UNIVERSITY OF GOTHENBURG

Revealing functional details of the mitochondrial Lon Protease by NMR spectroscopy

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Department of Chemistry and Molecular Biology

Thesis for the degree of Doctor of Philosophy in the Natural Sciences

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Cover: Structural illustration of full-length human Lon illustrating its hexameric structure with the three distinguished domains, proteolytic domain (orange), AAA+ domain (blue) and the amino terminal domain (green). (PDB ID: 7NGL).

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“Edukimi nuk humb te njeriu me mendje dhe zgjuarësi të kufizuar,
por mendja dhe zgjuarësia humbin te njeriu i paedukuar.”
Sami Frashëri

Avhandlingen dediceras till min familj för all deras stöd och kärlek

Abstract

The hexameric Lon protein is an ATP-dependent protease residing in the mitochondrial matrix. The Lon protein is the product of the nuclear *LONP1* gene and plays a crucial role in the maintenance and repair of mitochondrial DNA as well as being an essential regulator of the mitochondrial metabolism. Three main functional roles have been associated to Lon function: The degradation of oxidized dysfunctional proteins, proteolytic regulation of vital proteins such as the TFAM transcription factor, as well as co-chaperoning functions together with other proteins involved in the mitochondrial protein quality control machinery. Thus, Lon also plays an important role in different diseases due to its involvement in the mitochondrial oxidative stress response. Even though the vital importance of Lon is evident, the underlying functional mechanism and its mechano-chemical principles are not thoroughly known. To unravel these properties, I have started to address these outstanding questions by studying the structure of Lon as well as its underlying dynamical properties using nuclear magnetic resonance (NMR) techniques. Due to its large size, I used a divide-and conquer approach, of splitting the protein into its smaller functional units facilitating the subsequent detailed characterization by solution NMR spectroscopy. Lon can be divided into three functional subdomains its amino-terminal domain (proposed to function in substrate recognition and binding), its AAA+ domain (ATP-binding and substrate unfoldase domain), and its protease domain (proteolytic active domain) [1–3].

Sammanfattning på Svenska

Det mänskliga Lon-proteinkomplexet är ett ATP-beroende proteas som finns i den mitokondriella matrisen. Lon-proteinet är en produkt av den kärnkodade genen LONP1 och spelar en avgörande roll i underhåll och reparation av mitokondriellt DNA. Tre huvudsakliga funktionella roller har hittills associerats med Lon, nedbrytning av oxiderade dysfunktionella proteiner, proteolytisk reglering av vitala proteiner såsom TFAM (mitokondriella transkriptionsfaktorn) samt koopererande funktioner tillsammans med andra proteiner som är involverade i mitokondriell proteinkvalitetskontrollsmaskineriet. Följaktligen har Lon också en viktig roll i olika sjukdomar på grund av dess inblandning i den mitokondriella oxidativa stressresponsen som triggas av en mängd olika syndrom. Även om Lon:s avgörande betydelse i mitokondrien är uppenbar är dom underliggande funktionella mekanismerna och dess mekanokemiska principer inte fullständigt förstådda. För att uppdaga dessa egenskaper har jag börjat ta itu med dessa utestående frågor genom att studera strukturen av Lon samt dess underliggande dynamiska egenskaper med hjälp av kärnmagnetisk resonansspektroskopi (NMR). På grund av proteinets storlek använder jag en divide-and-conquer-teknik, där jag delar upp proteinet i dess mindre funktionella enheter och underlättar därmed den efterföljande detaljerade karaktäriseringen med hjälp av NMR-spektroskopi. Lon kan delas upp i tre subdomäner: den aminoterminala domänet (som antas fungera i substratigenkänning och bindning), AAA+-domänen (ATP-bindande och substrat-unfoldase-domänet) och dess proteasdomän (proteolytiskt aktiva domänet)

List of papers

This thesis is based on the studies of the following topics.

Paper I: **Sallova Y**., Lidman J., Burmann B.M. pH-dependent adaptations modulate the function of the proteolytic domain of the human mitochondrial Lon protease. *(Submitted)*

Paper II: **Sallova Y**., Lidman., Burmann B.M. Structure and dynamics of the amino-terminal domain of human Lon *(Manuscript)*

Paper III: **Sallova Y**., Lidman J., Burmann B.M. Dynamics and allosteric regulation of full-length human Lon as seen by NMR spectroscopy *(Manuscript)*

Paper IV: Lidman J., **Sallova Y**., Matečko-Burmann I., Burmann B.M. Structure and dynamics of the mitochondrial DNA-compaction factor Abf2 from *S. cerevisiae. (Under revision)*

Paper V: Castegnaro F.*, **Sallova Y**.*, Aspholm E.E., Burmann B.M. Solution NMR approaches to reveal the intricate details of the dynamic underlying protein quality control. *(Manuscript)*

Contribution report

Paper I: Me and my supervisor planned and designed the experiments together. I performed all the experimental work. Me, the second co-author and the supervisor analyzed the data together and wrote the manuscript jointly.

