffers allow one to adjust the pH of the solution from 6.5 to 8.5, and are compatible with the ESI ionization process. More acidic or basic pHs can be achieved by adding small amounts of formic acid or ammonia, respectively. In most studies, buffer concentrations between 10 and 500 mM are used, ensuring optimal ESI mass spectra quality. In protein-ligand binding studies the most important role of the buffer is to keep a protein stable and to minimize protein aggregation. Buffer exchange is an essential step for the native MS analysis since it provides a protein sample free of nonvolatile salts and therefore allows the acquisition of high-quality mass spectra and accurate mass measurements. [98, 106, 107]

1.4.2 Specific Instrumental Tuning for Native MS

Collision-induced dissociation of the complex ions when acquiring ESI mass spectra is one of the potential problems. The consequence is a decrease of the relative abundance of complex and protein ions and the underestimation of the calculated binding affinities. In the extreme case if no complex ions survive to detection, the dissociation will result in a false negative. [5]

It should be mentioned that on the other hand the formation of nonspecific clusters leads to an overestimation of the binding affinities.
Another effect is the stability of the complex ions in the gas phase, which is determined by the nature of the specific interactions that form the complex. Complexes stabilized in solution predominantly by weak or a small number of intermolecular interactions are prone to in-source dissociation. [104, 108, 109] It should also be mentioned that gas-phase stabilities do not necessarily reflect the solution binding affinities. It was already reported that complexes stabilized mainly by strong ionic interactions in solution exhibit higher gas-phase stabilities compared to complexes formed by hydrophobic interactions. [83, 110, 111]. Also, collisional heating of gaseous complex ions may occur at various stages, e.g., in the nozzle (or orifice)-skimmer region or during accumulation of ions within external rf multipole storage devices [75, 112]. The best way to pinpoint the occurrence of in-source dissociation is to observe changes in the ratio of the complex and free protein ions (R) resulting from changes in ion source parameters.

To ensure that noncovalent interactions survive the ionization process, parameters of the instrument that control the energy imparted on the ions should be optimized. [36, 37, 57] The regions between the ion source of the instrument at atmospheric pressure and the high vacuum region of the analyzer have to be properly adjusted. A too high energy imparted on the ions may lead to dissociation of the complex; on the other hand, too low energy may result in insufficient complex desolvation with peak broadening and therefore poor resolved spectra. It is necessary for every particular complex to find an optimal balance to conserve the intact noncovalent complexes and at the same time have an efficient desolvation without disrupting it.

As a rule, low temperature, a low potential across lens elements, and short accumulation times are essential to prevent the complex from dissociation and obtain reliable Kᵦ values. [5] As shown in Figure 1.7 the variation of the pressures in the rough-pumped/intermediate vacuum region at fixed cone/extractor voltages to gauge the effect on the intensity of the charge states for the complex of interest will help to maintain noncovalent complexes intact. [106]
Fig. 1.7 Influence of the pressure on the monomer : tetramer ratio of a 5 μM alcohol dehydrogenase (ADH), in 100 mM ammonium acetate at pH = 7. To increase the pressure the isolation valve (SpeediValve) in the source roughing line was closed stepwise. The bar indicates analyzer pressure (x 10⁸ mbar): a.) 6.3, SpeediValve fully open b.) 7.2 c.) 8.2 d.) 8.8 e.) 10 f.) 12 g.) 34. Magnification factors indicate the base peak signal intensity relative to spectrum (f). Adapted from [106]) with kind permission of Nature Publishing Group, Copyright© 2007.

The described parameters should be optimized in combination in order to get the best compromise between sufficient ion desolvation, good transmission of high m/z ions, and preservation of the noncovalent framework. The strength of the protein-ligand interactions can be influenced during the transfer from the solution phase to the gas phase of mass spectrometer. Great care has to be taken how to adjust instrument parameters and interpret the date when dealing with complexes that mainly involve hydrophobic interactions without electrostatic-based interactions.

1.4.3 pH and Temperature
The binding affinities of protein-ligand complexes in solution are in most of the cases sensitive to pH and temperature. Both the pH and the temperature of the solution may be changed during the ESI-MS measurement and therefore result in changes of
K_Ds, especially if low solution flow rates are used. [35] The dominant electrochemical reaction that occurs at a chemically inert electrode are oxidation (positive ion mode) and reduction (negative ion mode) of H_2O to the production of H_3O^+ and OH^-, respectively. At low solution flow rates (< 100 nL/min), the resulting pH can change by about >1 pH unit after 30 min of spraying. [75] To avoid this effect, ESI solutions with high buffer capacity should be used, and the spraying times should be limited to about 10 min.
1.5 Motivation and Scope of the Thesis

As described above ESI has been successfully applied as a tool to instigate a variety of noncovalent complexes. Despite the impressive progress in studying noncovalent protein-ligand interactions using nanoESI, open questions still remain. It is still debated, for example, whether weak noncovalent complexes can be detected in the gas phase and whether the relative peak intensities in ESI spectra reflect the solution-phase equilibria. Also another general aim of this thesis is the application of nanoESI-MS to gain insight about relevant question in the drug discovery process e.g., structural information, stoichiometry, mechanism of binding and finally determination of binding strengths. Many of the protein-ligand systems investigated so far with native MS are only model systems and of little relevance for drug discovery research. Therefore one of the goals of this thesis was to apply nanoESI for the investigation of more complex mechanisms, e.g., to study cooperative events and protein-protein interaction inhibition. We want to establish mass spectrometric measurements for drug discovery screening on several biological systems important for the pharmaceutical industry. Such measurements need to be put on a solid basis to be widely accepted. NanoESI based binding strengths should also be compared against results from standard assays. The major focus of this Ph.D. thesis is the binding of small molecule ligands to the biomolecules. The study of noncovalent complexes by nanoESI-MS will be organized in the following way:

Chapter 2 provides a brief overview of methods used to generate the results as well as the basic concepts of the ESI mechanisms, and discuss the principles of mass analysis in some commonly used mass spectrometers.

Chapter 3 continues to address the question whether we can apply nanoESI-MS for the investigation of a series of hydrophobically modified ligands interacting with serine protease trypsin. The basic question this study should answer is if we can observe the expected trend in the binding affinity of the inhibitors, when using ESI-MS as a read-out for the solution phase equilibrium. The length of the hydrophobic side chain of small competitive inhibitors should lead to the systematic differences in their binding affinities. A second question concerns the complex stability in the solution vs. in the gas phase. It was proposed by several groups that a correlation
between the gas-phase stability and binding affinities is expected only if electrostatic and H-bond interactions of protein-ligands play a dominant role in the complex stabilization, but not if hydrophobic interactions are one of the major driving force in stabilization. As part of this study we have also investigated the stabilizing effect of imidazole, which should protect protein-ligand complexes from in-source dissociation during the ESI-MS analysis process.

Chapter 4 deals with the questions if we can investigate the allosteric regulation of protein function and cooperativity in ligand binding using native MS. To study cooperative events is of major importance since it allows control of many of the cellular processes. We investigated the allosteric mechanism in the binding of ligands to a multimeric enzyme using nanoESI-MS. The investigated system is the tetrameric enzyme fructose-1,6-bisphosphatase (FBPase), a potential therapeutic target for glucose control in type-2 diabetes. A series of newly designed inhibitors that occupy the allosteric AMP binding site were examined, and the K_d and Hill coefficient (n) were determined via the titration method by nanoESI-MS. The nanoESI mass spectra allow one to deduce the stoichiometry and the cooperativity of the multimeric model system that proves to be positive in the case of the model system investigated.

DMSO is often used as a standard solvent for sample storage and handling of compounds in drug discovery, due to its favorable physicochemical properties. To date, little attention was given to how DMSO influences protein-ligand binding strengths. In Chapter 5 the application of nanoESI-MS for investigation of the DMSO influence on the binding affinities of different well-known protein-ligand complexes is described. The DMSO content chosen for the study is up to 7% (v/v) and close to that used in assays for HTS in drug discovery.

In the last decade the design and development of small molecules that are capable of inhibiting protein–protein interactions (PPI) are increasingly attracting attention, both in academia as well as in the pharmaceutical industry. In Chapter 6 the benefits of nanoESI-MS as a fast and label-free method for investigation of mechanism, stoichiometry, conformational changes and relative binding strengths of PPIs inhibition is described.
Chapter 7 concludes the thesis and gives future prospects for native mass spectrometry. References used are listed in a bibliography at the end of this thesis.
Chapter 2

2 Methods and Instrumentation

This chapter gives a general description of the instrumentation used in this work. Each subsection includes a connection to native MS as well as specific experimental challenges to overcome.
2.1 Fundamentals of Mass Spectrometry: An Overview

Mass spectrometry is an analytical tool that ionizes molecules and analyzes them as gas phase ions according to their mass-to-charge (m/z) ratios. [113] This method was for the first time described in 1898 by work of Sir J.J. Thomson. He investigated the quantitative measurement of the mass and charge of “cathode rays” (electrons) and for this purposes built the first mass spectrometer (parabola mass spectrograph). In 1906 he received the Nobel Prize for Physics in “in recognition of the great merits of his theoretical and experimental investigations on the conduction of electricity by gases”. [114] In 1918 Dempster introduced an instrument in which a strong magnetic field between two semicircular iron plates was produces in order to separate positive ion rays. [115] The first commercial instruments, available in the beginning of the 1940s, were based on magnetic deflection and electron impact ionization. At the end of the 1950s mass spectrometers were for the first time applied to elucidate the structure of natural products as well as to study fragmentation patterns. To the present day, a mass spectrometer consists of the same following components: a sample introduction device, an ionization source, a mass analyzer, a detector, and a computerized system for data treatment (Figure 2.1).

Fig. 2.1 Schematic overview of the mass spectrometer.

In the source region, which can be under vacuum or at atmospheric pressure, either positive or negative charged ions are generated. The mass analyzer and the detector operate under high vacuum, usually below $10^{-5}$ Torr to avoid the collisions between ions and residual gas molecules, which would result in reduced ion transfer. Each mass analyzer is specific in terms of its mass range and the resolving power. At present, the most frequently used mass spectrometers are quadrupoles or quadrupole ion traps. The physical fundamentals were described in the early 1950s by Steinwedel and the work of Paul, for which he was awarded the Nobel Prize in 1989. [116] With these instruments the mass resolution remains constant over a
defined mass range. The mass accuracy with Fourier-transform ion cyclotron resonance or orbitrap mass spectrometers is better than 1 ppm while with the time of flight (TOF) instruments a mass accuracy better than 5 ppm can be achieved. For the qualitative and quantitative analysis, triple quadrupole mass spectrometers are very popular. With the development of matrix-assisted laser desorption/ionization by Karas and Hillenkamp [21] in 1987 and of electrospray ionization by Fenn in 1988 (Nobel Prize in 2002) [24] a major milestone in the analysis of macromolecules especially for protein detection was reached. To date, significant progress in further development of mass spectrometers is being made, rendering these tools essential in life science. The next subchapters are focused on the instrumentation used for the native MS analysis.

2.2 Electrospray in Native MS

2.2.1 Ionization Techniques

Despite the early development of the mass spectrometer as an analytical method, a breakthrough in the field of intact biomolecules analysis did not occur until the 1970s. The original MS ionization techniques, e.g. electron impact and chemical ionization, were not suitable to ionize biological macromolecules. The implementation of ion desorption techniques (field- and plasma desorption) made mass spectral analysis of biomolecules possible. The application of these techniques was limited for the biomolecules analysis. [117-119] The two most relevant and also “softest” ionization techniques are ESI and MALDI.

2.2.2 Electrospray Ionization

ESI breakthrough occurred in 1988, when Fenn’s group presented mass spectra of intact high-mass proteins and synthetic polymers. [120, 121] The generation of multiply charged ions from a single analyte is of great interest especially in protein analysis, since it allows the detection of high-mass analytes at relatively low m/z ratios. Exactly this advantage is the distinct feature of ESI. Given the distribution of the charges accommodated by a macromolecular ion, its m/z value of a specific charge state \( n \), can be calculated as:
\[ \frac{m}{z} = \frac{M + n_i m_i}{n_i} \]  \hspace{1cm} (2.1)

where M is the mass of the macromolecule, \( m_i \) corresponds to a mass associated with a specific charge carrier (e.g., a proton); and \( n_i \) is the number of charges. In case that charging is mostly due to protonation, then equation 2.2 is simplified to:

\[ \left( \frac{m}{z} \right)_{n^+} = \frac{M + n}{n} \]  \hspace{1cm} (2.2)

Proteins are mostly analyzed in positive ion mode, by applying a positive spray voltage, resulting in the formation of \([M + nH]^{n^+}\) \[122\]. A spray of small charged droplets in the electrospray ionization process is generated by the nebulization of a solution in an electric field. The potential (typically low kV) is applied to the capillary or to an electrode in the solution, which flows through a stainless steel or fused silica capillary. The repulsion of the positive ions on the surface as well as the pull of the electric field gradient leads to the so-called Taylor cone formation. \[98, 123\] This small liquid filament, oriented towards the counter electrode, starts at the tip. Due to the unstable state the formation of small charged droplets occurs. Figure 2.2 displays a schematic overview of the nanoflow electrospray ionization process.

**Fig. 2.2** Schematic overview of the nanoflow electrospray ionization process. Top: The key elements of an atmospheric pressure nanoflow source and the corresponding desolvation stages. Bottom: Scheme of the ion desolvation process for a noncovalent protein dimer during transition from the solution nanodroplet to the gas-phase desolvated ion. Adapted from [124] with kind permission of Elsevier, Copyright© 2013.
Chapter 2 – Methods and Instrumentation

The step after nebulization is the reduction of the size of the charged droplets by solvent evaporation. When the droplet surface becomes unstable, Coulomb fission take place. The process of droplet shrinking and the subdivision goes until only isolated gas-phase ions escape. Electrospray ionization can be considered as an electrolysis, since in positive ion mode cations are enriched at the solution surface and negative ions move to the capillary. Especially at very slow flow rates, oxidation of the analytes may take place.

After this technique became more accepted in the MS community, a lot of effort was made to understand the ionization mechanism. Two different theories about the mechanisms for the formation of the gas phase ions from droplets have been proposed: the ion evaporation model [125] (IEM) for small molecules and the charged residue model [126] (CRM) for macromolecules. The CRM proposes that gas-phase ions are produced when only one ion is left within the droplet and no further solvent evaporation can take place. In other words, the droplets undergo evaporation and fission cycles. The remaining ion typically results in the formation of multiply charges. In case of the IEM, evaporation of the solvent leads to the droplets shrinking until the field strengths on the surface becomes so high that the ions are released from the droplet surface. Recently, a chain ejection model (CEM) has been proposed for disordered polymers. [123, 127] Briefly, the exposed large nonpolar surface areas of analyte molecules to solvent will migrate to the surface of droplet. The exposure of the one part of the polymer chain leads to the absorption of the charge carriers. The following step is the stepwise sequential ejection of the protein chain and the separation from the droplet.

Depending on the applied flow rates range on the electrospray one can distinguish between normal electrospray (>1 μL/min) and nano-electrospray (<1 nL/min). Nano electrospray, which was used for the noncovalent analysis in this thesis, will be described in the next subsection more in detail.
2.2.3 Nano Electrospray Ionization

In case of the conventional ESI source the analyte solution is supplied at a constant flow rate to the ion source. The biggest drawback in a traditional ESI source is the possible spray instabilities, which influence the reliability of the analysis. [122] For the first time, Wilm and Mann described in 1994 the benefits of using very low flow rates. [128] Miniaturization of the ESI technique led to development of the nano-electrospray ionization (nanoESI). [129] NanoESI generates droplets that are about 10 times smaller than droplets obtained with pneumatically assisted ESI due to the narrow bore (orifice 1-2 μm) of the spray capillary. It provides reduced flow rates (20-40 nL/min) affording a quite stable spray for about 30 min (the small orifice also prevents the formation of multiple Taylor cones at the tip of the capillary). Loading a small volume about 1-2 μl of a 1-5 μM sample is enough to perform a native MS analysis. [130, 131] Liquid flow is induced by applying high voltage to the capillary tip, which means that the solution is drawn from the capillary electrodynamically without the use of a conventional syringe pump. However, some back pressure to the spray capillary by a gas-tight syringe often is applied, which helps establishing the flow. The capillaries are loaded manually from the back and mounted directly in front of the MS sampling cone at a distance of only 0.5-2 mm. In addition to higher sensitivity as compared to conventional ESI, nanoESI is more efficient (better desolvation) and has much higher salt tolerance, by at least an order of magnitude. [132, 133] Several studies explained this finding in terms of the lower size (estimated to be one order of magnitude smaller than in conventional ESI, 150 nm compared to 1.5 μm in diameter) and the higher charge density of droplets emitted in nanoESI (Figure 2.3). This results in early fission events without extensive solvent evaporation, which would otherwise lead to a significant increase in salt ion concentration prior to fission. As a consequence, high salt amounts in the sample solution become less concentrated compared to conventional ESI.
Recently, a commercial automated nanoESI microfluidic chip system, which can also be used for noncovalent studies, has been development (NanoMate with ESI chip, Advion Biosciences, Ithaca NY, USA). [67, 134] The ESI chip is a microchip device that consists of a 20x20 array of micromachined nozzles. The samples are injected from a 96 or 384 well plate with each sample electrosprayed from a single-use nozzle etched in a silicon wafer. It is the middle-throughput approach combined with the benefits of nanoESI-MS. [66, 67] The system promises a 10-fold increase in signal stability compared with nanoflow capillaries and high nozzle-to-nozzle reproducibility. [68]

2.3 Mass Analyzers

The mass analyzer is the part of the mass spectrometer that allows the separation of the ions according to their m/z values. In the 20th century a lot of progress had been made to develop a variety of mass spectrometers. Mass spectrometers can be grouped into different types of operation mode: continuous mode (magnetic sector,
quadrupole), pulsed mode (time of flight), and ion trapping mode (quadrupole traps, Fourier transform ion cyclotron, orbitrap). [135]

In the following three subchapters, the time-of-flight (TOF) and the quadrupole (Q) mass analyzers will be discussed, together with the hybrid quadrupole-time-of-flight (QTOF) instrument, because most of the results presented in this thesis were obtained using this type of instrument.

2.3.1 Time-of-Flight Mass Analyzer

From the physical point of view the time-of-flight (TOF) is probably the simplest way to perform MS analysis. It is based on measuring the time that ions require to traverse a field-free drift tube of about 1 m length. [136, 137] TOF is a pulsed technique and a starting point is required. The motion of an ion is defined by its kinetic energy:

$$E_c = \frac{1}{2}m v^2$$  \hspace{1cm} (2.3)

(m= mass, v = speed)

If the initial velocity distribution of ions is neglected, then the velocity v of every ion is defined by its m/z and the acceleration potential $U_0$:

$$W = z e U_0 = \frac{m v^2}{2} \rightarrow v = \sqrt{\frac{2 z e U_0}{m}}$$  \hspace{1cm} (2.4)

If the acceleration pulse is very short, the time t that ions need to fly through field-free tube of length L and reach the detector is given by:

$$t = \frac{L}{v} = L \sqrt{\frac{m}{z} \cdot \frac{1}{2 z e U_0}}$$  \hspace{1cm} (2.5)

As can be seen from the equations, the speed of the ions or the time to fly is proportional to the square root of their m/z value. The ions formed, are accelerated by a strong voltage pulse (2-30 kV) in the direction of the detector. Ions with lower mass can reach the detector faster compared to higher mass ions.

In order to obtain the mass spectrum ions have to be converted into a usable signal by a detector. The most frequently used detectors for TOF mass analyzers are multichannel plate detectors (MCP). [138] MCP detectors allow simultaneous
multichannel detection. In brief, MCP consists generally of $10^6$ microscopic glass channels, ca. 5–50 μm in diameter. They are bound together and electrically connected with each other. The channels function is to operate as a continuous dynode electron multiplier. The advantages of MCP detector are high sensitivity and the elimination of the accompanying noise. The disadvantage is a relatively small dynamic range (generally two to three orders of magnitude). With the detector the response decreases with increasing m/z of the ions and limits therefore the detected mass range. [113] The most important drawbacks of the first TOF analyzers were the relatively poor mass resolution, which is affected by mainly by three factors: (i) the length of the ion formation pulse (time distribution), (ii) the size of the volume where the ions are formed (space distribution), and (iii) the variation of the initial kinetic energy of the ions (kinetic energy distribution).

One way to reduce the kinetic energy spread is to introduce a time delay between ion formation and acceleration, a technique called delayed extraction. The ions at first expand into a field-free region in the source and after a certain time delay (ns-μs range), a voltage pulse is applied to accelerate the ions out of the source. The extraction pulse applied after certain delays lead to greater acceleration of the ions with originally lower kinetic energies. This results that less energetic ions have higher kinetic energy and are able to reach the detector at the same time with initially more energetic ions. [113, 139]

The second way to improve the mass resolution of a TOF spectrometer is to use an electrostatic mirror also called a mass reflectron, placed in the drift region of ions. [140] The ions with higher energy penetrate deeper into the reflectron and thus spend more time within the reflector compared with the same m/z ions with the lower energy. Consequently the ions with the same m/z reach the detector at the same time due to the different trajectories. Ions of the same m/z values have then much lower energy dispersion. The advantage using the reflectron is the increased flight path without any need to change the physical instrument size. However, a “single-stage” reflectron are only able to perform first-order velocity focusing of ions initially located at the space focal plane. [122] The performance can be improved by second-order focusing when combining two stage or even multistage reflector
Chapter 2 – Methods and Instrumentation

designs. For this purpose a series of rings or less preferably grids at increasing potential are used. To make sure that all ions are reflected within the homogeneous portion of the electric field of the device the reflection voltage is set to about 1.05-1.10 times the acceleration voltage. Reflector instruments can be equipped with a detector placed behind the reflector and operated in the linear mode simply by switching off the reflector voltage. [140-142]

2.3.2 Quadrupole Mass Analyzer

The main idea behind the quadrupolar electrical fields is the “filtering of ions” according to their m/z ratios. [143-145] Such “mass filter” devices have the ability to select stable trajectories for ions of certain m/z ratios, while others become unstable. A quadrupole mass spectrometer is able to transmit ions within a narrow m/z range. A quad mass analyzer is usually formed by four hyperbolic or circular rods, which are placed in parallel and connected diagonally. A periodic potential of the following form is applied to each pair of electrodes:

$$\phi_o = \pm(U - V \cos \omega t)$$

(2.6)

where $U$ is a DC voltage and $V$ is an RF voltage of frequency $\omega$. A projection of this potential results in a periodic hyperbolic field configuration in the (x,y) plane:

$$\phi(x, y) = (U - V \cos \omega t) \cdot \frac{x^2 - y^2}{2r_0^2}$$

(2.7)

where $r_0$ is the distance between the central axis $z$ to the surface of electrodes. To achieve the highest mass resolving capability the filter has to be tuned so that the “point” corresponding to the ions of interest is close to the apex of the stability region.

For acquiring a mass spectrum acquisition, direct current (DC) as well as the rf potentials have to be varied, while their ratio is kept constant. The previously “unstable” ions of different m/z will become stable in the quadrupole at a given $U$ and $V$ combination and a mass spectrum can be obtained. [122, 139]
Fig. 2.4 Schematic diagram of a quadrupole. An approximate stable and unstable ion trajectory are shown. Adapted from [146] with kind permission of John Wiley and Sons, Copyright© 2012.

Quadrupoles can also be operated in RF-only mode if the DC component of the electrical field is set to zero. The frequency parameter $\omega$ determines the maximum transmission limits of quadrupole. The m/z range of quadrupole MS is usually limited to 4000 and up to m/z 32 000 in the RF-only mode. To analyze heavier ions for the native MS study the RF frequency have to be reduced while the RF voltage is increased. For example, reducing the RF frequency by the factor of two affords a twice higher m/z limit in the mass filter mode.

2.3.3 Hybrid Quadrupole Time-of-Flight (Q-TOF) Instruments

In addition to the above-mentioned analyzers the combination of more than one type of mass analyzers in a single instrument, so-called “hybrid mass spectrometers”, are gaining popularity. [146] Hybrid mass spectrometers profit from the combined advantages of each component and therefore often feature enhanced performance. Using a reflectron-TOF mass analyzer as the last mass analyzing stage has been particularly popular, due to the fast acquisition rate and high mass resolution. [147] The driving force for coupling different analyzers in native MS is the requirement of analyzers with extended m/z ranges (above m/z 4000). For native MS analysis, hybrid quadruple-TOF (Q-TOF) analyzers are often used since they combine high sensitivity (down to the attomolar range), high resolution (10,000+ FWHM), speed of acquisition, extended mass range and mass accuracy of 5-10 ppm. [148-150]

For the ion injection a pulsed extraction field is implemented in the ion modulator so that ions are pushed orthogonally to their initial direction into the TOF analyzer.
Hybrid instruments have additional potential for tandem MS measurements, providing additional structural information of noncovalent complexes. The quadrupole serves as a mass filter in MS/MS experiments. When tandem MS is not needed, it can be operated in RF-only mode. This allows acquisition of full-range mass spectra at high resolution using the TOF analyser. The collision energy is given in electrons volts (eV) and is therefore dependent on the charge of the ions. [113]

However, standard Q-TOF instruments have fundamental limitations in their performance for the analysis of proteins. The Q-TOF instrument used in this work was upgraded and is capable of isolating ions up to m/z 30,000 in the quadrupole and performing TOF MS/MS studies of these. The instrument was modified at first in the source region, because the resulting ions are too energetic to be efficiently transported into the transfer region of the instrument. Therefore the source pressure was increased by a factor of 10 by adding the throttle valve and a hexapole sleeve, which allows sufficient collisional cooling of the ions and their transit into the hexapole region. The quadruple mass range is modified by decreasing the RF frequency. The disadvantage is a reduction of the quadruple resolution, which is, however, more than enough to enable the selection of a specific charge state from a charge state distribution. Since the collision cell pressures and maximum collisional energy are too low for efficient CID, the pressure was increased by modifying the gas plumbing system. [151]

![Schematic diagram of Q-TOF Ultima mass spectrometer (Micromass/Waters).](image)

**Fig. 2.5** Schematic diagram of Q-TOF Ultima mass spectrometer (Micromass/Waters).
### 2.3.4 Ion Mobility Analyzer

Ion mobility (IM) spectrometry is a gas-phase separation tool comparable to electrophoresis in solution and can be combined with MS. This technique relies on the idea that gas-phase biomolecules with different structure can be separated based on their differential transport through an drift tube filled with inert neutrals in the presence of relatively weak electric field. [124, 152-160] It is possible from the ion transport properties measurements to generate ion size information. This would result in an orientitatonally averaged ion-neutral collision cross section (CCS).

![Image of Ion Mobility Analyzer](image)

**Fig. 2.6** Schematic representation of the commercially available TW-IM-MS/MS-QTOF mass spectrometer (Waters Synapt G2). The mobility cell is placed where the collision cell of the classic QTOF would be. The mobility cell is next to the trap. The transfer cell can serve as two independent collision cells for CID. A helium cell is put in front of the mobility cell and operated at high pressure. It should facilitate transport of ions into the 3 mbar nitrogen pressure mobility cell. The inset illustrates how two conformers are separated in TW-IM spectrometry. Adapted from [124] with kind permission of Elsevier, Copyright© 2013.

When thermal energy from collisions exceeds energy of the ions due to the electric field, the velocity, $v$ of the ion under the influence of an electric field $E$, is inversely proportional to its collisional cross-section $\Omega$:

$$v = \frac{3e}{16N} \frac{1}{\Omega} \left(\frac{2\pi}{\mu k_B T}\right)^{1/2} \cdot E \quad (2.8)$$
It the equation \( z \) represents the ion charge, \( \mu \) is the reduced mass of the ion and the buffer gas, \( k_B \) is the Boltzmann constant and \( N \) the number density of the buffer gas. Knowing the length of the drift cell and the drift time of the drift time of an ion specific information of small molecules are provided. The calculation of the collisional cross-section becomes possible when coupling the IM spectrometer with MS and measure m/z [146].

Recent studies show good correlation of many data sets between CCS value based on IM measurements and X-ray or NMR data sets for the same proteins and complexes in solution. Despite the measurements being carried out in the absence of bulk water, these studies suggest that IM reflect their solution properties and can be used as a possible technique for structural biology. [161-164] The recently introduced commercial instruments allow more widespread usage (Figure 2.6). It can be considered as a potential powerful tool for the drug discovery process for therapeutic small molecules. [165, 166]
Chapter 3

3 Quantifying Protein-Ligand Binding Constants Using Electrospray Ionization Mass Spectrometry: A Systematic Binding Affinity Study of a Series of Hydrophobically Modified Trypsin Inhibitors

This chapter is adapted from and reprinted with permission from:

Chapter 3 – Quantifying Protein-Ligand Binding Constants using ESI-MS of a Series of Hydrophobically Modified Trypsin Inhibitors

3.1 Overview
NanoESI-MS is used for determining binding strengths of trypsin in complex with two different series of five congeneric inhibitors, whose binding affinity in solution depends on the size of the P3 substituent. The ligands of the first series contain a 4-amidinobenzylamide as P1 residue, and form a tight complex with trypsin. The inhibitors of the second series have a 2-aminomethyl-5-chloro-benzylamide as P1 group, and represent a model system for weak binders. The five different inhibitors of each group are based on the same scaffold and differ only in the length of the hydrophobic side chain of their P3 residue, which modulates the interactions in the S3/4 binding pocket of trypsin. The dissociation constants (K_d) for high affinity ligands investigated by nanoESI-MS ranges from 15 nM to 450 nM and decreases with larger hydrophobic P3 side chains. Collision-induced dissociation experiments of five trypsin and benzamidine-based complexes show a correlation between trends in K_d and gas-phase stability. For the second inhibitor series we could show that the effect of imidazole, a small stabilizing additive, can avoid the dissociation of the complex ions and as a result increases the relative abundance of weakly bound complexes. Here the K_d values ranging from 2.9 to 17.6 μM – some 1–2 order of magnitude lower than the first series. For both ligand series the dissociation constants (K_d) measured via nanoESI-MS were compared with kinetic inhibition constants (K_i) in solution.

3.2 Introduction
ESI-MS is a powerful and increasingly utilized tool for the investigation of noncovalent interactions. [4] This soft ionization technique allows the transfer of noncovalent complexes from solution into the gas phase and their subsequent study by mass spectrometry. To this day, protein-protein, protein-small molecule, protein-DNA and DNA-small molecule complexes have been successfully detected and studied by this method. [99, 102, 167] Especially in drug discovery ESI-MS is of increasing importance for the investigation of protein-ligand interactions and determination of binding affinities. [69, 168] In recent years, binding affinities (K_d) have been successfully determined by ESI-MS for a variety of noncovalent protein-
ligand complexes. [35, 54, 99] Other MS-based methods have also been successfully applied for quantifying interactions, e.g., methods dubbed “Protein–Ligand Interactions in solution by MS, Titration and H/D Exchange” (PLIMSTEX) and “Stability of unpurified proteins from rates of H/D exchange” (SUPREX). [169-171]

Noncovalent interactions are of great importance in nature; for example they play a major role in stabilizing protein conformation. The hydrophobic effect plays an important role in protein folding, in the adhesion of lipid bilayers, nucleic acid structures, and protein-small molecule interactions. [172-174] Compared to the aqueous environment, the hydration shell is absent in the gas phase, and it’s not yet fully clear whether the conformation of noncovalent complexes remains unchanged during the transition from solution to vacuum. [175] Some forces such as hydrogen-bonding and electrostatic interactions between two oppositely charged molecules are strengthened in the gas phase, while hydrophobic interactions are weakened and therefore difficult to preserve during ionization and ion transfer. [176, 177] A number of research groups have reported investigations of noncovalent complexes where hydrophobic interactions play a dominant role for the complex stability. [74, 178, 179] In a very recent study, Klassen and co-workers [112, 180] demonstrated the application of ESI-MS to quantify binding strengths of β-lactoglobulin - fatty acid complexes in aqueous solution. For three short fatty acids, association constants smaller than expected, were found by the authors (K_a compared with data from a competitive fluorescence assay). They explained this with an in-source dissociation, which reduces the relative abundance of gaseous complex ions measured by ESI-MS.

In a previous study of the same research group it was shown that β-lactoglobulin retains the structure of its binding cavity even in the absence of a hydration shell. [181] The authors monitored the dissociation of the fatty acid-protein complexes in a BIRD experiment and extracted the temperature-dependent kinetic parameters. Their results show that the energy required for dissociation correlates with the length of the hydrocarbon fatty acid chain. Surprisingly, quantitative comparison of the dissociation rate constants in the hydrated and dehydrated states showed that the solvated complex is kinetically less stable than the corresponding gaseous ions at all temperature investigated. [97]
Even when carefully controlling the instrument parameters, complexes that are predominantly stabilized by nonpolar interactions are prone to dissociation in the gas phase. [182] This so-called in-source dissociation can lead to an artificially low binding constants based on the reduced abundance of the complex ions. [83] Stabilization by addition of imidazole to the nanoES solution was presented as a solution for this problem for several weakly bound complexes. [110, 183] The small imidazole molecule acts as a nonspecific, sacrificial ligand and can prevent dissociation of the specifically bound ligand during ES-MS analysis. This can also be thought of as enhanced evaporative cooling of the protein-ligand complex ions in the ion source. The extent of the stabilization depends strongly on the concentration of imidazole. At high imidazole concentrations (>1 mM), the ions of protein-fatty acid, protein-carbohydrate and protein-small molecule complexes can be stabilized, [110] although it is not always possible to prevent dissociation of very labile gas-phase complexes. [104, 109]

To determine binding constants, we used the ES-MS titration method, which has been found suitable for measuring binding strengths, not only in our laboratory but also in other groups. [71, 74, 80, 99, 184] It has also been validated against more established biophysical methods, such as isothermal titration calorimetry (ITC), surface plasmon resonance (SPR), and nuclear magnetic resonance (NMR) spectroscopy. [71, 79] The well-described titration method relies on detection of ions belonging to the complex versus bare proteins. The $K_D$ value can then be easily determined from a fit of the intensity ratio of bound and unbound protein as a function of the added ligand. This method assumes that no dissociation takes place during the transmission through the mass spectrometer. The second important assumption is that the intensity ratio observed in the gas phase correlates with the concentration ratio in solution. If a very low-mass ligand is bound to a high-mass protein, the ionization efficiency does not change for the complex vs. the bare protein, and this assumption is fulfilled in almost all cases. [99] As the ESI titration measurements can deliver a “snapshot” of the solution concentrations the $K_D$ values determined via nanoESI-MS reflect solution-phase binding affinities.

In this work we present a systematic nanoESI-MS study for quantifying the binding
strengths of trypsin in complex with two different series of competitive inhibitor. Closely related 4-amidinobenzylamide- (AMBA) and 2-aminomethyl-5-chlor-benzylamide-based (CMA) inhibitors were chosen to gauge how their hydrophobic side chains modulate the interaction (Table 3.1).

Table 3.1 Chemical structures of the scaffold of the investigated benzamidine- and CMA-type ligands. The residues (R) indicate the substructure of the inhibitor, which binds into the hydrophobic S3/4 pocket of trypsin. This part of the ligand was systematically varied in the size of the hydrophobic chain.

<table>
<thead>
<tr>
<th>R</th>
<th>H</th>
<th>I</th>
<th>Y</th>
<th>T</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abbr.</td>
<td>Gly</td>
<td>D-Ala</td>
<td>D-Val</td>
<td>D-Leu</td>
<td>D-Cha</td>
</tr>
</tbody>
</table>

The two different types of inhibitors are based on the same scaffold and each series varies only in the length of the hydrophobic P3 side chain. In essence, we expected that the binding affinity should increase with increasing length of the hydrophobic chain that is accommodated by the predominantly hydrophobic S3/4 cavity of the enzyme. We are using two compound model systems: benzamidine-type ligands that exhibit strong binding affinity to trypsin, while the CMA-based ligands show lower. The protein used in this study, bovine \( \alpha \)-trypsin, belongs to the family of the well-known serine proteases. Compounds that specifically inhibit these serine proteases can be used for the treatment of different diseases. For example, in the last years the first orally available thrombin and factor Xa inhibitors were approved as anticoagulants. [185, 186]

The basic question is whether we can observe the expected trend in the binding affinity of the inhibitors, when using ESI-MS as a read-out for the solution phase equilibrium. In the case of the benzamidine-based ligands, the binding affinity based on ESI titration measurements increases with increasing length of the hydrophobic P3 side chain, from 15 nM to 450 nM. A second question concerns the stability in
solution vs. in the gas phase. In some studies it was shown that the gas-phase stability reflects the binding properties in solution. [45, 49, 103] More frequently, however, a correlation between the gas-phase stability and the solution-phase stability is absent, [72, 105, 110, 182, 187-190] for example for leucine-zippers and acyl-CoA binding protein (ACBP) and a series of acyl CoA derivatives. [104] If binding properties in solution correlate with gas-phase stability it has to be assumed that the dominant interactions are very similar in solution and in the gas phase, and that solvent mediation play only a minor role (see Daniel at al. and Robinson et al. for comprehensive reviews on this topic [99, 190]). In our study the CID experiments for the benzamidine complexes show a correlation between the binding affinities in solution and the gas phase stability.

The complexes with the CMA inhibitors were prone to in-source dissociation. The main reason may be that complexes stabilized in solution by weak nonpolar interactions exhibit low gas-phase stability. [183] Therefore we have investigated the stabilizing effect of imidazole on this particular model system. Like Klassen and co-workers [83, 112] we found that the addition of imidazole to the nanoESI solution can protect protein-ligand complexes from in-source dissociation during the ESI-MS analysis process. Unlike in the case of the benzamidine series, the trend of higher binding affinity is clearly observed, with the order Gly < D-Ala < D-Leu. No independently measured $K_{d}$s are available for these systems. Therefore, binding affinities determined by MS were compared with inhibition constants (K) determined via an enzyme kinetic inhibition assay.
3.3 Experimental Section

3.3.1 Materials and Methods

Bovine pancreas α-trypsin (MW ≈ 23.300 Da), ammonium acetate, and CsI were purchased from Sigma Aldrich (Buchs, Switzerland). Imidazole (99.5 % purity) and DMSO were obtained from Fluka Chemie AG. Water was purified using a Milli-Q® Ultrapure water purification system by Millipore (Barnstead, IA / USA). All MS titration experiments were recorded under “native-like” conditions using 50 mM ammonium acetate buffer at pH = 7.8. Stock solutions of ligands were prepared at 40-50 mM concentration in DMSO. Prior to the measurement, the inhibitor solutions were diluted with Milli-Q® water to the desired concentration. The protein working solution was made from a 100 μM stock solution in ammonium acetate buffer. The exact trypsin concentration was determined using a UV spectrometer (NanoDrop 1000, Witec AG, Littau, Switzerland).

3.3.2 Mass Spectrometry

ESI spectra were acquired with a hybrid quadrupole time-of-flight mass spectrometer (Q-TOF ULTIMA, Waters/Micromass, Manchester, UK) in the positive ion mode. The instrument was controlled via the MassLynx version 4.0 software. In order to obtain a good signal-to-noise ratio, 100 scans were accumulated for one spectrum. The mass spectrometer is equipped with an automated chip-based nanoESI system (Nanomate 100, Advion Biosciences, Ithaca, NY, USA). It has a 96-well sample plate, a rack of 96 disposable, conductive pipet tips, and a nanospray chip containing 20 x 20 nozzles of 5 μm diameter. For investigation of noncovalent complexes, appropriate instrumental conditions have to be found. In this case the desolvation must be sufficiently complete in order to get narrow peaks for the detected species but not to dissociate the noncovalent complex. This can take an influence on peak broadening because of the adduct formation with salt and buffer molecules from the spray solution. The settings described below were found to be a good compromise between the intact complex detection and sufficient desolvation of analytes. For all nanoESI-MS measurements the voltage was set to 1.8-1.9 kV and a gentle backing pressure of 5 bar on the spray tip was used to assist the liquid sample flow. The source temperature was kept at 21 °C. To prevent dissociation of
the noncovalent complexes, the mass spectrometer was run with gentle desolvation parameters. The cone and first ion tunnel RF1 voltages, the parameters that control the kinetic energy of the ions in the source region of the mass spectrometer, were optimized to 40 V and 35 V. After this stage, the ion beam passed a hexapole collision cell filled with argon (Purity 5.0, PanGas). Collision-induced dissociation (CID) used in MS/MS experiments were preformed by adjusting the acceleration collision energy (CE) voltage until full dissociation of the parent complex ions was achieved. Calibration of the mass spectrometry instrument was performed using Csl clusters. The concentration of Csl was 2 μg/uL dissolved in water/2-propanol (1/1, v/v).

3.3.3 Data Processing
Before data processing, each mass spectrum was smoothed (Savitzky-Gollay smooth) with the MassLynx 4.0 software (Waters, UK). For the $K_D$ determination the measured relative peak height intensity (I) ratios ($R$) of the ligand-bound protein (P·L) to bare protein (P), $R = I (\text{P} \cdot \text{L})/I (\text{P})$, were calculated for each spectrum. For this calculation, all charge states were taken into account. The experimentally calculated $R$-values were plotted versus the total inhibitor concentration. The equation proposed by Daniel et al. [191] was used to determine the dissociation constant ($K_D$) from the titration curve fit:

$$\frac{I (\text{P} \cdot \text{L})}{I (\text{P})} = \frac{1}{2} \left( -1 - \frac{[\text{P} \cdot \text{L}]}{K_D} + \frac{[\text{L}]}{K_D} + \sqrt{4 \frac{[\text{L}]}{K_D} + \left( \frac{[\text{L}]}{K_D} - \frac{[\text{P} \cdot \text{L}]}{K_D} - 1 \right)^2} \right)$$

(3.1)

It was assumed here that the ionization efficiency for the bare protein and the complex is equal, which allowed us to use the intensity ratios of free protein over complex instead of their concentrations in solution. The $K_D$ calculations and the fitting of the titration curves were performed using MATLAB software (2010a, The MathWorks, Natick, MA, USA).

3.3.4 Kinetic Inhibition Assay
Kinetic inhibition of bovine trypsin was determined photometrically at 405 nm using the chromogenic substrate Pefachrom tPa (LoxoGmbH, Dossenheim, Germany) according to the protocols described by Stürzebecher et al. [192] under the following
conditions: 50 mM Tris/HCl (pH 8.0), 154 mM NaCl, 5% DMSO, and 0.1% polyethylene glycol (PEG) 8000 at 25 °C using different concentrations of substrate and inhibitor. $K_i$ values were determined at least in triplicate.
3.4 Results and Discussion

3.4.1 $K_d$-Determination of the Benzamidine-based Inhibitors by the NanoESI-MS

The benzamidine-based inhibitors in complex with trypsin represent a model system for the quantification of “high-affinity ligands”. Figure 3.1 provides an example of a nanoESI mass spectrum for a solution of 5 μM trypsin in the presence of d-Cha in 50 mM aqueous ammonium acetate solution under non-denaturing conditions (pH = 7.8).