Paper II: Me and my supervisor planned and designed the experiments together. I performed all the experimental work. Me, the second co-author and the supervisor analyzed the data together and wrote the manuscript jointly.

Paper III: Me and my supervisor planned and designed the experiments together. Me, the second co-author and the supervisor analyzed the data and wrote the manuscript jointly.

Paper IV: I supported and performed the cloning of the Abf2 constructs used in the manuscript.

Paper V: Me, the second shared first author and our supervisor planned the content and wrote the first draft of the chapter jointly. Me, the second shared first author, a third co-author and our supervisor wrote the final version jointly.

Abbreviations

A. U	–	Arbitrary Unit
AAA+	–	ATPases Associated with various cellular Activities
ATP	–	Adenosine triphosphate
BLI	–	Bio-layer interferometry
BTZ	–	Bortezomib
CPMG	–	Carr Purcell Meiboom Gill
CSP	–	Chemical shift perturbation
DNA	–	Deoxyribonucleic acid
E. coli	–	*Escherichia coli*
FITC	–	Fluorescein isothiocyanate-labeled
FRET	–	Förster resonance energy transfer
HMQC	–	Heteronuclear multi-quantum correlation
HSQC	–	Heteronuclear single-quantum correlation
IMS	–	Intermembrane space
IPTG	–	Isopropyl β-D-1-thiogalactopyranoside
NMR	–	Nuclear magnetic resonance
NOE	–	Nuclear Overhauser effect
PCR	–	Polymerase chain reaction
PDB	–	Protein Data Base
PQC	–	Protein quality control
ppm	–	Parts per million
PRE	–	Paramagnetic relaxation enhancement
SEC	–	Size exclusion chromatography
SEC-MALS	–	Size exclusion chromatography multi angle light scattering
SUMO	–	Small Ubiquitin-Related Modifier
TFAM	–	Transcription Factor A, Mitochondria
TOCSY	–	Total correlation spectroscopy
TROSY	–	Transverse relaxation optimized spectroscopy

Table of Contents

Scope of this thesis

The main focuses in this thesis are to unravel the structural and dynamical properties of the human mitochondrial Lon protease in solution using advanced high-resolution Nuclear Magnetic Resonance (NMR) spectroscopy in solution.

This thesis includes the following chapters.

Chapter I: Introduction into the biological role of the human Lon protease and the related literature involving mitochondrial protein quality control (MPQC)

Chapter II: Outlines the methodology used in the performed research.

Chapter III: Summary of the main findings and the resulting conclusions.

Chapter IV: A discussion of the future perspectives for Lon research.

Five papers are included in this thesis:

Paper I (submitted): This study focuses on the structural and dynamical properties of the isolated proteolytic domain of Lon. Our experiments could unravel the structural and dynamical adaptations underlying Lon protease pH-dependent activation.

Paper II (Manuscript): This study characterizes the amino-terminal domain of human Lon which plays an important role in the recognition and the initial binding of its substrates prior to subsequent unfolding and cleavage. The study lead the foundation for revealing novel insights into the dynamics and thus the functionality of this domain likely enabling future in-depth elucidation of its role within the context of full-length Lon.

Paper III (Manuscript): In this study the possibility of performing solution NMR spectroscopy on the 600 kDa full-length Lon is investigated, revealing unexpected domain dynamics, and laying the foundation for future in–depth characterization of this hexameric protein.

Paper IV (Under revision): In this paper the different dynamical properties of the two ABf2 HMG (high mobility group) DNA-binding domains are delineated. Thus, providing important novel insight into the ability of Abf2 to compact mitochondrial DNA in the absence of external energy such as ATP.

Paper V (Manuscript): This book chapter highlights the importance NMR spectroscopy has had in recent years in unraveling detailed structural and dynamical information about proteases as well as chaperone:client complexes involved in the cellular protein quality control (PQC) system.

Chapter I: The role of the human Lon protease in mitochondrial maintenance

Introduction

Almost all processes in living cells are driven by a diverse set of macromolecules that play a mandatory role in the maintenance of cells. These critical proteins are involved in many crucial mechanisms such as enzymatic catalysis [4], immune responses [5], DNA repair [6] among several other processes. The human body requires a steady supply of proteins to maintain the vital functions of its cells. However, the production of proteins is a complex process that is prone to errors. These errors lead to misfolded or dysfunctional proteins that can be harmful for the cell. Thus, it is of high importance to have a protein quality control system in place to ensure that the proteins are correctly folded and functional. The protein quality control system is a complex network of several essential macromolecules that work together to maintain protein homeostasis in the cells [7,8].