![Figure 3.1](image)

Fig. 3.1 Representative nanoESI mass spectra of 5 μM trypsin in the presence of the d-Cha-inhibitor obtained in positive ion mode under “native” conditions. Trypsin (T) was titrated with different concentrations of the d-Cha inhibitor a.) 0.5 μM b.) 2 μM c.) 2.5 μM d.) 5 μM. The signal for the noncovalent complex clearly increases with increasing amount of ligand present in solution. The full complexation is reached at 5 μM inhibitor concentration.

The narrow charge state distribution, predominantly 7+, 8+, 9+, is characteristic for native conditions, whereby 8+ is the most intense signal. In order to determine the dissociation constant via the titration method, a series of nanoESI experiments were performed with increasing inhibitor concentrations ranging from 0.5 to 5 μM. Figure 3.1a-d shows representative mass spectra obtained for the noncovalent trypsin-d-Cha-inhibitor complex using four different ligand concentrations. As expected, the ratio of the complex to free protein ion signals increases with higher total inhibitor concentration.
Already at 5 μM inhibitor concentration, full complexation is reached, because the benzamidine group binds strongly and specifically to the active site of the enzyme and occupies the S1 pocket of trypsin. Also, the amidinium group of benzamidine is a mimic of the guanidinium side chain of arginine. The stabilization of the benzamidine moiety in the S1 pocket of trypsin is based on the bridge linking the positively charged amidinium group and the carboxylate group of Asp189. [185] Three other hydrogen bonds to Ser190, Gly210 and a conserved water molecule contribute to the strong ligand binding. [193, 194] Moreover, additional van der Waals contacts between the P1 phenyl ring and residues of the S1 pocket of trypsin enhance the stability of the complex. The proline moiety of the inhibitor occupies the S2 pocket. [195] (Table 3.1).

**Fig. 3.2** Representative nanoESI-MS spectra for three different complexes at the same inhibitor concentration (2.5 μM). The ratio of the complex over the free protein expressed in R-values are a.) D-Cha R = 2.6; b.) D-Val R = 1.6; c.) Gly R = 0.9 and confirmed the binding affinity order Gly < D-Val < D-Cha by the spectra.

Once the optimized conditions were found for the D-Cha-inhibitor/trypsin complex, the protein was titrated against four other inhibitors. Spectra obtained with trypsin and equal concentrations (2.5 μM) of three different inhibitors (Gly, D-Val and D-Cha) are shown as examples in Figure 3.2.
Based on the length of the hydrophobic side chain, d-Cha should show the strongest binding followed by d-Val and Gly. In other words, at the same inhibitor concentration the highest complex to the free protein ratio should be observed for d-Cha. The ratio between d-Cha-trypsin and free protein ions was 2.6, while d-Val and Gly-trypsin generated ratios of 1.6 and 0.9. The trend in increased binding affinity of these three inhibitors expressed by R-values is clearly confirmed by the spectra. These different hydrophobically modified residues bind into the hydrophobic S3/4 pocket of trypsin and are responsible for the observed increasing affinity. This pocket is supposed to favor binding of ligands possessing bulky aromatic moieties. [196]

The stabilizing effect of imidazole, explained in detail below, was also tested on this particular model system. To test the influence of imidazole, a small stabilizing solution additive, we chose the d-Val-inhibitor. However, upon addition of 10 mM imidazole to the nanoES solution, no change of the complex ions to the free protein was observed (data not shown). The only observable effect of imidazole was a shift of the charge state distribution to lower charge states. Since the ESI titration measurements were performed under “soft” sampling conditions and the benzamidine inhibitors bind specifically into all trypsin pockets, we assume that complexes are stable and not prone to dissociation in the gas phase.

The dissociation constants $K_d$ for all trypsin-inhibitor complexes were determined as described in the “Data Evaluation” section. Five different titration curves for the benzamidine-based inhibitors in complex with trypsin are shown in Figure 3.3. As can be seen on the curves a larger slope reflects a higher binding affinity and vice versa.
Fig. 3.3 NanoESI-MS titration curves for the binding of five different inhibitors to trypsin. The ligand concentration ranges from 0.5 to 5 μM, while the protein concentration was kept constant. This titration method relies on the relative abundance of bare protein and complex ions measured by nanoESI-MS assuming that the intensity ratio observed in the gas phase correlates with the concentration ratio corresponding to the free protein and complex concentration in solution.

The $K_D$-values based on ESI measurements and the $K_I$-values measured for five different inhibitors are compared in Table 3.2. $K_D$s for binding with trypsin were in the range of 15 nM to 449 nM. The largest jump in $K_D$ value was observed going from 79 nM to 15 nM, when the hydrophobic P3 side chain binding in the S3/4 pocket increased in size from a D-Leu group to D-Cha. The comparison with inhibition constants ($K_I$s) was not to validate the $K_D$ values, but to see whether they follow a similar trend as that found for the $K_I$ values; depending on assay conditions, $K_D$s and $K_I$s need not be identical. It should also be noted that most of the $K_I$s for thrombin are smaller than those for trypsin.
**Table 3.2** List of calculated dissociation constants ($K_D$) for five benzamidine based ligands and trypsin determined by the nanoESI titration method, and $K_i$ values from a kinetic inhibition assay determined in solution for trypsin and thrombin.

<table>
<thead>
<tr>
<th>benzamidine series (R)</th>
<th>$K_D$ [nM]</th>
<th>$K_i$ [nM]</th>
<th>$K_i$ [nM]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nanoESI-MS trypsin$^a$</td>
<td>kinetic inhibition assay trypsin$^b$</td>
<td>kinetic inhibition assay thrombin$^b$</td>
</tr>
<tr>
<td>d-Cha</td>
<td>15.0 ±2.5</td>
<td>0.95 ± 0.18</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>d-Leu</td>
<td>79.3 ± 5.9</td>
<td>1.43 ± 0.18</td>
<td>0.89 ± 0.12</td>
</tr>
<tr>
<td>d-Val</td>
<td>87.1 ± 15.2</td>
<td>0.52 ± 0.11</td>
<td>1.29 ± 0.35</td>
</tr>
<tr>
<td>d-Ala</td>
<td>193.5 ± 17.8</td>
<td>1.95 ± 0.77; $5$ ± 3$^c$</td>
<td>5.2 ± 0.8</td>
</tr>
<tr>
<td>Gly</td>
<td>449.7 ± 75.9</td>
<td>15.9 ± 3.06; 37 ± 3$^c$</td>
<td>3.7 ± 0.6</td>
</tr>
</tbody>
</table>

$^a$The error is based on 95% confidence interval of the fitting curve  
$^b$The error is given as the standard deviation calculated from at least three different measurements  
$^c$Second independent measurement

The reason is the known preference of thrombin for inhibitors with proline as a P2 residue, which fits perfectly below the thrombin specific 60-insertion loop making strong hydrophobic contacts to residues Tyr60A and Trp60D. The $K_i$ values for trypsin determined via the kinetic inhibition assay increased with larger hydrophobic side chain with exception of d-Val (**Table 3.2**). However, the kinetic inhibition constants for d-Cha, d-Leu and d-Val are very similar and in the low nM-range.

The discrepancy between $K_D$ and $K_i$ values can be easily explained by different assay conditions: for the MS-based $K_D$ determination, ammonium acetate was used as a buffer, while for the kinetic inhibition assay, a buffer solution of 50 mM Tris/HCl (pH 8.0), 154 mM NaCl, 5% DMSO, and 0.1% polyethylene glycol (PEG) was used. Different buffer solutions may have a substantial influence on the binding affinities for benzamidine-trypsin complexes. Since benzamidine inhibitors are very potent, the $K_i$ determination using chromogenic substrate are close to the instrument limit of detection. The consequence is that even marginal measurement inaccuracies would have significant influence on the $K_i$ values. This might be a possible explanation for observing higher binding affinity for d-Val instead of d-Cha. However, with the exception of d-Val, the $K_D$s and the $K_i$ values that were determined show that relative binding affinities of the different inhibitors can be successfully determined, and that the $K_D$ and $K_i$ values decrease with larger hydrophobic side chain.
3.4.2 Gas-Phase Stability vs. Binding Affinity

Collision-induced dissociation (CID) experiments were performed to gain additional information about the stability of different noncovalent trypsin-inhibitor complexes in the gas phase. For the MS/MS measurements the 8+ complex ions were selected and dissociated during the transmission through the mass spectrometer. The collision energy offset was varied until the selected parent ions of different noncovalent complexes were completely dissociated. Figure 3.4 shows MS/MS spectra of three different Gly-, D-Leu-, D-Cha- ligand-trypsin complexes with a collision energy offset of 20 V.

![CID experiments of different noncovalent trypsin and benzamidine based inhibitors.](image)

**Fig. 3.4** CID experiments of different noncovalent trypsin and benzamidine based inhibitors. For the MS/MS measurements the 8+- charged complex precursor ions (*) were selected. The collision energy was set to 20 V. At the same collision energy, the D-Cha-complex stays 53 % intact, for D-Leu and Gly complexes 45 % and 34 % survive.

Dissociation of the precursor ions (complex) yielded the P7+ and P8+ ions. The extent of dissociation of three different complexes at the same collision energy offset is obvious in the spectra: 53 % of the D-Cha-inhibitor/trypsin-complexes remained intact, while of the D-Leu and Gly complexes 45 % and 34 % survive, respectively. Since the increase in binding affinity of the different benzamidine inhibitors is based on the differences in their hydrophobic P3 side chain, a correlation between the
solution-phase binding affinity and the gas-phase stability for D-Cha, D-Leu and Gly complexes could be established by a single measurement.

Several groups have tried to find a correlation between the gas-phase stability and the type of interactions involved in a complex. As already mentioned in the Introduction, it is important to note that the gas-phase stabilities of noncovalent complexes generally do not correlate with solution binding affinities.

![Graph showing the percentage of intact complex against collision energy offset](image)

**Fig. 3.5** Normalized percentage of the intact complex $100 \times \frac{l_{T+L}}{l_{T+L}+l_{T}}$ plotted against the collision energy offset. A correlation between the gas phase stability and binding affinity is observed: With an increasing binding affinity more energy is necessary to dissociate the complex.

In our work, $CE_{50}$ values, the collision energy offset where 50% of the complexes are dissociated, were used as a measure for the gas-phase stability of the complex. The dissociation curves for the different noncovalent complexes are shown in **Figure 3.5** where the normalized percentage of the intact complex $100 \times \frac{l_{T+L}}{l_{T+L}+l_{T}}$ was plotted against the collision energy offset. $CE_{50}$ values for the five different complexes reveal different dissociation rates during their transmission through the mass spectrometer. While 50% of the D-Cha complex dissociates at collision energy setting of only 21 V, only 16% of the Gly complex stays intact at the same collision energy. The data indicate that a correlation between the gas-phase stability and the binding affinity in solution exists, showing a general trend towards higher stability with increasing hydrophobic P3 side chain. This correlation between solution-phase and gas-phase stability of the complexes suggests that polar and/or electrostatic
contacts dominate the noncovalent interaction, which is additionally modulated by a contribution from hydrophobic contacts.

3.4.3 $K_D$-Determination of the CMA Series by NanoESI-MS Titration Method

The CMA-based inhibitors serve as a model system for weak binders in complex with trypsin. As shown in the spectra in Figure 3.6a, there is no change in the charge state distribution compared to the tighter binding benzamidine-based inhibitors. The +7, +8, +9 ions are predominantly observed in the spectrum.

![Figure 3.6](image)

**Fig. 3.6** Representative nanoESI-MS spectra for 25 μM Gly-inhibitor in complex with trypsin. The ratio (R) between complex and bare protein decreases with increasing accumulation time after a.) 30 sec (R = 0.4); b.) 60 sec (R = 0.26); c.) 180 sec (R = 0.21).

In Figure 3.6a-c the mass spectra of 25 μM Gly-inhibitor/trypsin-complex was acquired using an accumulation time of 30, 60 and 180 seconds. We found that the relative abundance of protonated complex ions gradually decreased during acquisition of spectra. The longer accumulation time led to a decrease of 90% in the ratio of complex and bare protein, which would result in an artificially low binding affinity. The main reason of this observation is probably the in-source dissociation of the complex, as suggested previously by Klassen and co-workers. [83] One way to circumvent this limitation would be to use very short accumulation times. However, in order to achieve better signal-to-noise ratios in the mass spectrum, longer acquisition times are desirable. Even with the gentlest sampling conditions, the
dissociation of the trypsin-CMA-inhibitor complexes could not be prevented. Therefore, other methods for stabilizing protein-ligand complexes were considered. It has recently been shown that ESI solution additives, such as imidazole, can protect the protein-ligand complex from dissociation.

The stabilization effect of imidazole in the presence of 25 μM Gly-inhibitor is illustrated in Figure 3.7.

![Figure 3.7](image)

**Fig. 3.7** ESI mass spectra acquired for 25 μM Gly-inhibitor in the presence of 10 mM imidazole. The relative complex abundance is doubled (the ratio of the complex over the free protein expressed in R-values is 0.75) increased compared with Figure 3.6.

The nanoESI measurements were acquired under identical conditions as those in Figure 3.7 but now in the presence of 10 mM imidazole in the ES-solution. Upon its addition to the nanoES solution, nonspecific adducts between the imidazole molecules and trypsin-ligand complexes can be formed. The nonspecific interactions should be kinetically less stable compared to specific trypsin-inhibitor complexes. After loss of the nonspecific interactions in the hexapole region, the internal energy of trypsin-ligand ions in the source should be lowered, thereby stabilizing the complex. As shown in the spectra, the addition of imidazole to Gly-inhibitor/trypsin nanoES solution results in a double increase in the relative abundance of the complex ions. The important effect of this small molecule is the constant relative complex abundance measured over the longer accumulation time (data not shown). Also, the addition of imidazole reduces the average charge state distribution of the
Chapter 3 – Quantifying Protein-Ligand Binding Constants using ESI-MS of a Series of Hydrophobically Modified Trypsin Inhibitors

Gly-inhibitor/trypsin complex from \( n = 9-7 \) to \( n = 8-6 \) (Figure 3.7). This stabilizing effect can be explained through enhanced cooling from imidazole evaporation that delays the dissociation. Coulomb repulsion can be minimized due to the reduced net charge state of the protein that stabilizes the complex. [39] Due to the high gas-phase basicity of imidazole (217 kcal mol\(^{-1}\)), it is able to strip protons from the protonated protein ions in the gas phase. [197] As mentioned above, this observed charge stripping might play an important role in stabilizing of the complex, because the lower charge states are less susceptible to collisional dissociation. However, the appearance of the lower charge states is probably not the main mechanism for the complex stabilization, since SF\(_6\) provides similar complex stabilization without any shift of charge state distribution. [183]

Another explanation for observing a drop in relative abundance of complex ions might be due to electrochemical reactions that occur where the electrode contacts the solution of the ES ion source. Products of such electrochemical reactions can alter the solution composition and affect the relative abundance of CMA complexes in solution and protonated complex ions during acquisition of spectra. As already shown [198] the solution composition and the resulting nanoESI spectra can be time dependent, with changes in the spectra being ascribed to on-going (electro)chemical reactions upstream in the capillary. It is conceivable that addition of imidazole can buffer a shift of the solution phase equilibrium, although the detailed mechanism of how this occurs is unknown.

This observation indicates that imidazole is a suitable additive for protecting the CMA- complexes during the nanoESI process. Because the stabilization effect of imidazole on Gly-trypsin system appeared rather effective, we decided to perform further titration experiments with other CMA- inhibitors in the presence of imidazole. All titration experiments for CMA-complexes were carried out in positive ion mode. The only difference to the previous experiments is a higher concentration of the ligand (1–30 \( \mu \)M), required to observe the weaker interactions and to obtain useful free protein-to-complex ratios.

The dissociation constants \( K_D \) for all trypsin-inhibitor complexes were determined by fitting the five different titration curves as described above (data not shown). The
results, given in Table 3.3, show higher dissociation constants compared to the benzamidine series, with $K_0$ values between 2.9 and 17.6 µM – some 1–2 order of magnitude lower than the benzamidine complexes. The reason for lower binding affinity compared to the previously described benzamidine based inhibitors is the substitution by the CMA anchor group, which is involved in the binding to the S1 pocket of trypsin.

Table 3.3 List of calculated dissociation constants ($K_0$) for five CMA based ligands determined by the nanoESI titration method and kinetic inhibition assay ($K_i$) determined in solution. For both methods the binding affinity increasing in order Gly < d-Ala < d-Leu.

<table>
<thead>
<tr>
<th>CMA serie (R)</th>
<th>$K_0$ [µM]</th>
<th>$K_i$ [µM]</th>
<th>$K_i$ [nM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>nanoESI-MS trypsin$^a$</td>
<td>kinetic inhibition assay trypsin$^b$</td>
<td>kinetic inhibition assay thrombin$^b$</td>
<td></td>
</tr>
<tr>
<td>d-Cha</td>
<td>17.63 ± 1.2</td>
<td>6.6 ± 0.8</td>
<td>0.052 ± 0.005</td>
</tr>
<tr>
<td>d-Leu</td>
<td>2.9 ± 0.2</td>
<td>0.4 ± 0.09</td>
<td>0.259 ± 0.024</td>
</tr>
<tr>
<td>d-Val</td>
<td>12.4 ± 0.2</td>
<td>6 ± 0.2</td>
<td>0.788 ± 0.070</td>
</tr>
<tr>
<td>d-Ala</td>
<td>8.4 ± 1.4</td>
<td>3.1 ± 1</td>
<td>2.2 ± 0.4</td>
</tr>
<tr>
<td>Gly</td>
<td>24.4 ± 0.49</td>
<td>9.9 ± 0.1</td>
<td>1.5 ± 0.1</td>
</tr>
</tbody>
</table>

The error is based on 95 % confidence interval of the fitting curve
The error is given as the standard deviation calculated from at least three different measurements

Moreover, unlike as for the benzamidine complexes, no trend in $K_0$ with increasing side-chain could be observed. However, an increased binding affinity is clearly observed in the order Gly < d-Ala < d-Leu (Table 3.3). Since the x-ray data for these complexes are not available, we assume that the different trend of binding affinities is probably due to the different anchor groups of the different ligand series.

The relative difference in the $K_0$ values determined by nanoESI-MS and the characterized $K_i$ values are equivalent, considering the error margins of the methods. These results confirm the same trend observed in the kinetic inhibition assay as under the experimental MS conditions, demonstrating the ability of quantitative ESI-MS measurement to clearly distinguish between ligand affinities.
Chapter 3 – Quantifying Protein-Ligand Binding Constants using ESI-MS of a Series of Hydrophobically Modified Trypsin Inhibitors

3.5 Conclusions

In conclusion, we have applied nanoESI-MS for the investigation of a series of hydrophobically modified ligands interacting with trypsin. The size of the hydrophobic side chain (R) that binds in the S3/4 pocket of trypsin was systematically increased. The different substituents (R = Gly, d-Ala, d-Val, d-Leu, d-Cha) have a significant influence on the binding constants. The quantification of binding affinities was possible using the titration method. In the case of the benzamidine series the trend to higher binding affinity with increasing hydrophobic P3 side chain is strong, ranging from 450 nM to 15 nM. The binding affinities measured by ESI-MS titration and kinetic inhibition constants for the benzamidine-trypsin complexes show, with the exception of d-Val, the same relative ordering. Collision-induced dissociation experiments across the benzamidine type series clearly show the correlation between the binding affinity and the gas phase stability. More collision energy is necessary to dissociate the complex with higher binding affinity and vice versa.

The CMA-inhibitors served as a model system for a series of less potent complexes, which are prone to in-source dissociation. This effect causes a reduced relative abundance of the gaseous complex ions and leads therefore to artificially lower binding affinities in the measurements. Upon addition of imidazole, a stabilizing solution additive, the relative abundance of the non-dissociated could be increased. Compared to benzamidine-inhibitors the CMA ligands did not show the clear trend towards higher binding strengths with longer side-chains. The increased binding affinity is observed for Gly < d-Ala < d-Leu.

For the CMA series the relative difference for $K_D$ and the characterized $K_i$ values are equivalent, which demonstrates the ability of quantitative ESI-MS to distinguish between ligand affinities.
Chapter 4

4 Determination of Protein-Ligand Binding Constants of a Cooperatively Regulated Tetrameric Enzyme Using Electrospray Mass Spectrometry

This chapter is adapted from and reprinted with permission from:
Chapter 4 – Determination of Protein-Ligand Binding Constants of a Cooperatively Regulated Tetrameric Enzyme Using ESI-MS

4.1 Overview

This study highlights the benefits of nano electrospray ionization mass spectrometry (nanoESI-MS) as a fast and label-free method not only for determination of dissociation constants ($K_D$) of a cooperatively regulated enzyme, but also to better understand the mechanism of enzymatic cooperativity of multimeric proteins. We present an approach to investigate the allosteric mechanism in the binding of inhibitors to the homotetrameric enzyme fructose-1,6-bisphosphatase (FBPase), a potential therapeutic target for glucose control in type-2 diabetes. A series of inhibitors binding at an allosteric site of FBPase were investigated to determine their $K_D$s by nanoESI-MS. The $K_D$s determined by ESI-MS correlate with IC$_{50}$ values in solution very well. The Hill coefficients derived from nanoESI-MS suggest positive cooperativity. From single-point measurements we could obtain information on relative potency, stoichiometry, conformational changes and mechanism of cooperativity. A new X-ray crystal structure of FBPase tetramer binding ligand 3 in a 4:4 stoichiometry is also reported. NanoESI-MS based results match the current understanding of the investigated system and are in agreement with the X-ray structural data, but provides additional mechanistic insight on the ligand binding, due to the better dynamic resolution. This method offers a powerful approach for studying other proteins with allosteric binding sites as well.

4.2 Introduction

Allosteric regulation of protein function is of major importance since it allows control of many cellular processes. The regulation of allosteric cooperative systems can be achieved by conformational changes in multimeric proteins induced by binding of substrates or ligand molecules. [10, 12] The binding of one ligand increases (positive cooperativity) or decreases (negative cooperativity) the affinity of the protein toward a second ligand. [199] Understanding mechanisms of allosteric regulation is important and can be used in several fields, e.g., in small molecule drug discovery or in better understanding of biochemical pathways. An allosteric binding site may be more species-specific than the active site of the protein. [11, 200, 201] The quantitative determination of binding strengths of such protein-ligand complexes is
of high importance for the design and optimization of novel potential therapeutics in drug discovery.

Several techniques for investigating allosteric mechanisms are in use, e.g., physicochemical methods such as X-ray crystallography, NMR spectroscopy, surface plasmon resonance (SPR) or isothermal titration calorimetry (ITC) or biochemical methods. All of these methods have their advantages and drawbacks in terms of high sample consumption, throughput, dynamic range; some require immobilizing of one of the binding partners. [5, 61, 64, 65, 202] It is not possible to gain all necessary information by applying only one technique, so that combinations of several methods is necessary to reveal the nature of allosteric mechanisms.

Another powerful and increasingly utilized tool for the detection and characterization of noncovalent protein-ligand interactions is nanoESI-MS. [4, 99, 167] This method can be used for determining composition, stoichiometry, subunit interactions, and architectural organization of noncovalent protein-ligand complexes. [39, 190] Current research suggests that proteins in the gas phase are in a folded conformation, which is similar to the native conformation in solution, i.e. they still possess binding pockets comparable to that of the protein in solution and will bind ligands. [203, 204] In recent years, more research groups have become active in this field. Especially ESI-MS is becoming increasingly important in drug discovery, for the investigation of protein-ligand interactions and determination of binding affinities. [69, 134, 168] Dissociation constants (Kd) have been successfully determined by ESI-MS for a range of noncovalent protein-ligand complexes. [4, 35, 54, 71, 83, 112, 148, 205]

This work emphasizes the advantages of the nanoESI-MS method for studying allosteric regulation of protein-ligand interactions illustrated with the tetrameric protein FBPase. It gives the possibility of directly visualizing ligation states and conformational changes of tetrameric FBPase present in solution as well as determining binding strengths. To our knowledge, only a single publication has appeared on cooperative mechanisms of ligand binding to well-studied multisubunit enzymes using ESI-MS. [206] The relative abundance of all the distinct enzymatic species in order to deduce the cooperativity of the system has been determined in
this work. Therefore, there is a clear need to better establish ESI-MS as a complimentary tool in drug discovery to gain deeper understanding of allosterically regulated biological systems.

In this study we investigate the allosteric mechanisms of the binding of different inhibitors to homotetrameric FBPase. FBPase is an AMP allosterically regulated enzyme consisting of four identical subunits (MW= 36,700 Da) organized as a dimer of dimers. [207-209] Enzymatic activity is regulated by a change of the quaternary structure between the active (R) and inactive (T) conformational state. FBPase is a potential therapeutic target for glucose control in type-2 diabetes implicated in glucose sensing and in regulating insulin secretion in β-cells. [210-212]

Several small molecule FBPase inhibitors have progressed to clinical trials to treat type-2 diabetes such as CS-917 and MB07803 discovered by Metabasis. We investigated a series of inhibitors against FBPase that occupy the allosteric AMP binding site, which appears to be the most attractive binding site for drug discovery efforts. [212] In the first step, a series of titration experiments at constant FBPase and different inhibitor concentrations were performed using nanoESI-MS. [71, 74, 99] For the quantitative determination of \( K_0 \) values by ESI-MS the Hill equation was applied. We have also monitored the effect of the natural, mixed-competitive substrate inhibitor fructose 2,6-bisphosphate (F-2,6P\(_2\)) on the binding properties of the allosteric inhibitors to FBPase.

Beside the quantification of binding strengths we could obtain information about stoichiometry and mechanism of cooperativity from single-point measurements. This method also shows the oligomeric species that exist in solution. Moreover, the nanoESI-MS assay has advantages in terms of simplicity (label-free measurements), selectivity (possibility of using additional stages of MS combined with ion activation methods), sensitivity (low sample consumption) and speed (mass spectra can be acquired in less than a minute). [5]

All results obtained with nanoESI-MS match the current understanding of this system and prove that this technique is as a powerful tool for investigating other allosterically regulated proteins.
4.3 Experimental Section

4.3.1 Materials and Methods

All solvents and caesium iodide (CsI) were purchased from Sigma Aldrich (Buchs, Switzerland). Human liver FBPase as well as small molecule inhibitors (see Table 4.1) were provided by F. Hoffmann-La Roche Ltd (Basel, Switzerland).

Table 4.1 Numbers, chemical structures and the corresponding IC_{50} values of the investigated inhibitors in complex with FBPase.

<table>
<thead>
<tr>
<th>Number</th>
<th>Structure</th>
<th>Molecular weight</th>
<th>IC_{50} [μM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image1.png" alt="Structure 1" /></td>
<td>429.68</td>
<td>0.33</td>
</tr>
<tr>
<td>2</td>
<td><img src="image2.png" alt="Structure 2" /></td>
<td>493.79</td>
<td>0.14</td>
</tr>
<tr>
<td>3</td>
<td><img src="image3.png" alt="Structure 3" /></td>
<td>506.40</td>
<td>0.05</td>
</tr>
<tr>
<td>4</td>
<td><img src="image4.png" alt="Structure 4" /></td>
<td>423.43</td>
<td>1.66</td>
</tr>
<tr>
<td>5</td>
<td><img src="image5.png" alt="Structure 5" /></td>
<td>437.46</td>
<td>0.53</td>
</tr>
</tbody>
</table>

Fructose 2,6-bisphosphate was obtained from Fluka Chemie AG. Water was purified using a Milli-Q® Ultrapure water purification system (Millipore, Barnstead, USA). All MS titration experiments were recorded under “native-like” conditions using 500 mM ammonium acetate buffer at pH = 7.5. Prior to mass spectrometric analysis the protein stock solution (1mg/ml) in 20 mM Tris-HCl, 150 mM NaCl, 1 mM DTT (pH = 7.5) was desalted and buffer exchanged (PD MiniTrap G-25, GE Healthcare,
Chapter 4 – Determination of Protein-Ligand Binding Constants of a Cooperatively Regulated Tetrameric Enzyme Using ESI-MS

Buckinghamshire, UK) with the non-denaturing buffer. For protein denaturation, ZipTip columns containing C$_4$-resin (Millipore, Molsheim, France) were used. The exact protein concentration was determined using a UV spectrometer (Genesys 10S UV-VIS, ThermoScientific, Bremen, Germany) by measuring the absorbance at 280 nm. Human liver FBPase (Swissprot Database reference PO9467, entry F16P_HUMAN) was expressed in E. coli and purified according to a protocol similar to that described in El-Maghrabi et. al. [213]

4.3.2 Mass Spectrometry
Electrospray ionization analyses were performed with a hybrid quadrupole time-of-flight mass spectrometer (Q-TOF ULTIMA, Waters/Micromass, Manchester, UK) in the positive ion mode. The instrument was controlled via the MassLynx software (version 4.0). Ions were formed using nanoflow platinum-coated borosilicate electrospray capillaries (Proxeon, Odense, Denmark). For all nanoESI-MS measurements, the capillary voltage was set to 2.1 kV and a gentle backing pressure of 0.3-0.5 bar was applied to assist the liquid sample flow. The source temperature was kept at 21 °C. In order to get narrow peaks of the detected species without dissociating the noncovalent complex, appropriate instrumental conditions had to be found. The precise settings can have an influence on peak shape. Because of adduct formation with salt and buffer molecules from the spray solution peaks might be broadened. The mass spectrometer was run with the following gentle desolvation parameters: the cone and first ion tunnel RF1 voltages, parameters that control the kinetic energy of the ions in the source region of the mass spectrometer, were set to 80 V and 60 V. After this stage, the ion beam passed a hexapole collision cell filled with argon (Purity 5.0, PanGas). The collision energy offset was used to optimize desolvation and set to 30 V. The pressure in the source was increased to 5.5 mbar, using a Speedivalue (Edwards Ltd., Sussex, U.K.) connected between the rotary pump and source pumping line. The pressure in the analyzer region (pressure of argon in the collision cell) was adjusted to $1.1 \times 10^{-4}$ mbar and the TOF pressure was $7.47 \times 10^{-7}$ mbar. The ion transmission was optimized for an m/z range between 1,000 and 11,000 Da. The following mass range settings of the quadrupole (“MS profile”) were used: 3,000 (lower m/z), 5,000 (peak m/z transmission) and 10,000 (upper m/z).
Optimized dwell time settings were used to maximize the intensity of the ion m/z range within a scan, by dwelling on the appropriate quad MS profile. The scan time and interscan times were 1 and 0.1 sec, respectively.

Calibration of the mass spectrometry instrument was performed using caesium iodide (CsI) clusters. CsI was dissolved in water/2-propanol (1/1, v/v) at a concentration of 2 µg/µL.

### 4.3.3 Crystallographic Experiments

The crystallographic experiments were performed using the procedures described in detail in the Table 4.2 and by Hebeisen et al. [211].

**Table 4.2** Crystallographic data collection and structure refinement statistics.

<table>
<thead>
<tr>
<th>Data Collection</th>
<th>PDB ID 4MJO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength (Å)</td>
<td>1.00</td>
</tr>
<tr>
<td>Resolution¹ (Å)</td>
<td>2.4 (2.51 - 2.4)</td>
</tr>
<tr>
<td>Unique reflections²</td>
<td>115242 (13485)</td>
</tr>
<tr>
<td>Completeness (%)¹</td>
<td>96.5 (89.9)</td>
</tr>
<tr>
<td>Rmerge (%)¹</td>
<td>9.7 (39.9)</td>
</tr>
<tr>
<td>&lt;I/σ&gt;¹</td>
<td>12.5 (3.8)</td>
</tr>
<tr>
<td>Space group and unit cell</td>
<td>P2₁ a=65.97Å b=286.31Å</td>
</tr>
<tr>
<td></td>
<td>c=83.69Å and β=97.79°</td>
</tr>
<tr>
<td><strong>Refinement</strong></td>
<td></td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>2.4 (2.462-2.4)</td>
</tr>
<tr>
<td>Rcryst¹,³</td>
<td>21.1 (27.8)</td>
</tr>
<tr>
<td>Rfree¹,⁴</td>
<td>26.7 (36.6)</td>
</tr>
<tr>
<td>Mean B-value (Å²)</td>
<td>31.6</td>
</tr>
<tr>
<td>R.m.s. deviations from ideality</td>
<td>0.011 / 1.4</td>
</tr>
<tr>
<td>Bond lengths (Å) / angles (°)</td>
<td></td>
</tr>
<tr>
<td>Main chain dihedral angles (%)</td>
<td>89.3 / 10.5 / 0.2 / 0.0</td>
</tr>
<tr>
<td>Most favored/allowed/generous/</td>
<td></td>
</tr>
<tr>
<td>disallowed⁵</td>
<td></td>
</tr>
</tbody>
</table>

¹ Values in parentheses refer to the highest resolution bins. [214]
² Rmerge=Σ|I-<(I)|/ΣI where I is intensity.
³ Rcryst=Σ|Fo-Fc|/ΣFo where Fo is the observed and Fc is the calculated structure factor amplitude.
⁴ Rfree was calculated based on 5% of the total data omitted during refinement.
⁵ Calculated with PROCHECK [214]
In brief, the crystal was grown in a 1 μl hanging drop from a mixture of precipitant solution (0.1 M ammonium acetate and 12% polyethylene glycol 3350 in 0.1 M HEPES, pH 7) with human liver FBPase preincubated with the inhibitor. Harvested crystals were flash cooled (using glycerol as cryo protectant) and data was collected at the Swiss Light Source (SLS) beam line X10SA. Standard crystallographic programs from the CCP4 software suite [215] were used to refine the x-ray structure. The atomic coordinates were deposited at the Protein Data Bank with the PDB ID 4MJO.

### 4.3.4 Data Processing

Before data processing, each mass spectrum was smoothed (Savitzky-Golay smooth) with the MassLynx 4.0 software. In order to obtain a good signal-to-noise ratio, at least 50 scans were accumulated for one spectrum. The ratios of the measured peak heights of the ligand-bound protein (PL<sub>n</sub>) to the sum of the bare protein (P) and (PL<sub>n</sub>) were calculated for each spectrum:

\[
\frac{\text{Bound}}{\text{Total}} = \frac{[\text{PL}_n]}{[P] + [\text{PL}_n]} \tag{4.1}
\]

The experimentally determined ratios were plotted against the total added inhibitor concentration (L₀), which was necessary to reach full complexation. For the quantitative determination of binding constants (K₀) and Hill coefficient (n) the Hill equation was applied. For our particular model system, it reads:

\[
\frac{\text{Bound}}{\text{Total}} = \frac{[L]^n}{K_D + [L]^n} \tag{4.2}
\]

When fitting titration curves, it first was assumed that added ligand concentration (L₀) is equal to (L). For the extraction of the ligand concentration (L) Mathematica (V5.2, Wolfram Research, Oxfordshire, UK) was used. We have used the following model system for the extraction of (L):

\[
P + 4L \rightleftharpoons PL_4 \tag{4.3}
\]

\[
[L_0] = [L] + 4[PL_4] \tag{4.4}
\]

\[
[P_0] = [P] + [PL_4] \tag{4.5}
\]

\[
K_D = \frac{[L]^4[P]}{[PL_4]} \tag{4.6}
\]
By simultaneously solving Eqns (4.4) to (4.6) we received the equation:

\[
K_D = \frac{[L]^4([L]-[L_0]+4[P_0])}{-[L]+[L_0]}
\]  
(4.7)

For a set of given parameters, five solutions for (L) exist. The procedure we applied was to sort out all impossible solutions containing, e.g. imaginary numbers or concentrations higher than the maximal possible ones. As result, a single solution remains. In order to calculate K_D, titration curves were fitted using MATLAB (2010a, The MathWorks, Natick, MA, USA).
4.4 Results and Discussion

4.4.1 NanoESI-MS Analysis of FBPase

To preserve the tetrameric form of FBPase and of the ligand complex, appropriate instrumental conditions had to be set in order to avoid in-source dissociation. Not adequately adjusted instrument parameters would lead to determination of lower binding strengths. The pressures in the source and analyzer region of the instrument have a big influence on detection of intact noncovalent interactions. The pressure in the source was increased to 5.5 mbar, using a Speedivalve (Edwards Ltd., Sussex, U.K.) connected between the rotary pump and source pumping line.

Most of the common buffers used for protein purification and storage are not compatible with ESI-MS and lead to severe ion suppression and peak broadening. Therefore a buffer exchange was conducted prior to analysis. [202] For the investigation of noncovalent complexes by nanoESI-MS, aqueous ammonium acetate solutions, with limited amounts of non-volatile salts or detergents, are often used. The stability of FBPase and the resolution and S/N of nanoESI spectra were higher at higher buffer concentrations, therefore we decided to use 500 mM ammonium acetate, which was eventually used in all measurements.

Prior to any addition of small molecule inhibitors, homotetrameric human FBPase was analyzed under “native” and denaturing conditions. Figure 4.1 shows an example of nanoESI mass spectra for a solution of denatured and native FBPase. In the first spectrum, the broad charge state distribution that appears at a lower m/z range indicates the presence of completely unfolded monomer. In contrast, Figure 4.1b displays the spectrum under non-denaturing buffer conditions at pH = 7.5.

The narrow charge state distribution, predominantly 22+, 23+, 24+, is characteristic for native conditions, and is consistent with a compact conformation. Many studies have demonstrated that the charge state distribution depends on the protein conformation in solution. [34, 216] This conformational analysis of the enzyme by ESI-MS is important, since proper folding is crucial for ligand binding in solution. The consideration of any possible conformational difference of inhibitor-bound and unbound enzyme is of importance as well.
The pH also plays an important role in protein activity, e.g., it was already reported by other biophysical methods that at neutral pH FBPase should only exist as a tetramer of four identical monomers. [217]

![Fig. 4.1](image)

**Fig. 4.1** a.) NanoESI mass spectra of FBPase showing A. unfolded monomer measured under denaturing conditions and b.) native intact tetramer using non-denaturing buffer; * indicates an impurity.

This could be confirmed by the nanoESI spectrum: FBPase was exclusively detected as an intact tetramer: no monomers, dimers or trimers were observed.

### 4.4.2 Binding of Small Molecule Inhibitors to FBPase

The sulfonylurea class of small molecules has previously been identified as potent inhibitors of FBPase. In our study we investigated five different inhibitors, that are based on (hetero)aromatic bioisosters of aminothiazoles. [210]

The addition of ligand 2 to tetrameric FBPase was monitored by nanoESI-MS in 500 mM ammonium acetate solution (**Figure 4.2**). Upon addition of ligand 2 no shift of the charge state distribution is observed in the spectrum, the 22+, 23+ and 24+ ions are still observed. The spectrum shows that FBPase forms a noncovalent complex by binding four ligands. Interestingly, even upon addition of small amounts of the ligand (2.5 μM) no ligation states with one, two or three ligands were detected. We assume that due to the insufficient desolvation of the ions formed, the resolution of the signals of different ligation states is not high enough. This assumption could be confirmed in the case of ligand 3, which has a higher molecular weight compared to the other four investigated ligands and therefore the ligation states could just be resolved.
Fig. 4.2 Representative nanoESI mass spectra of 2.8 μM FBPase tetramer (empty circles) in complex with 4 equivalents of ligand 2 (filled circles) obtained in positive ion mode under "native" conditions. Three titration curves are shown adding different ligand concentration to the FBPase a.) 5 μM b.) 7.5 μM c.) 10 μM. The signal for the noncovalent complex clearly increases with increasing amount of ligand present in solution. The full complexion is reached at 15 μM inhibitor concentration.

In the case of the ligand 3-FBPase complex, two different ligation states, with two and four ligands bound (Figure 4.3a), were detected. This observation is consistent with a dimer-of-dimer assembly of the FBPase tetramer. In Figure 4.3d, a new X-ray crystal structure of FBPase tetramer in complex with four molecules of ligand 3 is reported. It clearly shows binding of this ligand in the allosteric AMP sites. The ESI-MS results are in agreement with this structural data, but complement it in the sense that dynamic information is available, and therefore additional insight into the mechanism of ligand 3 binding to FBPase.

The ratio between the two observed ligation states with native MS is an obvious indicator for the positive cooperativity of the investigated system. Since the ligand binding to FBPase is cooperatively regulated, the binding of one ligand will enhance the affinity of the other ligands. The detected positive cooperativity was shown by applying a Hill analysis and estimating the Hill coefficient, and will be discussed in the later section.
**Fig. 4.3** NanoESI-MS spectra for inhibitors 3, 4 and 2 at equal concentration (5 μM) in complex with 2.8 μM FBPase. By calculating the ratio of the complex over the free protein it is possible to rank the binding affinities. a.) R = 1.38; b.) R = 0.39; c.) R = 0.74, which confirmed the binding affinity order $4 < 2 < 3$ by the spectra. In the case of ligand 3, not only the ligation state of four bound ligands, but also of two are observed in the spectrum. d.) Cartoon of the X-ray structure of human liver FBPase tetramer with bound ligand 3 (space filling representation) in the allosteric AMP sites. The four monomeric subunits are colored differently. The inset, rotated by approximately 90° around the y-axis, shows the interaction of inhibitors across the tetramer interface. **X** indicates the four catalytic sites of FBPase; PDB ID 4MJO.

### 4.4.3 $K_D$ and Hill Coefficient Determination of the FBPase-Inhibitor Complexes

In order to determine the dissociation constant via the titration method, a series of nanoESI experiments were performed with increasing inhibitor concentration ranging from 0.5 to 15 μM. **Figure 4.2** displays representative nanoESI spectra obtained for the noncovalent inhibitor 2-FBPase complex using three different ligand concentrations. As expected, increased complex signal intensity was observed with higher total inhibitor concentration. At 15 μM inhibitor concentration, full complexation was reached (data not shown).

The comparison of three example spectra obtained with 2.8 μM FBPase and equal concentration (5 μM) of three different inhibitors are shown in **Figure 4.3**. The spectra represent the interaction of FBPase in complex with the ligand 2, 3 and 4. By performing the experiments at constant concentrations of enzyme and of different small molecules, it is possible to rank binding affinities by calculating the ratio ($R$)
between the complex and free protein. R-values for FBPase complexes with inhibitors 2, 3, and 4 were found to be 0.74, 1.38 and 0.39, respectively, where a higher R stands for a higher binding affinity. The IC\textsubscript{50} values in solution also suggest that ligand 3 has the strongest binding affinity, followed by 2 and 4. [210] Thus, by single-point nanoESI-MS measurement it is possible to rank binding affinities of these three different complexes. It is noteworthy that a longer incubation time of the complex in solution (2 h) gave the same complex to protein ratio (data not shown).

In the next step, titration experiments for all inhibitors over a range of concentrations were carried out. Since all inhibitors are based on the same scaffold, similar behavior of the complexes in the gas phase is expected. Therefore, the conditions optimized for the inhibitor 2-FBPase complex were applied for other complexes. Since the inhibitor-bound protein complex and the bare protein have the similar mass it was also assumed that the ionization efficiencies are equal. The dissociation constants K\textsubscript{D} for investigated complexes were determined by applying a Hill-type analysis (see experimental section). For the K\textsubscript{D} determination it was important to take the abundance of all detected complex and protein ions into account. The second free parameter in this fitting procedure was the Hill coefficient, which describes the cooperativity of binding with FBPase.

In the first step, the Hill equation was applied under the assumption that the added inhibitor concentration (L\textsubscript{0}) is equal to the ligand concentration in equilibrium (L). This assumption allows a very rapid estimation of binding strengths. Five different titration curves of the complexes are shown in Figure 4.4. The signal ratio of the detected complex and the sum of the free protein- and the complex-signal was plotted against the total ligand concentration (L\textsubscript{0}). All titration curves showed a typical sigmoidal shape indicating positive cooperativity.

The K\textsubscript{D}- and IC\textsubscript{50}-values for five different complexes are given in Table 4.3. Their inhibitor potency toward FBPase is ranging from K\textsubscript{D} = 3.75 μM to 8.3 μM. The assumption L\textsubscript{0}=L allows fast determination of binding affinities and makes it suitable for ranking the K\textsubscript{D}-values.
Fig. 4.4 Titration curves of five different FBPase complexes. Here it was assumed that $L_0 = L$. In order to determine the $K_d$ the curves were fitted applying Hill equation.

Table 4.3 List of $K_d$ values and Hill coefficients ($n$) for five inhibitors in complex with FBPase determined via nanoESI-MS titration method. $IC_{50}$ values determined in solution are listed for comparison.

<table>
<thead>
<tr>
<th>Number</th>
<th>$IC_{50}$ [$\mu$M]</th>
<th>$K_d$ [$\mu$M]$^{a,b}$</th>
<th>$n^{a,b}$</th>
<th>$K_d$ [$\mu$M]$^{b,c}$</th>
<th>$n^{b,c}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.33</td>
<td>3.55 ± 0.38</td>
<td>3.82 ± 1.65</td>
<td>6.66 ± 1.35</td>
<td>2.28 ± 1.0</td>
</tr>
<tr>
<td>2</td>
<td>0.14</td>
<td>1.47 ± 0.14</td>
<td>3.10 ± 1.02</td>
<td>6.0 ± 0.85</td>
<td>2.1 ± 0.6</td>
</tr>
<tr>
<td>3</td>
<td>0.05</td>
<td>0.60 ± 0.03</td>
<td>5.59 ± 1.60</td>
<td>3.75 ± 0.56</td>
<td>2.32 ± 0.93</td>
</tr>
<tr>
<td>4</td>
<td>1.66</td>
<td>4.49 ± 0.29</td>
<td>4.14 ± 1.37</td>
<td>8.38 ± 1.05</td>
<td>2.5 ± 1.0</td>
</tr>
<tr>
<td>5</td>
<td>0.53</td>
<td>4.24 ± 0.37</td>
<td>3.79 ± 1.50</td>
<td>7.6 ± 0.98</td>
<td>2.28 ± 0.78</td>
</tr>
</tbody>
</table>

$^a$ Data for extracted $L$

$^b$ Data for assumption $L=L_0$

$^c$ The error is given as the standard deviation calculated from three different measurements and is based on 95% confidence interval
More accurate binding affinities were determined by extracting the ligand concentration in equilibrium. The titration curves with computed [L] from the data are shown in Figure 4.5. The larger slope of the titration curves compared to the previous curves reflects a higher binding affinity. The \(K_{dS}\) determined by calculation of the ligand concentration in equilibrium are two to six times smaller than without extraction.

![Figure 4.5](image)

**Fig. 4.5** Titration curves obtained for five different FBPase complexes. The added ligand concentration was varied until full complexation was reached, while the FBPase concentration was kept constant. The curves were fitted with the Hill equation, whereby the \(L\) in equilibrium was extracted.

Compared to the \(IC_{50}\) values measured by kinetic inhibition assay in solution, the \(K_d\) values based on the ESI-MS measurements suggest up to one order of magnitude
lower binding affinity. Although the relative values for $K_D$ and $IC_{50}$ correlate well for the different complexes.

The Hill coefficient ($n$) determined by curve fitting reflect the extent of cooperativity among multiple ligand binding sites and open a possibility to estimate the number of binding sites. For the complexes studied here, Hill coefficient should in an ideal case be 4. Assuming that $L_0 = L$, $n$ clearly indicates a positive cooperativity for all measured complexes ($n = 2.1-2.5$). When using the calculated ligand concentration, the Hill coefficients are higher and range from 3.1-5.59, which matches the 4 FBPase ligand-binding sites. Except for one overestimated Hill coefficient of 5.59, all other coefficients are slightly underestimated. It is known that even in the case of marked positive cooperativity, the Hill coefficient provides only a minimum estimate of the number of binding sites. [218] This is, for example, the case for the binding of four oxygen molecules to haemoglobin, where a Hill coefficient between 1.7 and 3.2 was obtained. [219] Other studies also reported very similar results to ours. Adams et al. determined a Hill coefficient of 2.4 for the inhibition of FBPase by adenosine monophosphate (AMP) using a spectrophotometric assay. [217] Other studies give values between $n = 2.2$ and $n = 3.3$ for the same system. [220-223] The nanoESI-MS approach thus allows one to deduce the cooperativity of the system, which proves to be positive in the case of FBPase.

### 4.4.4 Monitoring Effect of Substrate Binding to the Complex

FBPase is inhibited allosterically by AMP and competitively by F-2,6P2. [224-226] It was reported in other studies that F-2,6P2 synergistically increases the binding affinity of the inhibitor. For example, the binding of AMP is up to an order of magnitude higher in the presence of F-2,6P2. [220, 223, 227] We have used native MS to directly monitor the modulation of inhibitor binding to FBPase in the presence of the natural ligand.

Experiments with the ligand 2-FBPase complex in the presence of the natural substrate site inhibitor F-2,6P2 were performed. In Figure 4.6 the spectra in the absence and in the presence of 10 μM F-2,6P2 are shown. The mass shift of the complex relative to the bare protein tetramer corresponds to the Δm from both the ligand 2 and the F-2,6P2. The signal for the ligand 2-FBPase complex is about three
times higher, and clearly shows a higher binding affinity in the presence of the substrate in solution. Measured IC₅₀ values in solution for FBPase-2 complex in the presence of F-2,6P₂ also show two-fold higher potency of compound 2. [210]

![Diagram](image)

**Fig. 4.6** NanoESI mass spectra of 2.8 μM FBPase in complex with 2.5 μM ligand 2 in the presence a.) of 10 μM and in the absence b.) of substrate F-2,6 P₂.

We could show that the native MS approach is suitable to directly monitor not only the binding of an inhibitor to multiple allostERIC binding sites of tetrameric FBPase, but also enhancement of the natural substrate on inhibitor binding to FBPase.
4.5 Conclusions

Understanding allosteric mechanisms of target proteins is an important aspect in drug discovery. In this work we have investigated the allosteric regulation of inhibitors binding to an oligomeric enzyme using nanoESI-MS. The investigated protein was tetrameric FBPase protein, a potential target for glucose control in type-2 diabetes. To our knowledge, this is the first application of native mass spectrometry to this therapeutic target and determination of ligand binding affinities to a cooperatively regulated enzyme.

For $K_D$ determination, a series of titration experiments was conducted, and the Hill equation was applied. The nanoESI based $K_D$ values are in good agreement with $IC_{50}$ values measured in solution. In addition, the Hill coefficients obtained show positive cooperativity of this system. These results are in very good agreement with Hill coefficients obtained by other techniques. The effect of the natural substrate competitor F-2,6P$_2$ on synthetic inhibitor binding to FBPase was monitored. The obtained inhibitor-FBPase complex signal was about three times higher in the presence of F-2,6P$_2$, indicating enhancement of binding affinity of the inhibitor.

Other nanoESI-based results also match the current understanding of the investigated system in terms of conformation and stoichiometry, which is in agreement with the X-ray structural data of ligand 3 in complex with FBPase. Compared to the X-ray structure better dynamic resolution of the MS technology was shown, since not only the ligation state of four bound ligands, but also of two are observed in the spectrum.

The aim of the study was to highlight the benefits of nanoESI-MS as a method of choice or as a complementary technique to gain deeper understanding of allosteric mechanisms for therapeutic drug targets. It is fast, label-free, and sensitive method that readily allows not only the determination of dissociation constants, but also provides information on stoichiometry, conformational changes and cooperativity which is in sharp contrast to conventional methods such as enzymatic inhibition assays with labelled substrates. We believe that this study may be used as a basis for future investigation of other important cooperatively regulated drug targets.
Chapter 5

5 Influence of Dimethylsulfoxide on Protein-Ligand Binding Affinities

This chapter is adapted from and reprinted with permission from:

5.1 Overview

Because of its favorable physicochemical properties, DMSO is the standard solvent for sample storage and handling of compounds in drug discovery. To date, little attention was given to how DMSO influences protein–ligand binding strengths. In this study we investigated the effects of DMSO on different noncovalent protein–ligand complexes, in particular in terms of the binding affinities, which we determined using nanoESI-MS. For the investigation, three different protein–ligand complexes were chosen: trypsin–Pefabloc, lysozyme–tri-N-acetylchitotriose (NAG₃), and carbonic anhydrase–chlorothiazide. The DMSO content in the nanoESI buffer was increased systematically from 0.5 to 8 %. For all three-model systems, it was shown that the binding affinity decreases upon addition of DMSO. Even 0.5–1 % DMSO alters the $K_D$ values, in particular for the tight binding system carbonic anhydrase–chlorothiazide. The determined dissociation constant ($K_D$) is up to 10 times higher than for a DMSO-free sample in the case of carbonic anhydrase–chlorothiazide binding. For the trypsin–Pefabloc and lysozyme–NAG₃ complexes, the dissociation constants are 7 and 3 times larger, respectively, in the presence of DMSO. This work emphasizes the importance of effects of DMSO as a co-solvent for quantification of protein–ligand binding strengths in the early stages of drug discovery.

5.2 Introduction

The discovery and characterization of protein-ligand interactions (e.g., with other proteins, carbohydrates, lipids, DNA, or small molecules) is crucial for understanding biochemical reactions and pathways as well as for design of new therapeutics for treatment of different human diseases. [5]

High-throughput screening (HTS) is a major tool for drug discovery to investigate large numbers of pharmaceutical compounds against a target. Since the compound screening process has to be automated, the success depends on the content and quality of the chemical library, the choice of the screening assay, etc. [228] Dimethyl sulfoxide (DMSO) is an important and frequently used solvent for sample storage and handling in the initial stages of HTS in drug discovery. This powerful dipolar solvent has favorable properties in terms of dissolving ability, low chemical
reactivity, relatively low toxicity, and low vapor pressure. [228] The DMSO concentration used as a solvent in HTS varies from 1 to 10 % (v/v). [229, 230] We therefore asked the question how different DMSO contents influence protein-ligand binding strengths, which were determined here with the nano electrospray ionization-mass spectrometry (nanoESI-MS) titration method. [4, 39, 190, 228] To date, little attention was given to which extent the addition of DMSO would influence the measured binding affinity of noncovalent complexes. To our knowledge, only one group has investigated noncovalent complexes in the presence of 0.1–1 % DMSO. [228] Therefore, there is a clear need to understand the effects of DMSO as a co-solvent for quantification of protein-ligand binding strengths in the early stages of drug discovery. In the last years, an increasing number of research groups have become active in this field. ESI-MS based drug discovery in particular is becoming more important for the investigation of protein-ligand interactions and determination of binding affinities. [69, 168] Binding affinities have been successfully determined by ESI-MS for a variety of noncovalent protein-ligand complexes. [35, 54] The advantages of the nanoESI-MS method for studying the effects of DMSO on protein-ligand interactions are the direct visualization of ligation states and of conformational changes of the protein. Other commonly used techniques for quantification of protein-ligand binding in vitro, such as surface plasmon resonance (SPR) spectroscopy, isothermal titration calorimetry (ITC), enzyme-linked immunosorbent assays, or nuclear magnetic resonance spectroscopy. Each of these analytical methods has particular strengths and weaknesses. Compared to the above-mentioned standard methods, the ESI-MS assay has advantages in terms of simplicity (label-free measurements), selectivity (possibility of using additional stages of MS combined with ion activation methods), sensitivity (low sample consumption), and speed (mass spectra can be acquired in less than a minute). [61-65]

It has already been shown that the effects of DMSO on protein structure and function can be extremely varied since it can act as an stabilizer, a denaturant, an activator, as an inhibitor, or as a cryoprotector. [231] In a recent study, it has been shown that addition of DMSO results in destabilization of the investigated proteins and can also change the binding property of the protein. [228] Yang et al. have investigated the unfolding mechanism of dimeric bacterial NAD$^+$ synthetase using
differential scanning calorimetry in the presence of 2.5 % DMSO. Although no effect on the enzyme catalytic activity was observed, 2.5 % DMSO already led to changes in the dimer stability to and its partial unfolding. [232]

Therefore using DMSO as co-solvent can give misleading results in the drug discovery process. We note that in most other structural biology-type studies, the DMSO concentration used for protein characterization was between 10 % and 70 %, which is significantly higher than the concentration used for HTS in drug discovery. [231, 233, 234]

For the investigation of DMSO related effects, three different noncovalent protein-ligand complexes were chosen: trypsin-Pefabloc, lysozyme-NAG₃, and carbonic anhydrase-chlorothiazide. This study involves measurement of dissociation constants (Kₒ) of ligands to the proteins in the absence and presence of different DMSO amounts. Depending on the measured complex, the DMSO amount was increased systematically from 0.5 to 8.0 %. In the first step, the Kₒ values were measured without DMSO and compared to reference Kₒs. The determined Kₒs were found to be in good agreement compared to the reference Kₒ values measured in solution.

Upon addition of DMSO, we found that the binding affinity decreases for all three investigated model systems. The determined Kₒ values based on nanoESI-MS are up to 10 times higher compared to the DMSO-free sample for carbonic anhydrase–chlorothiazide binding and 7 and 3 times higher for the trypsin-Pefabloc and lysozyme-NAG₃ complexes. This study thus highlights the dependence of the relative binding affinities of noncovalent complexes on DMSO concentration and the importance of these effects in the early stages of drug discovery. Our results can serve as a basis for a better understanding of changes of binding affinities of many other protein-ligand complexes upon addition of DMSO.
5.3 Experimental Section

5.3.1 Materials and Methods

Bovine pancreas α-trypsin, bovine carbonic anhydrase II (CA), chicken egg white lysozyme, ammonium acetate, and CsI were purchased from Sigma Aldrich (Buchs, Switzerland). Pefabloc TH was obtained from Biochemica (Düsseldorf, Germany), NAG₃ from Carbosynth Ltd. (Berkshire, UK) and chlorothiazide from Enzo Life Science (Lausen, Switzerland). DMSO was obtained from Fluka Chemie AG. Water was purified using a Milli-Q® Ultrapure water purification system (Millipore, Barnstead, USA). All MS titration experiments were carried out with “native-like” conditions, using 10-20 mM ammonium acetate buffer at pH=7-7.4. Stock solutions of inhibitors were prepared at 100-300 mM concentration in the buffer solution. Prior to mass spectrometric analysis the protein stock solution was desalted and buffer exchanged (PD MiniTrap G-25, GE Healthcare, Buckinghamshire, UK) with the non-denaturing buffer. The exact protein concentration was determined using a UV spectrometer (Genesys 10S UV-Vis, ThermoScientific, Bremen, Germany) by measuring the absorbance at 280 nm.

5.3.2 Mass Spectrometry

ESI spectra were acquired with a hybrid quadrupole time-of-flight mass spectrometer (Q-TOF ULTIMA, Waters/Micromass, Manchester, UK) in positive ion mode. The instrument was controlled via the MassLynx version 4.0 software. Ions were formed using nanoflow platinum-coated borosilicate electrospray capillaries (Proxeon, Odense, Denmark). For all nanoESI-MS measurements the capillary voltage was set to 2.1 kV and a gentle backing pressure of 0.3 bar on the spray tip was used to assist the liquid sample flow. The source temperature was kept between 21 °C and 35 °C.

In order to prevent dissociation, all noncovalent complexes were measured under gentle desolvation parameters. Desolvation must be sufficiently complete in order to get narrow peaks for the detected species but not so harsh as to dissociate the noncovalent complex. The choice of desolvation condition will influence peak widths, due to adduct formation with salt and buffer molecules from the spray solution. The cone voltage was kept at 40-60 V for all measurements. The RF1
voltage, the potential applied to the first ion tunnel in the linear flight path before the quadrupole, was varied from 35-60 V. After this stage, the ion beam passed a hexapole collision cell filled with argon (Purity 5.0, PanGas) as a collision gas. The collision energy offset was used to optimize desolvation and was set to 10-15 V. Each mass spectrum was acquired for 2-3 minutes in order to get a good signal-to-noise ratio. The mass spectrometer instrument was calibrated using caesium iodide (CsI) clusters. CsI was dissolved in water/2-propanol (1/1, v/v) at a concentration of 2 μg/μL.

5.3.3 Data Processing

Before data processing, each mass spectrum was smoothed (Savitzky-Gollay smooth) with the MassLynx 4.0 software (Waters, UK). For the $K_D$ determination the measured relative peak heights ($I$) were used. The ratio ($R$) of the ligand-bound protein (P·L) to bare protein (P), $R = I (P·L) / I (P)$, was calculated for each spectrum. For this calculation, all charge states were taken into account. We used two equations that are based on the same binding model system to determine the $K_D$s from the relative peak ratios. In the first case, experimentally calculated $R$-values were plotted versus the total inhibitor concentration. The equation derived by Daniel et al. [191] was then used to determine the dissociation constant ($K_D$) from a fit to the titration curve.

The second approach is based on a single-point ESI-MS measurement for calculating the $K_D$s using the following equation: [235]

$$\frac{1}{K_D} = K_A = \frac{R}{[L]_0 - \frac{R}{1+R}[P]_0} \quad (5.1)$$

In both cases, it was assumed that the ionization efficiencies for the bare protein and the complex are equal, which allowed us to use the intensity ratios of free protein over complex instead of their concentrations. The $K_D$ calculations and the fitting of the titration curves were performed using the MATLAB software (2010a, The MathWorks, Natick, MA, USA).
Titration curve fitting as well as the single-point determination led to equal $K_D$s (tested for lysozyme-NAG$_3$ and carbonic anhydrase-CTA binding affinities). Due to its simplicity, we decided to use the single-point method to determine binding affinities.
5.4 Results and Discussion

5.4.1 Lysozyme-NAG₃ Binding

Lysozyme is a well known and thoroughly studied protein, which catalyses the hydrolysis of beta-1,4-glycosidic linkages in some gram-positive bacterial walls. [236, 237] Prior to any addition of DMSO, the lysozyme-NAG₃ complex was analyzed under “near-native” conditions in aqueous 20 mM ammonium acetate solution at pH = 7. We first determined the dissociation constant in the absence of DMSO and validated our method by comparing the dissociation constant with reference values. Figure 5.1 shows nanoESI mass spectra for solutions containing the lysozyme-NAG₃ complex. A narrow charge state distribution, predominantly 7+ (m/z= 2043.56), 8+ (m/z= 1788.30) ions, appears at fairly high m/z range, characteristic for native conditions, and consistent with a compact conformation of lysozyme in solution.

![Fig. 5.1 Representative nanoESI mass spectra of 4.5 μM lysozyme in complex with NAG₃ obtained under “near native” conditions (20 mM AmAc, pH = 7). Lysozyme (Lys) was titrated with different NAG₃ concentrations a.) 2.5 μM b.) 10 μM c.) 12.5 μM d.) 17.5 μM.](image)

Also lysozyme remains disulfide intact since the calculated mass of 7+, 8+ ions (m/z = 2043.57 and 1788.13) is consistent with the observed. Higher charge states and also broader charge states distributions are generated from solutions in which protein denaturation has occurred. [216, 238, 239] Many studies have demonstrated that the charge state distribution correlates with the protein conformation in solution. [240]
Chapter 5 – Influence of Dimethylsulfoxide on Protein-Ligand Binding Affinities

For the determination of the \( K_D \) values via the titration method, a set of nanoESI experiments were performed at different NAG\(_3\) and constant lysozyme concentrations. **Figure 5.1a-d** displays representative mass spectra obtained for the noncovalent complex at four different ligand concentrations. The complex peak increases with higher total NAG\(_3\) concentration. In order to determine the \( K_D \) it is important to take the abundance of all detected complex and protein ions into account. In case of the lysozyme-NAG\(_3\) complex, it was previously reported that the \( K_D \) is charge state dependent. [80] However, in our case, the detected intensity ratio of complex and free protein ions was found to be virtually identical for all charge states. The \( K_D \) determined by a set of titration experiments was 9.24 \( \mu \)M. This value is in agreement with other values determined for lysozyme binding NAG\(_3\) in solution. According to the literature, \( K_D \) values of 8.6 \( \mu \)M using fluorescence, 6 \( \mu \)M using diffusion MS and 10 \( \mu \)M using nanoESI-MS were obtained. [74, 109, 241]

Since we could reproduce literature \( K_D \)S and prove the ability of nanoESI-MS to measure an accurate binding affinity for the Lys-NAG\(_3\) complex, we next investigated the influence of DMSO on the protein-ligand interaction. The DMSO amounts added to the nanoESI solution were 1, 2, 3, 4, 5, 7 and 8 % (v/v). **Figure 5.2** shows spectra measured at the same protein and ligand concentrations but in the presence of different amounts of DMSO.

DMSO has two effects on the investigated complex, a shift towards higher charges and a decrease of the relative intensity of the complex signal. In the absence of DMSO, the dominant charge state is +7 followed by +8. In the presence of 3 % DMSO in the ESI solution, the charge state envelope shifts towards higher charge state (lower m/z), within 8+ being the predominant signal. Upon addition of 8 %, DMSO +10 ions are additionally generated. At this stage it is important to mention that upon addition of DMSO, the pH of the nanoESI solution remains unchanged. This shift in charge state upon adding DMSO is already well known, for example, DMSO has been used as a supercharging reagent. [240, 242, 243] It is proposed that the increase in charge, observed for some proteins, is due in part to unfolding in the final stages in the electrospray droplet. [244] Using m-nitrobenzyl alcohol (m-NBA), another well-known supercharging agent, it has been shown that production of higher charge states does not result in loss of protein-ligand interactions, indicating
that the protein is in a near native condition and its ligand-binding cavity is intact. [245] There is also strong evidence that folded and compact proteins can survive in the gas phase, at least on a millisecond time scale. [246] However, the effect of charge state on conformation of proteins is still a hotly debated issue. [240]

**Fig. 5.2** NanoESI-MS spectra for Lys-NAG₃ complex measured under the same ligand (10 μM) and protein concentration (4.5 μM) with different amounts of DMSO added: a.) 0 % DMSO b.) 3 % DMSO c.) 5 % DMSO d.) 8 % DMSO. The ratio of complex to bare protein decreases from a to d.

In case of the Lys-NAG₃ complex, a slight shift towards higher charge can be detected in the presence of DMSO. No denaturation of the protein is observed, which would show up in the form of signals in the lower m/z range.

The spectra in **Figure 5.2** show a second effect of DMSO on the lysozyme-NAG₃ complex: the decrease of relative complex abundance with higher DMSO amount. The complex ratio in the presence of 3 % DMSO decreases by about 45 % compared to a DMSO free sample. By adding 8 % DMSO, the complex abundance is half of that without DMSO.

The Kₛₛ for all lysozyme-NAG₃ complexes were determined as described in the “Data Evaluation” section. Two different titration curves, in the absence and in the presence of 7 % DMSO are displayed in **Figure 5.3**. A larger slope reflects a higher binding affinity and *vice versa*. 
Fig. 5.3 NanoESI-MS titration curves for lysozyme-NAG\textsubscript{3} complex in absence and presence of 7 \% DMSO. The ligand concentration ranges from 2.5 to 17.5 \( \mu \text{M} \), while the protein concentration was kept constant.

5.4.2 Trypsin-Pefabloc Binding

Bovine \( \alpha \)-trypsin belongs to the family of the well-known serine proteases. Inhibitors that specifically bind to these serine proteases can be used for the treatment of different diseases. For example, in recent years, the first orally available thrombin and factor Xa inhibitors were approved as anticoagulants and are of high interest in drug discovery. [185, 186] Representative mass spectra of the noncovalent trypsin-Pefabloc complex in the absence and in the presence of different DMSO amounts are shown in Figure 5.4. The most abundant charge state for the DMSO-free solution is +8 and 9+, where +8 is the most intense signal. The calculated mass for observed ions is +8 and 9+ is m/z = 2917 and 2594.09 (mass based also on sodium and ammonia adducts) is consistent with the expected mass. The measured dissociation constants for trypsin-Pefabloc are given in Table 5.1. The \( K_\text{D} = 0.59 \, \mu \text{M} \) for the complex without DMSO is in good agreement with that found in solution, 0.69 \( \mu \text{M} \). [192]
Addition of only 0.5 % DMSO causes the charge states +8 and +9 to be equally intense. For the spectra obtained in the presence of 2 % and 8 % DMSO, the predominant signal is charge state +9, while the intensity of +8 charge state decreases with higher DMSO content. As in the case of lysozyme-NAG₃, the charge state envelope is shifted towards higher values, with +10 ions being generated at a DMSO concentration of 0.5 %. Our results also show higher dissociation constants upon addition of DMSO e.g. upon addition of DMSO the Kᵦ value is up to 7 times larger. This will be discussed in detail below.

### 5.4.3 Carbonic Anhydrase-CTA Binding

Carbonic anhydrase catalyzes the hydration of carbon dioxide to carbonic acid. It has a divalent zinc ion that is a noncovalently attached co-factor and essential for catalytic activity. [247] Figure 5.5a provides an example of a nanoESI mass spectrum for a solution of 4 µM carbonic anhydrase in the presence of 2 µM CTA in 10 mM aqueous ammonium acetate solution under native conditions (pH = 7.4). As can be seen on the spectra, the +9 at m/z = 2914.3 and +10 charge states at m/z = 3237.1 are generated and in agreement with expected mass. In order to determine the dissociation constant without DMSO, a set of nanoESI experiments were performed at different inhibitor concentrations. As expected, the ratio of the complex to free
protein ion signals increases with higher total inhibitor concentration. The
dissociation constant was determined to be 0.058 μM, in good agreement with the
reference value of 0.075 μM. [248]

![NanoESI mass spectra of 4 μM carbonic anhydrase (CA) in complex with 2 μM CTA
obtained under “native” conditions (10 mM ammonium acetate, pH = 7.4).](image)
a.) without and b.) upon addition of 3 % DMSO. In the presence of DMSO, two protein peaks appear, with
and without coordinated zinc.

In the next set of experiments, the DMSO amount was varied between 1 and 5 %.
**Figure 5.5b** shows a spectrum of carbonic-anhydrase-CTA complex in the presence of
3 % DMSO. Unlike in the case of lysozyme-NAG₃ and trypsin-Pefabloc binding, no
charge state shift compared to the spectrum without DMSO is observed.
Interestingly, two different peaks of the bare protein are detected, carbonic
anhydrase with and without zinc bound. It is important to mention that both the
protein ions with and without zinc should be taken into account to determine the
dissociation constant; otherwise the results are not consistent.
5.4.4 Influence of DMSO on the $K_D$ of the Investigated Complexes

In Figure 5.6 the $K_D$s determined are plotted against the added DMSO percentage. A monotonic increase of the measured dissociation constant is observed with higher DMSO concentration for all three investigated complexes. The determined $K_D$ values based on nanoESI measurements measured in the absence and at six different DMSO concentrations are shown in Table 5.1.

![Fig. 5.6 Dissociation constant for the three investigated complexes plotted against added DMSO amount.](image)

The $K_D$s for the lysozyme-NAG$_3$ complex are in the range of 9 μM to 33 μM. The $K_D$ values increases by only 2 μM when going from 0 to 1 % DMSO. When adding 2, 3, 5, 7 and 8 % DMSO, the $K_D$ values increase each time by about 5 μM.

<table>
<thead>
<tr>
<th>DMSO %</th>
<th>$K_D$ [μM] by nanoESI-MS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lys+NAG$_3$</td>
</tr>
<tr>
<td>0</td>
<td>9.24 ± 0.58</td>
</tr>
<tr>
<td>0.5</td>
<td>1.14 ± 0.1</td>
</tr>
<tr>
<td>1</td>
<td>11.3 ± 0.3</td>
</tr>
<tr>
<td>2</td>
<td>12.9 ± 0.7</td>
</tr>
<tr>
<td>3</td>
<td>17.9 ± 0.3</td>
</tr>
<tr>
<td>5</td>
<td>23.5 ± 0.5</td>
</tr>
<tr>
<td>7</td>
<td>28.2 ± 0.2</td>
</tr>
<tr>
<td>8</td>
<td>33.3 ± 1</td>
</tr>
</tbody>
</table>

Table 5.1 List of calculated dissociation constants ($K_D$) for lysozyme-NAG$_3$, trypsin-Pefabloc, carbonic anhydrase-CTA complexes in the absence and in the presence of different DMSO amounts, determined by nanoESI titration method. The error is given as the standard deviation calculated from at least three different measurements.

103
In a recent study, the size, shape, structure, and the interactions of lysozyme in the ternary system lysozyme/DMSO/water was reported for DMSO volume fractions ($\phi_{\text{DMSO}}$), ranging from 0 (pure water) to 1 (pure DMSO). [233] Using different biophysical methods, it was shown that lysozyme remains in a folded conformation for $\phi_{\text{DMSO}} < 0.7$ and unfolds above $\phi_{\text{DMSO}} > 0.7$.

In another study, Balaram and co-workers reported that from 20-25 % (v/v) (≈6 % mole fraction) DMSO, lysozyme proceeds slowly from the native to a partially unfolded state. The change in conformation at low DMSO concentrations was interpreted to be a consequence of preferential binding of the organic co-solvent near the active lysozyme site and on the protein surface. [249] A number of other studies using different biophysical methods like rotatory dispersion, optical and infrared spectra, also support this finding, but only at DMSO concentrations higher than 10 % (v/v).[233, 234, 250, 251] Near-UV CD Spectroscopy experiments have shown that lysozyme becomes more compact in solution containing up to 50% DMSO, but unfolds between 50 and 70 % DMSO. [252] In our study, the DMSO concentration does not exceed 8 %. In this range we could not observe denaturation of the protein, just a slight shift towards higher charge state. However, the binding affinity is 3.2 times lower when adding only 8 % DMSO.

In the case of trypsin-Pefabloc binding, a trend of lower binding affinity upon addition of DMSO is also observed (Table 5.1): $K_\text{D}$ increases from 1.1 to 4.3 μM, up to 7.5 times higher than for a DMSO-free solution. It was already reported for the model system human growth hormone receptor and low-molecular mass compound containing 0 to 1 % DMSO that the ability of the enzyme to bind an inhibitor is significantly weakened with increasing amounts of DMSO, even when its enzymatic activity was not affected. [228] The charge state envelope at all measured DMSO amounts was identical, i.e., the decrease in relative complex abundance cannot be explained by further protein destabilization. The authors explained this effect by DMSO acting as a competing ligand. An analogous phenomenon was already reported for of alcohol dehydrogenase. [253, 254] However, the differences of the dissociation constants were not calculated.

As shown in Table 5.1, the $K_\text{D}$ values for carbonic anhydrase-CTA in the presence of 1 and 2 % DMSO are very similar. Also, in the presence of 5 % DMSO no increase in $K_\text{D}$
compared to the solution containing 3 % is observed. Nevertheless, the binding affinity in the presence of DMSO is up to 10.5 times lower compared to DMSO-free solutions. The detected loss of zinc might be an additional reason for the lower binding affinity in the presence of DMSO, since it is required for the enzyme activity. As described elsewhere, the DMSO/water microstructure is strongly dependent on the DMSO volume fraction. This co-solvent acts as a strong H-acceptor. It is a poor solvent for the polar side groups and for the backbone of proteins, although it is a good solvent for relatively apolar side groups, e.g., amino acids with aromatic rings, such as tryptophan. Addition of DMSO can apparently shift the equilibrium between protein and protein-ligand complex and therefore lead to an underestimation of the binding affinity.

The partial loss of protein-ligand interactions can occur due to the destabilization of the protein, destruction of the ligand-binding pocket, DMSO can interfere with the ligand, or it can itself act as a competing inhibitor.

Additional experiments of three proteins with DMSO alone were performed as well in order to exclude the change in charge state distribution. The charge state distribution of three proteins in presence of DMSO is consistent with the measurements of the complexes. Additional figures of three proteins with DMSO alone are shown below.

![Mass spectra](image)

**Fig. 5.7** Representative nanoESI mass spectra of 4.5 µM lysozyme obtained under “near native” conditions (20 mM AmAc, pH = 7 with different amounts of DMSO added a.) 0% DMSO b.) 5 % DMSO c.) 8 % DMSO.
Fig. 5.8 NanoESI mass spectra of 4 μM trypsin (15 mM ammonium acetate, pH = 7) upon addition of DMSO a.) 0 % DMSO b.) 0.5 % DMSO c.) 8 % DMSO.

Fig. 5.9 NanoESI mass spectra of 4 μM carbonic anhydrase (CA). a.) without and upon addition of b.) 3 % DMSO and c.) 5 % DMSO.
Chapter 5 – Influence of Dimethylsulfoxide on Protein-Ligand Binding Affinities

5.5  Conclusions

We have applied nanoESI-MS to investigate the influence of DMSO on binding affinities of protein-ligand complexes. Three different noncovalent complexes were chosen for our study: trypsin-Pefabloc, lysozyme-NAG₃ and carbonic anhydrase-CTA. In the first set of the experiments, we determined $K_D$ values without the addition of DMSO in order to validate our method by comparing them with reference values. In the second set of experiments the DMSO amount was systematically increased up to 8 % (v/v). This DMSO content is close to that used in assays for HTS in drug discovery. In all three model systems studied we could show that DMSO has a significant influence on protein-ligand binding. Even 0.5-1 % DMSO alter the $K_D$ values, in particular for the tight binding system carbonic anhydrase-chlorothiazide. In case of carbonic anhydrase, DMSO has also influence on the zinc coordination. Upon addition of DMSO we observed a monotonic decrease of the binding affinities (increase of $K_D$) for all three investigated complexes. The advantage of using nanoESI-MS is the direct visualisation of the ligation state upon addition of DMSO. This allows one to deduce from only one measurement the protein-ligand binding affinities. The determined dissociation constants ($K_D$s) were up to one order of magnitude higher than for DMSO-free samples. The addition of DMSO can shift the equilibrium between protein and ligand and therefore lead to an underestimation of the binding affinities, which is probably true for cases other than the complexes investigated in this study. We believe, that this trend may be fairly universal because the binding modes in the three models investigated cover the range found in most noncovalent protein-inhibitor complexes.

One should therefore be aware that using DMSO in in the early stages of drug discovery can influence the protein-ligand interactions and therefore lead to erroneous binding affinities.
Chapter 6

6 Direct Monitoring of Protein-Protein Inhibition Using Nano Electrospray Ionization Mass Spectrometry

This chapter is adapted from:

6.1 Overview
Dissociation of the TNF-alpha trimer caused by the small-molecule inhibitor SPD304 was monitored using native ESI-MS and ion mobility spectrometry. Upon addition of inhibitor, our data clearly indicates partial dissociation of the protein into dimers and monomers. The IMS-MS analysis shows that dimeric ions have their own characteristic drift time distributions, which are different from those of the dimer ions originating in the gas phase due to collision-induced dissociation. We show that only one equivalent of the inhibitor binds to the trimeric form. We also investigated inhibition of the heterodimer formation of the survival protein Bcl-xL and cell death-promoting regions of the proteins Bak and Bad, using the small inhibitors ABT737 and ABT263. We found that the ABT737 is more potent compared to ABT263 in preventing the heterodimerization between Bcl-xL with Bak and Bad derived BH3 peptide. We could also monitor the mode of binding, which in this case is competitive. These results indicate that native ESI-MS can be widely used to study the inhibition of other relevant protein-protein interactions (PPIs), and provide a good basis for further improvement and identification of small-molecules PPI inhibitors.

6.2 Introduction
Protein-protein interactions (PPIs) are of fundamental importance in most biological processes - from intercellular function to programmed cell death. [17-19] The controlled disruption of PPIs with small-molecule inhibitors is of high interest in current drug discovery due to the large number of protein-protein interactions involved in signalling pathways related to cancer and many other human diseases. In the last decade, significant progress in the design and development of potential small inhibitors of PPIs has been made. [19, 255, 256] Therefore, understanding mechanisms of protein-protein disruptors can be used in several fields, e.g., in small molecule drug discovery, in order to design and optimize the novel potential therapeutics.

The conventional tools and methodologies for investigating PPIs include physicochemical methods such as X-ray crystallography, NMR spectroscopy, surface
Chapter 6 – Direct Monitoring of Protein-Protein Inhibition Using NanoESI-MS

plasmon resonance (SPR), isothermal titration calorimetry (ITC), fluorescence spectroscopy, or biochemical methods. All these techniques have particular strengths and weaknesses in terms of sample consumption, throughput, dynamic range; some require immobilizing of one of the binding partners. [5, 61, 64, 65, 202] Another powerful and increasingly utilized method to detect and characterize noncovalent interactions is nano electrospray ionization mass spectrometry (nanoESI-MS). [4, 167, 191] It was shown by many research groups that proteins in the gas phase are in a folded conformation, which is similar to the native conformation in solution, and that they are therefore able to bind inhibitors and provide a “snapshot” of the solution phase equilibrium. [39, 190, 203] In recent years, nanoESI-MS has become increasingly used in drug discovery, for the investigation of protein-ligand and protein-protein interactions. [35, 69, 134, 168] This technique can address key questions about composition, stoichiometry, subunit interactions, and architectural organization of noncovalent complexes. [257] The present work emphasizes the advantages of the native MS approach for direct monitoring of protein-protein inhibitions. Pioneering work in the detection of protein-protein interactions inhibition via ESI-MS was carried out by Grygon and co-workers [258] Beside the quantification of protein-protein interactions it offers the possibility to directly visualize ligation states and conformational changes upon addition of small disruptor molecule in solution.

In this work we have also applied ion mobility (IM) spectrometry, which is a gas-phase separation tool comparable to electrophoresis in solution, and which can be combined with MS. This is a technique that allows ions to be separated by a weak electric field in a gas environment according to their mobility. From the ion transport properties measurements, ion size information can be generated. This results in an orientationally averaged ion-neutral collision cross sections (CCS). [158] Recent studies show good correlation of many data sets between CCS values based on IM measurements and X-ray or NMR data sets for the same proteins and complexes in solution. Although these measurements are carried out in the absence of bulk water, these studies suggest that IM data reflects condensed phase properties and can be used as a technique for structural biology. Some of the recent reviews summarizing
the developments of IM-MS to rapidly measure changes in protein structure, oligomeric state, and binding stoichiometry from complex mixtures are by Niu et al., [158] Hall and Robinson [240], Konijnenberg et al. [124].

In this contribution we first investigated the alpha tumour necrosis factor (TNF-alpha), a cytokine involved in systemic inflammation and in immune regulation, and therefore a therapeutic target for many diseases. The known inhibitor SPD304 was used to induce dissociation of the trimeric TNF-alpha, as monitored by nanoESI-MS. [259] In addition we performed ion mobility mass spectrometry (IM-MS) experiments. NanoESI- and IM-MS results are in agreement, and, upon ligand addition, show dissociation of the trimer into dimers and monomers. The IMS-MS analysis shows that dimeric ions have their own characteristic drift time distributions, which are different from dimer ions generated in the gas phase due to CID. Therefore dissociation occurs due to SPD304-promoted dissociation of TNF-alpha trimers in solution. The mode of inhibitor binding to the TNF-alpha was studied as well.

The second system investigated in this study is the interaction between the anti-apoptotic Bcl-2 family protein Bcl-xL and two different pro-apoptotic binding partners, Bak and Bad. The pro-apoptotic has similarity to the anti-apoptotic group in a single alpha helix called the BH3 region, which is essential for binding to Bcl-xL and also required for the proapoptotic effect. [260-265] Heterodimerization between members of the Bcl-2 family proteins plays a key role in the regulation of programmed cell death. In a first step we investigated the heterodimerization between Bcl-xL and the BH3 domain of Bak and Bad derived synthetic peptides, which bind with high affinity in vitro; it had also been shown that the Bak BH3 peptide alone could induce apoptosis in various cell lines. [262] Titration experiments at constant Bcl-xL and different peptide concentrations were first performed using nanoESI-MS. Results are in agreement with these from other biophysical methods. In a second step, we investigated the recently introduced small BH3 mimetic inhibitors ABT737 and ABT263 that are designed to disrupt the above-mentioned cancer-linked protein–protein interactions. These small-molecule inhibitors have been found to occupy the BH3 binding groove of anti-apoptotic Bcl-2
family members, preventing them to antagonize pro-apoptotic proteins and induce apoptosis, thereby enhancing programmed cell death of cancer. [261, 266] NanoESI-based results show that ABT737 prevent the heterodimerization of Bcl-xL-Bak as well as Bcl-xL-Bad binding more efficiently compared to ABT263. We also observed competition of the small molecule inhibitors with the BH3 derived peptide for the same Bcl-xL binding pocket, clearly indicating the mechanism of binding.

All nanoESI-MS based results obtained show that this technique is a valuable tool for investigation of PPI inhibition. In addition to the quantification of binding strengths of PPIs, we could gain information about stoichiometry, conformational changes, binding mechanism, and relative binding strengths of the small PPI inhibitors from single-point measurements. Key advantages of native MS are its simplicity (label-free measurements), selectivity (possibility of using additional stages of MS combined with ion activation methods), sensitivity (low sample consumption), and speed (mass spectra can be acquired in less than a minute).
6.3 Experimental Section

6.3.1 Materials and Methods

All solvents and caesium iodide (CsI) were purchased from Sigma Aldrich (Buchs, Switzerland). The pET29 plasmid bearing the coding sequence of Bcl-x\(_L\) (amino acids 1-209, A45-84) was a kind gift from Prof. Ho Sup Yoon (Nanyang Technical University, Singapore). [264] The Bcl-x\(_L\) protein expression has been previously described. [260] The expression and purification protocol of TNF-alpha (A.Corti) [267] was shortened and optimized by introduction of the N-terminal (His)\(_\theta\) tag. This allows for use of a Ni-NTA affinity purification step that significantly shortens the entire purification protocol. Owing to the Ni-NTA step, troublesome and time-consuming hydrophobic chromatography and desalting at 65% ammonium sulphate steps can be skipped. This results in a higher yield of purified protein; the His-tag also allows immobilizing TNF on different media (e.g. BiaCore chip or Ni-NTA beads). The BH3 peptide domains of the Bad (NLWAAQRYGRELRRMSDK) and the Bak protein (GQVGRQALIIGDDINR) were obtained from Genscript (NJ, USA) and Anaspec (Fremont, USA), respectively. The small-molecule inhibitor SPD304 was purchased from Cayman Chemicals (MI, USA), ABT737 and ABT263 from Selleckchem (TX, USA). Water was purified using a Milli-Q\textsuperscript* UltraPure water purification system (Millipore, Barnstead, USA). Prior to mass spectrometric analysis the Bcl-x\(_L\) protein stock solution (224 \(\mu\)M) in 50 mM Heps, 100 mM NaCl, 10 % glycerol, 1mM EDTA, 1mM DTT, 0.1 % Nonidet-40 substitute (pH = 7.5) and the TNF-alpha protein stock solution in 50 mM phosphate buffer, 100 mM NaCl, 2.5 mM EDTA (pH = 7.7) were desalted and buffer exchanged (PD MiniTrap G-25, GE Healthcare, Buckinghamshire, UK) against the ammonium acetate buffer. The stock solutions of Bad and Bak as well as small molecule inhibitors were dissolved in DMSO at a concentration of 10 mM and further diluted in ammonium acetate to desired concentration. All MS titration experiments were recorded under “native-like” conditions using 50 mM ammonium acetate buffer (pH = 7.7) for TNF-alpha-SPD304 and 300 mM (pH = 7.5) for Bcl-x\(_L\)-peptide-inhibitor complex. To ensure the integrity of the protein complexes we kept the pH of the ammonium acetate buffer the same as that of the buffer used for protein expression and storage, which was previously optimized. In all experiments
the DMSO concentration did not exceed 1 % (v/v). For TNF-alpha denaturation, ZipTip columns containing C$_4$-resin (Millipore, Molsheim, France) were used. The exact TNF-alpha and Bcl-x$_L$ concentration was determined using a UV spectrometer (Genesys 10S UV-VIS, ThermoScientific, Bremen, Germany) by measuring the absorbance at 280 nm.

### 6.3.2 Mass Spectrometry

NanoESI-MS analyses were performed with a hybrid quadrupole time-of-flight mass spectrometer (Q-TOF ULTIMA, Waters/Micromass, Manchester, UK) in positive ion mode. The instrument was controlled via the MassLynx software (version 4.0). Sample solutions were directly infused with gold/palladium-coated borosilicate glass offline nanoESI emitters (Thermo Fisher Scientific, Reinach, Switzerland) using a commercial nanoESI ion source (Waters/Micromass, Manchester, UK). The capillary voltage was set to 1.8 kV and a gentle backing pressure of 0.3-0.5 bar was applied to assist the liquid sample flow. The source temperature was kept at room temperature. Instrumental conditions had to be adjusted in order to get narrow peaks of the detected ions without dissociating the noncovalent complex. The precise settings have an influence on the peak shape: due to adduct formation with salt and buffer molecules from the spray solution, peaks might be broadened. The mass spectrometer was run with the following gentle desolvation parameters: the cone and first ion tunnel RF1 voltages, parameters that control the kinetic energy of the ions in the source region of the mass spectrometer, were set to 50 and 50 V for Bcl-x$_L$; and 70 and 60 V for TNF-alpha experiments, respectively. After this stage, the ion beam passed a hexapole collision cell filled with argon (purity 5.0, PanGas, Zurich, Switzerland). The collision energy offset was used to optimize desolvation and set to 22 V. The pressure in the source was increased to 5.5 mbar, using a “speedivave” (Edwards Ltd., Sussex, UK) connected between the rotary pump and source pumping line. All instrument parameters used (e.g. capillary voltage, cone voltage, RF1 voltage, collision energy) were carefully adjusted and optimized to be as soft as possible for all investigated protein-complexes. Collision-induced dissociation (CID) used for TNF-alpha MS/MS experiments were performed by adjusting the
acceleration collision energy (CE) offset until full dissociation of the parent ions was achieved. The ion transmission was optimized for a m/z range between 100 and 9,000 Da for TNF-alpha, and 100-5,000 Da for Bcl-xL. The scan time and interscan times were 1 and 0.2 sec, respectively.

IMS-MS experiments were performed on the Synapt G2-S HDMS (Waters, Manchester, UK). Ions were produced by a commercial NanoLock Spray ionization source (Waters, Manchester, UK) using offline capillary emitters (see above). A capillary voltage of 0.8-1.3 kV and a backing pressure of 0.25-0.3 bar were applied to generate the nano-electrospray. The sampling cone voltage and the source offset were set to 20 and 80 V, respectively. The traveling-wave ion guides were tuned to minimize unwanted fragmentation of ions during ion transfer, trapping, ion mobility separation, and mass analysis. For instance, the trap DC bias, helium cell DC offset, and IMS bias were lowered to 40, 30 and 0.5 V, respectively. The trap gas flow was increased to 5.5 ml min⁻¹ to facilitate transmission of high-m/z ions. The trap and transfer collision energies were set to 10 and 5 V, respectively, and trap and transfer CID was induced by increasing the corresponding voltage offsets. Ion mobility separations were carried out using IMS wave velocity (WV) ramping of 1600 to 200 m s⁻¹ (unless specified differently) and wave height (WH) amplitude of 40 V. Nitrogen (purity 5.0, PanGas, Zurich, Switzerland) was used as IMS buffer gas. The spectra were acquired in the range of m/z 50-8000 using scan time of 2 s and interscan delay of 0.01 s. Typically, at least 50 scans were combined to produce a spectrum.

Calibration of the mass spectrometry instrument was performed using caesium iodide (CsI) clusters. CsI was dissolved in water/2-propanol (1/1, v/v) at a concentration of 2 μg/μL.

### 6.3.3 Data Processing

Before data processing, each mass spectrum was smoothed (Savitzky-Gollay smooth) with the MassLynx 4.0 software (Waters, UK). For the dissociation constant (K_D) determination of the Bcl-xL-Bad and Bcl-xL-Bak complexes the measured relative peak heights (I) were used. The peak height ratio (R) of the Bad- and Bak-bound
Bcl-x\textsubscript{L} complex (P:\textcdot L) to bare protein (P), \( R = \frac{I(\text{P}\cdot \text{L})}{I(P)} \), was calculated for each spectrum. For this determination, all charge states were taken into account. The ratio of the sum of all detected complex species divided by the sum of the free protein was determined. The experimentally calculated relative peak heights were plotted versus the total added Bad or Bak concentration. The equation derived by Daniel et al [191] was used to determine the \( K_0 \) values from fitting a titration curve. The \( K_0 \) calculations and the fitting of the titration curves were performed using the MATLAB software (2010a, The MathWorks, Natick, MA, USA).
6.4 Results and Discussion

6.4.1 NanoESI-MS Analysis of TNF-alpha

Prior to the addition of the inhibitor to TNF-alpha, the proper instrumental conditions had to be adjusted to preserve the trimeric protein structure. Therefore the trimeric human TNF-alpha was analysed under denaturing and “native” conditions using the Q-TOF ULTIMA. In Figure 6.1 nanoESI mass spectra for a solution of denatured and native TNF-alpha are shown. The first spectrum measured under denatured conditions generates a broad charge state distribution. Under this condition the completely unfolded monomer that appears in the lower m/z range is detected. In contrast, Figure 6.1b displays the spectrum under “native-like” conditions in 50 mM ammonium acetate and 1 % DMSO at pH = 7.7.

![Fig. 6.1 NanoESI mass spectra of TNF-alpha showing a.) unfolded monomer measured under denaturing conditions and b.) native intact trimer using 50 mM ammonium acetate buffer in 1 % DMSO at pH = 7.7.](image)

The observed narrow charge state distribution, predominantly + 11, + 12, + 13, is typical for non-denaturing conditions, and is consistent with a compact conformation in solution. In addition to the trimeric TNF-alpha ions, we can observe minor monomeric peaks at + 7, + 6, as well. Many studies have demonstrated that the charge state distribution depends on the protein conformation in solution. [34, 206, 216] Native nanoESI-MS analysis of the protein is relevant, since proper TNF-alpha folding is crucial for the later interaction with the inhibitor in solution.

In addition we performed CID experiments in order to confirm the trimeric TNF-alpha assembly and gain additional information about the protein stability in the gas
phase. For the MS/MS measurements the +14 trimeric ions were selected and dissociated during transmission through the mass spectrometer. For this, the CE offset was varied in 10 V steps from 15 to 100 V, until the selected trimeric ions were completely dissociated. In Figure 6.2 two different CID spectra at a CE offset of 30 and 100 V are shown.

![CID spectra](image)

**Fig. 6.2** CID experiments of the trimeric TNF-alpha: a.) For the MS/MS measurements the +14- charged protein precursor ions (*) were selected. The collision energy offset was set to 30 V; b.) At the collision energy offset of 100 V the selected trimeric +14 ions dissociate into monomers and dimers.

Dissociation of the precursor ions yielded the dimeric and monomeric protein ions. These CID experiments provide additional evidence for the trimeric TNF-alpha assembly. In should be mentioned that different charge state distribution are generated in the absence and in the presence of 1 % DMSO. This effect is described in the next subsection more in detail.

### 6.4.2 Monitoring the Disruption of the TNF-alpha by NanoESI- and IM-MS

SPD304 has previously been identified as potent inhibitor against TNF-alpha. We monitored the influence of SPD304 on TNF-alpha using nanoESI- and ion mobility-MS. At this point it is noteworthy to state that the described experiments were run in 1 % DMSO (v/v). It has been shown that this DMSO amount will not significantly influence the binding of the small molecule to the protein as observed by nanoESI-MS. [268] However, it is still necessary to perform experiments in the presence and
in the absence of DMSO. This should be considered in order to properly evaluate any possible conformational difference, resulting in a different charge state distribution, of the complex and the bare or dissociated protein. Also, DMSO may lead to partial dissociation of the protein. **Figure 6.3** illustrates IMS-MS analysis of TNF-alpha under “native ESI-MS” conditions. The shown results should provide additional structural information based on the separation of gas-phase ions based on their differential transport through an environment of inert neutrals. [158]

We show the 2D IMS drift time vs. m/z plots with corresponding mass spectra and drift time distributions. We first performed experiments using a 4.5 μM TNF-alpha solution in 75 mM ammonium acetate buffer at pH = 7.7 and the same protein concentration in the presence of 1 % (vol.) DMSO. In the presence of 1 % DMSO, an overall charge state reduction can be observed; +11, +12, +13 compared to +13, +14, +15, +16. The appearance of a small amount of the monomers is also observed in the presence of DMSO. The trimeric form is compact in both cases. As a next experiment, we have investigated the influence of the inhibitor by adding 100 μM SPD304 to 4.5 μM trimeric TNF-alpha in 75 mM ammonium acetate solution at pH = 7.7 in 1 % DMSO. The same charge state distribution is detected for the TNF-alpha upon inhibitor addition. Again, three different compact charge states representing the TNF-alpha trimer are observed. The appearance of dimer ions and the increase of monomer peak intensities is clearly seen, indicating the dissociation of the trimeric protein form in solution. The advantage of IM-MS in this case is the clear separation of dimer and trimer ions due to their different drift times. The peak maxima in the drift time distributions are represented with the respective bin numbers. As can been seen in **Figure 6.3**, the +13 charge state of the trimeric protein appears in all three cases (without DMSO, with DMSO, and upon ligand binding). The +13 charge state shows the same drift time distribution in all three cases. These results indicate clearly that the dissociation upon ligand addition already happens in solution and not due to partial dissociation of the trimer in the gas phase.
Fig. 6.3 IMS-MS analysis of TNF-alpha under “native ESI-MS” conditions. Shown are 2D IMS drift time vs. m/z plots with corresponding mass spectra (top traces) and drift time distributions (right traces). Peaks corresponding to monomeric, dimeric, and trimeric TNF-alpha ions are labelled as $M$, $D$, and $T$, respectively, and their charge states are indicated. Peak tops in drift time distributions are labelled with the respective bin numbers. Along with the integral drift time distributions (black traces), some selected-ion drift time distributions are shown in colour. Peak labels are color-coded accordingly. a.) 4.5 μM TNF-alpha solution in 75 mM ammonium acetate buffer pH 7.7. b.) Same as in a.) in the presence of 1 % (vol.) DMSO: note overall charge state reduction and the appearance of small amount of the monomers. c.) Same as in a.) in the presence of 1 % (vol.) DMSO and 100 μM SPD304: note the appearance of dimer ions and the increase of monomer peak intensities. Parts of the mass spectra in b.) and c.) are shown at 5-fold magnification.
Under this aspect, we have also investigated the dependence of the drift time distribution of the TNF-alpha trimer 13+ ion on the trap collision energy applied (Figure 6.4).

![Figure 6.4](image)

**Fig. 6.4** Dependence of the drift time distribution of TNF-alpha trimer 13+ ion on the trap collision energy applied. a.) 4.5 μM TNF-alpha in 75 mM ammonium acetate buffer pH 7.7. b.) Same as in a.), in the presence of 1 % (vol.) DMSO. c.) Same as in a.), in the presence of 1 % (vol.) DMSO and 100 μM SPD304. The peak intensity is normalized.

This charge state was chosen since it is generated in all three cases (with and without DMSO and in the presence of the inhibitor). The selected 13+ ions were interrogated by changing the trap collision voltage in the ion trap just prior to the mobility cell. The increased voltage accelerates the ions such that they encounter neutral gas molecules with greater kinetic energy in the ion trap. Nearly identical collision-induced unfolding profiles registered for the TNF-alpha T13+ ion electrosprayed from various solution conditions (buffer, 1 % DMSO, 1 % DMSO + 100 μM ligand) is observed. The drift time distribution of T13+ ion is narrow and unimodal in all three cases, with the peak maximum in bins 85-86 up to a trap collision energy offset of 30 V. At a collision energy of 40 V, unfolding starts, which is manifested by a slight broadening of the drift time distribution and a minor shift of the peak towards shorter drift time, due perhaps to a gas-phase collapse of the trimer. As the collision energy increases to 50 and 60 V, the drift time distribution broadens dramatically, shifts towards higher drift times, and becomes multimodal, with several more or less overlapping peaks. Dissociation into monomer and dimer ions with asymmetric charge partitioning is observed simultaneously in mass spectrum (data not shown). At high trap collision energies, the drift time distribution coalesces into a single peak at bin 108. This behavior resembles a two-state, all-or-
Fig. 6.5 IMS-MS analysis of TNF-alpha ions produced under “native ESI” conditions from 4.5 µM protein solution in 75 mM ammonium acetate buffer (pH 7.7) containing 1% (vol.) DMSO and 100 µM SPD304 (marked as ligand). The samples were analyzed at various transfer collision energy offsets: 5 V a.), 30 V b.), 90 V c.), and 130 V d.). Peaks corresponding to monomeric, dimeric, and trimeric TNF-alpha ions are marked as M, D, and T, respectively, and their charge states are assigned. Note the presence of TNF-alpha dimer ions D8+, D9+, and D10+ (marked in red) even at low transfer collision energy offsets (a, b). At high collision energy offsets (c, d), collision-induced dissociation (CID) of TNF-alpha ions occurred in the transfer region of the mass spectrometer, after IMS separation (fragments marked with blue text). Thus, fragment ions have the same drift time, as the respective parent ions (some dissociation channels are indicated with blue arrows). Note that D8+, D9+, and D10+ ions have their own characteristic drift time distributions, which are different from those of the dimer ions originating in the gas phase due to CID. Therefore, D8+, D9+, and D10+ ions must have been present in the sample prior to IMS-MS analysis, i.e. they occurred due to SPD304-promoted dissociation of TNF-alpha trimers in solution.
none protein-unfolding behavior. The most important conclusion is that the behavior of T13+ is the same in all three cases, i.e. there are no stabilizing or de-stabilizing effects found in the gas phase when the protein is incubated with DMSO or ligand. In Figure 6.5, the IMS-MS analysis of TNF-alpha ions produced under “native ESI” conditions from 4.5 μM protein solution in 75 mM ammonium acetate buffer (pH 7.7) containing 1% (vol.) DMSO and 100 μM SPD304 is shown.

The sample is analysed at various transfer collision energy offsets. The ions were interrogated by changing the transfer collision voltage in the transfer region just after the mobility cell. TNF-alpha dimer ions D8+, D9+, and D10+ are present even at low transfer collision energy offsets (Fig. 6.5a and b). At high collision energy offsets (Fig. 6.5c and d), collision-induced dissociation (CID) of TNF-alpha ions occurred in the transfer region of the mass spectrometer, after IMS separation. Thus, fragment ions have the same drift time, as the respective parent. The D8+, D9+, and D10+ ions have their own characteristic drift time distributions, which are different from those of the dimer ions originating in the gas phase due to CID. Therefore, D8+, D9+, and D10+ ions must have been present in the sample prior to IMS-MS analysis, i.e. they occurred due to SPD304-promoted dissociation of TNF-alpha trimers in solution.

In addition, we have performed nanoESI-MS measurements on the Q-TOF ULTIMA. Figure 6.6 shows the influence of adding of 100 μM SPD304 to 4.5 μM trimeric TNF-alpha on the nanoESI mass spectra in 50 mM ammonium acetate solution at pH = 7.7 in 1% DMSO. Interestingly, here we can observe a wider charge state distribution in the spectrum compared to the above-mentioned results. Additional charge states, the +14, +15 and +16 ions, are generated compared to the spectrum without inhibitor (see Figure 6.1b). However, it would be quite speculative to state that this shift in charge state distribution towards lower m/z indicates a “less compact” trimeric protein structure in the presence of SPD304. This “more open” trimeric form may go hand in hand with a partial dissociation of the protein into dimers and monomers, which indicates a conformational change in the protein structure. The observation that the dimer abundance is lower compared to monomers is probably due to a lower ionization efficiency of the dimeric form. A very interesting result is that TNF-alpha forms a noncovalent complex by binding one inhibitor molecule. No
ligation states with two or three ligands were detected.

![Graph showing relative intensities](image)

**Fig. 6.6.** a.) Representative nanoESI mass spectra of 4.5 μM TNF-alpha in the presence of 100 μM SPD304 under “native” conditions at pH = 7.7 in 50 mM ammonium acetate and in 1 % DMSO; binding of SPD304 (filled rhombus) dissociates the trimeric protein into dimers and monomers. b.) Zoom of the spectrum; dimer peaks and binding of SPD304 to the trimeric TNF-alpha is detected.

A X-ray structure reveals that a one equivalent of the inhibitor molecule displaces a subunit of the trimer and leads to the formation of a dimeric protein form. Biophysical experiments as well as biochemical and cell-based assays have shown that the inhibitor was capable to dissociate TNF-alpha trimer in solution and also the interaction between intact trimeric protein, which lead to subunit dissociation. [259] The ESI-MS and IM-MS results are in agreement with this structural data, but complement them in the sense that we gain additional insight into inhibitor binding to TNF-alpha.

### 6.4.3 K<sub>D</sub> Determination of the Bcl-x<sub>L</sub>-Bak and Bcl-x<sub>L</sub>-Bad Complexes

As a second system we investigated the heterodimerization between members of the Bcl-2 family of proteins, which is very important in regulating programmed cell death. The subsequent influence of small molecule disruptors on these interactions was monitored as well. In the first step, before addition of small disruptors ABT737 and ABT263, we carried out measurements with Bcl-x<sub>L</sub> (amino acids 1-209, Δ45-84) in complex with the synthetic peptides of the Bak- and Bad BH3 domain. In Figure 6.7a the nanoESI mass spectra of the bare protein in the presence and in the
absence of Bak in 300 mM aqueous ammonium acetate at pH = 7.5 are shown. A narrow charge state distribution, predominantly 7+, 8+ ions, appears at fairly high m/z. This is characteristic for native conditions, and consistent with a compact conformation of Bcl-x\textsubscript{L} in solution. In order to determine the dissociation constant via the titration method, a set of nanoESI experiments was performed with increasing Bak concentrations ranging from 0.5 to 3 \textmu M, at a constant Bcl-x\textsubscript{L} concentration. Figure 6.7a displays representative nanoESI spectra obtained for the Bcl-x\textsubscript{L}-Bak complex at three different ligand concentrations.

![NanoESI spectra and titration curve for Bcl-x\textsubscript{L}-Bak complex](image)

**Fig. 6.7 a.)** Representative nanoESI mass spectra of 3 \textmu M Bcl-x\textsubscript{L} in complex with Bak (filled circle) obtained in positive ion mode under “native” conditions. In the first spectrum adduct formation due to the small residue of HEPES buffer is detected. Titration experiments are shown adding different Bak concentration to the Bcl-x\textsubscript{L}. The signal for the noncovalent complex clearly increases with increasing Bak concentration present in solution. b.) NanoESI-MS titration curves for Bcl-x\textsubscript{L}-Bak complex. The Bak concentration ranges from 0.5 to 3 \textmu M, while the protein concentration was kept constant.

As expected, increased complex signal intensity was observed with higher total Bak concentration. At 3 \textmu M Bak concentration, full complexation was reached (data not shown). Titration experiments for Bcl-x\textsubscript{L}-Bak binding over a range of concentrations were performed. We can detect different complex/free protein ratios for different charge states. This phenomenon is well known and has already been mentioned for
different noncovalent complexes, although no clear explanation can be found in the literature. [5, 80, 257] In order to determine the $K_D$ we took the abundance (peak intensities) of all detected complex and protein ions into account. The titration curve is shown in Figure 6.7b. The signal ratio of the detected complex and the sum of the free protein and the complex signal was plotted against the total ligand concentration ($L_0$). The $K_D$ determined by a set of titration experiments was 314 ± 35 nM. This value is in very good agreement with other values determined for Bcl-xL•Bak in solution. In the literature, $K_D$ values of 480 nM and 340 nM using a fluorescence polarization-based competition assay were obtained. [263, 264]

For Bcl-xL in complex with the Bad BH3 derived peptide we performed titration experiments as well. The charge state distribution is comparable with that obtained for Bcl-xL•Bak. However, higher Bad concentrations were needed to reach full complexation. Therefore the titration experiments were performed from 2 to 20 μM (data not shown). The $K_D$ determined for Bad binding to Bcl-xL is 4.45 ± 0.3 μM. Depending on the length of the synthetic Bad peptide $K_D$ values ranging from 50 μM to the low nanomolar range were reported using fluorescence polarization competition assay. [269]

For the Bcl-xL•Bak and Bcl-xL•Bad disruption with ABT737 and ABT263, we performed the experiments at concentrations where mainly the complex peak is observed in the spectrum. In case of Bcl-xL•Bak, the ratio was 1:1 (eq) and for the Bcl-xL•Bad binding 1: 6.6 (eq). The experiments are described in detail in the next section.

6.4.4 Monitoring the Bcl-xL-Bak and Bcl-xL-Bad Inhibition Using Small Disruptors ABT737 and ABT263

We used native MS to directly monitor the inhibition of the Bcl-xL•Bak and Bcl-xL•Bad heterodimers in the presence of the small inhibitors ABT737 and ABT263. These compounds were shown to inhibit binding of peptide and induce apoptosis. [266]

Experiments with the small disruptor ABT737 of the Bcl-xL-Bak heterodimer were first carried out. In Figure 6.8a the spectra of 3 μM Bcl-xL in complex with 3 μM Bak (full complexation reached), in the presence at different ABT737 concentrations ranging from 1.25 to 12.5 μM are shown. With higher inhibitor concentration we can
clearly monitor the increasing disruption of the Bcl-x<sub>L</sub>-Bak interaction. Upon addition of the small inhibitor the disrupted Bcl-x<sub>L</sub>-Bak complex generates additional peaks of the bare Bcl-x<sub>L</sub> protein and a Bcl-x<sub>L</sub>-ABT737 complex. This observation gives us additional information about the mechanism of binding of the ABT737, which is in this case is competitive. The small disruptor ABT737 is able to displace the Bak derived peptide from the BH3 binding pocket of Bcl-x<sub>L</sub>. No peaks where all three species form a complex were detected, which confirm our interpretation of a competitive mechanism. In a recent study it was described that ABT binds to the BH3 pocket of Bcl-x<sub>L</sub>, breaking its hold on Bak. [261, 266] At 12.5 μM ABT737 the major +8 peak is Bcl-x<sub>L</sub> in complex with ABT737, only a minor undisrupted +8 heterodimer peak remains.

![Fig. 6.8 NanoESI mass spectra of 3 μM Bcl-x<sub>L</sub> in complex with 3 μM Bak (filled circle) in the presence of different concentrations of small disruptor (filled rhombus) a.) ABT737 and b.) ABT263. The heterodimer signal clearly decreases with increasing inhibitor concentration present in solution.](image)

For the native MS measurements of the Bcl-x<sub>L</sub>-Bak disruption in the presence of the small inhibitor ABT263, the same instrument conditions were used. In Figure 6.8b the spectra of 3 μM Bcl-x<sub>L</sub> in complex with 3 μM Bak in the presence at different ABT263 concentration between 6.25 μM and 25 μM are shown. The mode of binding is, as in the previous case, competitive, although a significant difference in the
inhibition of PPI compared to ABT737 was detected. In order to disrupt half of the heterodimer, 17.5 μM of ABT263 had to be present in solution. As shown in Figure 6.8, 6.25 μM ABT737 disrupt three times more Bcl-xL•Bak complex compared to ABT263 at the same concentration.

This observation lets us conclude that ABT263 is a less active inhibitor compared to ABT737. These data are consistent with those generated in a TR-FRET assay, which also indicate that ABT737 is more active than ABT26. [266]

In addition, the in vitro efficacies of ABT737 and ABT263 were studied in a recent study. The authors have shown that ABT737 is more active than ABT263 in inducing apoptosis in chronic lymphocytic leukemia (CCL) cells, because ABT263 was more strongly bound by albumin compared to ABT737, which accounted for the differential sensitivity of CLL cells. [270] However, the activities in our assay using purified protein are not affected by albumin binding.

In a second step, we also studied the influence of ABT737 and ABT263 on the Bcl-xL•Bad interaction. For this the experiments with the small inhibitors with 3 μM Bcl-xL in complex with 20 μM Bad were performed. Figure 6.9 displays nanoESI spectra at different ABT737 and ABT263 concentrations. Again, with higher inhibitor concentration the stronger disruption of the Bcl-xL•Bad interaction is detected. The disrupted Bcl-xL•Bad complex dissociates into ions representing the bare Bcl-xL protein and the Bcl-xL•ABT737 or Bcl-xL•ABT263 complexes. We found again that the ABT737 is more potent compared to ABT263 in preventing the heterodimerization between Bcl-xL and Bad derived BH3 peptide in solution. To completely dissociate the dimerization, a 2.5 times higher concentration of ABT263 was required, corresponding to the 18 μM ABT263 and 7 μM ABT737 inhibitor concentration.
Fig. 6.9 NanoESI mass spectra of 3 μM Bcl-xL in complex with 20 μM Bad (filled circle) in the presence of different concentration of small disruptor (filled rhombus) a.) ABT737 and b.) ABT263.

The heterodimer ratios (Bcl-xL-Bak/ Bcl-xL or Bcl-xL-Bad/ Bcl-xL) upon addition of the total ABT263 and ABT737 concentration are plotted in Figure 6.10.

Fig. 6.10 Plotted ratios (bound heterodimers/ unbound bare Bcl-xL and Bcl-xL-ABT737 complex) against the different inhibitor concentration in order to dissociate the heterodimer

Compared to the Bcl-xL-Bak, no significant difference in the inhibitor efficiency is observed in preventing the Bcl-xL-Bad interaction; ABT737 and ABT263 seem to have a very similar influence in disrupting both investigated heterodimers. For the Bcl-
Bad disruption with ABT263, no significant difference in dissociation of the heterodimer with lower ABT263 concentration is observed. Therefore less data points are plotted compared to other three investigated systems. We could show that the native MS approach is suitable to directly monitor not only PPI inhibition, but also the relative binding strengths and the nature of binding.
6.5 Conclusions

In this study we investigated the inhibition of protein-protein interactions using nanoESI-MS. As a first system we investigated the dissociation of the trimeric TNF-alpha in the presence of the inhibitor SPD304. Ion mobility experiments were performed as well. The inhibitor promotes subunit disassembly of the trimeric form into monomers and dimers. Only one molecule inhibitor binds to the trimeric TNF-alpha. The SPD304-promoted dissociation into dimers ions must have been present in the sample prior to IMS-MS analysis, since the dimeric ions have their own characteristic drift time distributions, which are different from those of dimer ions originating in the gas phase due to CID.

As a second system we investigated the inhibition of the heterodimer formation of the survival protein Bcl-xL and death-promoting regions of proteins Bak and Bad. Recently developed small-molecule inhibitors for the above-mentioned interaction, ABT737 and ABT263, were used to detect the disruption of the heterodimers. In the first step we determined the dissociation constants of the Bcl-xL in complex with Bak or Bad derived peptide domain applying titration method. The ratio of the protein-peptide wherein the complex peak was generated was used for further experiments with small inhibitors. We found that ABT737 is more active inhibitor compared to ABT263 in disrupting the heterodimerization between Bcl-xL and Bak and also Bad derived BH3 peptide. The small disruptor ABT737 as well as ABT263 is able to displace the Bak and Bad derived peptide from the BH3 mainly hydrophobic pocket of the Bcl-xL. This observation indicates a competitive mode of binding.

The nanoESI-based results for both investigated systems are in agreement with our biophysical methods in terms and can therefore be used as a suitable/appropriate technique for studying PPI inhibition. Due to the advantages of the nanoESI approach in terms of speed, absence of label and sensitivity, we believe that can be widely used for better understanding and development of small inhibitors of PPIs. This method allows the monitoring of ligation states, provide information of mechanisms, on stoichiometry and relative binding potency.
Chapter 7

7 Conclusions and Outlook

This chapter gives a brief summary of the results of this thesis and potential directions for future.
This thesis tried to extend the work in ESI-MS based methods designed to answer questions related to the investigation of noncovalent protein-ligand interactions as well as applications in drug discovery research.

In our first study we have applied nanoESI-MS for the investigation of a series of hydrophobically modified ligands interacting with serine protease trypsin. The basic question this study addresses is whether we can observe the expected trend in the binding affinity of the inhibitors, when using ESI-MS as a read-out for the solution phase equilibrium. The two different series of competitive inhibitor used in this work are based on the same scaffold and vary only in the length of the hydrophobic P3 side chain (R = Gly, d-Ala, d-Val, d-Leu, d-Cha). This hydrophobic side chain should lead to systematic differences in their binding affinities. For all closely related inhibitor-trypsin complexes we could successful in reproduce the trends found by a kinetic binding assay in solution with the dissociation constants $K_D$ determined by nanoESI-MS. A second question concerns the stability in solution vs. in the gas phase. It was proposed by several groups that a correlation between the gas phase stability and binding affinities is expected only if electrostatic and H-bond interactions of protein-ligands play a dominant role in the complex stabilization, but not if hydrophobic interactions are the major driving force in stabilization. Therefore, collision-induced dissociation experiments were also performed to gain information about the correlation between the binding affinities in solution and the gas phase stability. As part of this aspect we have also investigated the stabilizing effect of imidazole, which should protect protein-ligand complexes from in-source dissociation during the ESI-MS analysis process. Collision-induced dissociation experiments across the first series clearly show a correlation between the binding affinity and the gas phase stability. For the second inhibitor series we could show that upon addition of imidazole, a stabilizing solution additive, the relative abundance of the non-dissociated could be increased. The CMA ligands did not show the clear trend towards higher binding strengths with longer side-chains, since the increased binding affinity is observed with the order Gly < d-Ala < d-Leu.

In the following chapter we continued to investigate allosteric regulation of protein function. We investigated the allosteric mechanism in the binding of newly
developed inhibitors to of enzyme fructose-1,6-bisphosphatase (FBPase), a potential therapeutic target for glucose control in type-2 diabetes. In this study, a series of inhibitors that occupy the allosteric AMP binding site were investigated to determine the dissociation constants (Kd) by nanoESI-MS. Measured IC50 values values in solution are in good agreement with nanoESI based KdS titration. In addition, we extracted the Hill coefficient, which describes the number of inhibitor molecules required to bind to the FBPase. The nanoESI mass spectra allow one to deduce the cooperativity of the multimeric model system that proves to be positive in case of the investigated model system. This study highlights the benefits of ESI-MS as a fast and label-free method not only for determination of dissociation constants of cooperatively regulated enzyme, but also to better understand the mechanism of enzymatic cooperativity.

Chapter 5 describes how DMSO influences protein-ligand binding strengths. Three different noncovalent complexes were chosen for our study: trypsin-Pefabloc, lysozyme-NAG3 and carbonic anhydrase-CTA. The DMSO content chosen for the study is up to 8 % (v/v) and close to that used in assays for HTS in drug discovery. In all three-model systems studied, we could show that DMSO has a significant influence on protein-ligand binding. The advantage of using nanoESI-MS is the direct visualisation of the ligation state upon addition of DMSO. This allows one to deduce from only one measurement the protein-ligand binding affinities. The study highlights the dependence of the relative binding affinities of different noncovalent complexes on DMSO concentration and the importance of these effects in the early stages of drug discovery.

In the last decade the design and development of small molecules that are capable to inhibit the protein–protein interactions (PPI) are increasingly attracting attention, both in academia as well as in the pharmaceutical industry. Our study highlights the benefits of nanoESI-MS as a fast and label-free method for investigation the mechanism, stoichiometry, conformational changes and relative binding strengths of heterodimer formation of the survival protein Bcl-xL and cell death-promoting regions of the proteins Bak and Bad, using the small inhibitors ABT737 and ABT263.
Dissociation of the TNF-alpha trimer caused by the small-molecule inhibitor SPD304 was monitored as well. Ion mobility separation coupled with MS (IM-MS) could provide interesting structural information of large biomolecule, e.g., to assess the size, shape, binding, stoichiometry, stability, and structure of protein-ligand complexes. IM-MS has potential in monitoring small molecule binding to biomolecules inducing conformational changes of the target proteins. For example, it would be possible to study the binding of the small inhibitors of the allosterically regulated FBPase using the new generation of the Synapt G2-S TWIMS mass spectrometer available in our group. One could investigate the correlation between the change in the conformation of the FBPase and the binding affinities. Other relevant biological systems, where conformational changes upon ligand binding are expected, could be investigated as well.

In the case of the DMSO study reported in Chapter 5, IM-MS could provide additional information about the conformational changes of the protein-ligand system upon increasing DMSO content. For instance, we observed a slight shift towards higher charge state upon DMSO addition. However, it is difficult to judge from the spectra whether this shift indicates the partial protein dissociation. IM-MS could provide additional information on the investigated systems. Determination of \( K_0 \) values via nanoESI titration method is currently possible from the mid \( \mu \)M down to the mid nM range, since the sample concentration used range from low \( \mu \)M to the high nM range. Lower \( K_0 \) values may be difficult to obtain since the saturation curves become undistinguishable; on the other hand, above \( 10^{-3} \) M the fraction of complex formed is to low to be accurately detected in an ESI mass spectrum. This limitation renders nanoESI unsuitable for determination of any desirable \( K_0 \) in drug discovery process. Therefore there is a clear need to extend on the one hand nanoESI assays for measuring very high binding affinities, and on the other hand for measuring very low binding affinities. Fragment molecules (fragment based drug discovery, FBDD) emerged as a valuable method for finding lead compounds in drug discovery process. The binding of such a fragments to a particular target is expected to be in a high \( \mu \)M range. Development of a nanoESI
approach for screening fragments binding that bind with low affinity could make
native MS as a viable tool, due to its sensitivity and speed.
Another drawback in the context of fragment-based drug screening is the low
molecular weight of the ligands, especially in the case when the relative differences
between the molecular weight of the small molecule inhibitors and the investigated
protein is high. Therefore, it may be challenging to resolve the complex peak from
the bare protein peak on high-mass QTOF instruments. The determination of the
binding affinities as well as the complex characterization is not possible. For
instance, in the case of the FBPase-small inhibitor study, it was very challenging to
resolve the peaks of binding of two inhibitors, due to the low mass difference of the
complex and the bare protein. (FBPase ≈ 150 kDa, FBPase-inhibitor ≈ 151 kDa).
Therefore we could determine the $K_D$ value in the case when all four ligand were
bound to the protein, but it would also be useful to have the possibility to determine
micro-$K_D$s ($K_D$ with two bound ligands) to get more information about the
cooperativity. New generations of even more sensitive mass analyzers with higher
resolution would help to answer the above-mentioned question. Recently, the mass
range of the Orbitrap mass spectrometer has been extended for the analysis of
native protein complexes. [271] Due to the high resolution of the Orbitrap it offers
completely new possibilities and applications for native MS. Results on an Orbitrap
mass spectrometer tuned to analyze biopharmaceutical products like antibodies and
much larger protein complexes up to 800-1000 kDa in mass have already been
introduced. However, the development of such a commercial instrument is still in
progress and its application is therefore a long-term project.
It would also be interesting to compare our nanoESI-MS results for the analysis of
protein-protein interactions inhibitions with MALDI-MS. In our group MALDI-MS
based analysis of intact proteins is done using when optimized cross-linking reagents
in combination with ultrahigh-mass detection systems. Cross-linking of the subunits
prevents the complex from dissociating during sample preparation or laser
desorption. One could compare nanoESI-MS based relative binding affinities with
MALDI experiments. IM-MS measurements could also provide additional
information.
Noncovalent protein-ligand complexes show different charge states of the complex and bare protein ions in native ESI mass spectra. In order to determine binding strengths, the ratio of the complex and the free protein should be calculated for each charge state. However, in some of our studies, e.g., the investigation of Bcl-xL and the Bak peptide, we realized the discrepancy in ratios of different charge states. This observation was also reported in other studies when studying noncovalent protein-ligand complexes. This effect renders the proper evaluation of binding strengths challenging. However no explanation for this phenomenon can be found in the literature. A future project could be to investigate a set of different protein-ligand systems, e.g., with different size, polarity. Such a study could help to find out in which cases the charge state difference occurs.

Native MS is not compatible with buffers used for protein storage and expression like HEPES, TRIS, NaCl. Therefore additional buffer exchange steps, usually against ammonium acetate or ammonium bicarbonate, are necessary before the MS analysis. Sometimes we faced the problem that even after extensive protein purification, the protein quality was not sufficient for native MS analysis. This results in the detection of broad peaks due to adduct formation with residual buffer. Therefore, a development of new nanoESI sources, which are more tolerant to biological buffers, could circumvent this problem.

One of the major drawbacks of noncovalent complexes studied by ESI-MS is that careful tuning of the instrumental parameters is required for every particular protein sample. Also, the subsequent data processing need to be adapted in order to determine K_D values. The data evaluation is still done manually and is therefore time consuming. Especially when the peaks are not sufficiently resolved or if the peaks overlap, a proper evaluation is difficult. This can lead to over- or underestimation of K_D values. In our group we had a semester project, which attempted to solve this problem. A MATLAB script was written for this purpose. However, each spectrum has to be uploaded manually. In order to use native MS as a tool for drug discovery, automated and uniform programs for data processing should be developed.

In conclusion, native MS is still a new technology, where development and application power is still in progress. Despite some drawbacks, we strongly believe
that this technique has potential to be used in HTS drug discovery in the pharmaceutical industry. In this thesis, successful investigation of several complex protein-ligand systems relevant for industrial research have been successfully investigated. One can anticipate an increasing contribution of native MS, probably in combination with IM-MS, to more difficult targets such as membrane proteins.
References

8 References


References


References


References


References


References


References


References


Appendix

A – Abbreviations

ACN  Acetonitrile
AMBA 4-amidinobenzylamide
AMP Adenosinmonophosphat
CE Collision energy
CHAPS 3-[(3-Cholamidopropyl)dimethylammonio]-1- propanesulfonate
CID Collision-induced dissociation
CMA 2-aminomethyl-5-chlor-benzylamide
CRM Charged residue model
CSS Collision cross section
CTA Chlorothiazide
CW Continuous wave
Da Dalton
DC Direct current
DMSO Dimethyl sulfoxide
DNA Deoxyribonucleic acid
DTT Dithiothreitol
ESI Electrospray ionization
ESSI Electrosonic spray ionization
HEPES 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
FAB Fast atom bombardment
FBDD Fragment based drug discovery
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBPase</td>
<td>Fructose-1,6-bisphosphatase</td>
</tr>
<tr>
<td>IC_{50}</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>IMS</td>
<td>Ion mobility spectrometry</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal titration calorimetry</td>
</tr>
<tr>
<td>K_A</td>
<td>Equilibrium association constant</td>
</tr>
<tr>
<td>K_D</td>
<td>Equilibrium dissociation constant</td>
</tr>
<tr>
<td>Ki</td>
<td>Inhibition constant</td>
</tr>
<tr>
<td>KNF</td>
<td>Koshland-Nèmethy-Filmer</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass-to-charge ratio</td>
</tr>
<tr>
<td>MCP</td>
<td>Multichannel plate</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Tandem mass spectrometry</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>MWC</td>
<td>Monod-Wymann-Changeux</td>
</tr>
<tr>
<td>NAG_3</td>
<td>Tri-N-acetylchitotriose</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>QTOF</td>
<td>Quadrupole time-of-flight</td>
</tr>
<tr>
<td>RF</td>
<td>Radio frequency</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>TCEP</td>
<td>Tris(2-carboxyethyl)phosphine</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoressigsäure</td>
</tr>
<tr>
<td>TRIS</td>
<td>2-Amino-2-(hydroxymethyl)-propan-1,3-diol</td>
</tr>
<tr>
<td>TritonX</td>
<td>Octyl phenol ethoxylate</td>
</tr>
<tr>
<td>TOF</td>
<td>Time-of-flight</td>
</tr>
<tr>
<td>TW</td>
<td>Traveling wave</td>
</tr>
<tr>
<td>TW-IMS</td>
<td>Traveling-wave ion mobility spectrometry</td>
</tr>
<tr>
<td>UV/Vis</td>
<td>Ultraviolet/visible light</td>
</tr>
<tr>
<td>x-ray</td>
<td>Rontgen rays</td>
</tr>
</tbody>
</table>