Protein quality control system in mitochondria

Protein quality control is a critical process in cells that ensures proper folding, assembly, and degradation of proteins. Mitochondria, the organelles responsible for the energy production in cells, in terms of ATP, have a unique protein quality control system that is essential for their function and survival [9,10]. One of the critical components of mitochondrial protein quality control is the molecular chaperone system, which facilitates the correct folding and assembly of newly synthesized proteins [11,12]. Mitochondria house several molecular chaperones, including Hsp70 and Hsp60 [13,14], that help prevent protein misfolding and aggregation by supporting the correct fold of their substrates. Additionally, the mitochondrial chaperones are involved in the degradation of misfolded or damaged proteins through the mitochondrial quality control protease (MQCP) system [15,16]. Another critical component in the MPQC system are mitochondrial proteases [17]. The proteases found in mitochondria include serine proteases [18–20], metalloproteases [21,22], and peptidases [23,24]. Proteases are enzymes that have the ability to cleave peptide bonds between amino acid residues, and they play crucial roles in a wide range of cellular processes, including protein degradation, maturation, and regulation. Mitochondria, contain several types of proteases that are critical for maintaining mitochondrial function and ensuring the quality control of mitochondrial proteins [25]. Mitochondrial proteases are classified into three main groups: matrix-localized, membrane-bound and inner membrane space (IMS)

proteases. Matrix-localized proteases including Lon and ClpXP are ATP-dependent, which are responsible for degrading and processing mitochondrial proteins in the matrix. Membrane-bound proteases include i-AAA and m-AAA proteases [26] (part of the AAA+-family) as well as PARL [27] and OMA1 [28] and in the IMS HtrA2 is found with its apoptosis inhibiting functions [29,30].

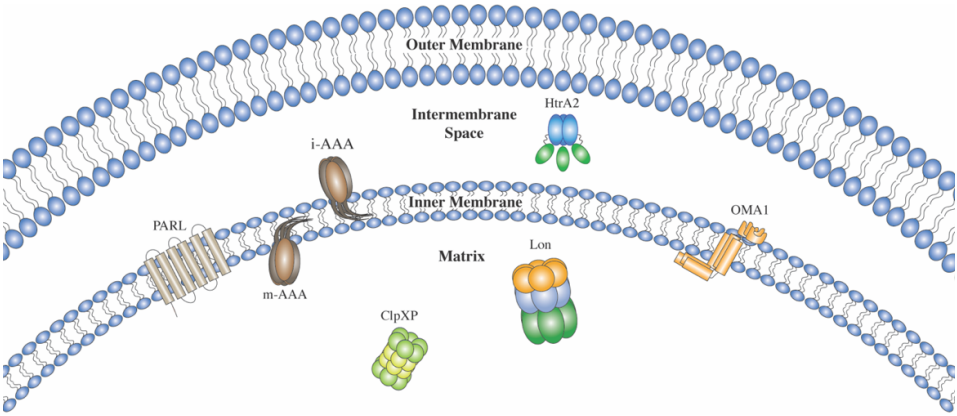

Figure 1: The organization and localization of some proteases involved in mitochondrial quality control. In the mitochondrial matrix, the most abundant proteases Lon (in selenium/blue/green) and the Clp protease complex (CLPXP) (green/yellow/green) are shown. In the intermembrane, four membrane bound proteases are depicted, PARL (in light brown), m-AAA and i-AAA (in dark brown) as well as OMA1 (in orange). In the IMS High temperature requirement A2 (HtrA2) protease is depicted in blue/green.

Serine proteases

Serine proteases are highly prevalent enzymes in the human genome [31], comprising approximately one third of all known enzymes. They participate in various cellular processes such as inflammation, apoptosis, and protein quality control. The catalytic triad of SA clan is typically composed of histidine, aspartic acid, and serine while the catalytic dyad is composed of lysine and serine or histidine and serine [32]. The serine residue serves as the nucleophile, attacking peptide bonds through acyl-enzyme intermediates [20]. ClpP and Lon, two mitochondrial matrix AAA+ proteases are a part of the

AAA+ proteases

AAA+ adenosine triphosphatases (ATPases) contain of a wide variation of molecular machines involved in a vast range of functions in the cell, such as protein unfolding, transportation of vesicles and organelle assembly. In many cases the ATPase domain of these proteins assemble into ring shaped hexamers in their mature state. One major class of AAA+ ATPases found in mitochondria are the ATP-dependent AAA+ protease class [33–35]. These proteases are responsible for the degradation of misfolded or damaged proteins in the mitochondrial matrix, as well as the regulation of mitochondrial dynamics and biogenesis. The AAA+ proteases are highly conserved in all kingdoms of life and play crucial roles in diverse cellular activities [36,37]. It has been shown in earlier studies that mutations or dysfunction found in mitochondrial proteases are potentially dangerous and in some cases fatal for the cells [38,39]. There is also an correlation between the dysfunction of the mitochondrial proteases with a range of human diseases, including neurodegenerative disorders [40], metabolic disorders [41], and several types of cancer [42]. Therefore, understanding the functions and mechanisms of these proteases in mitochondria is crucial for the future development of novel effective therapies addressing these diseases. ClpP and Lon are two major matrix localized AAA+ proteases in mitochondria as mentioned before that have been extensively studied. They are essential for mitochondrial function and have been implicated in the response to stress and disease [43,44].

Lon Protease Family

The Lon protease family is a group of enzymes that cleave peptide bonds in proteins to generate smaller peptides or amino acids and are present in archaea, bacteria, and eukaryotes [45]. Lon proteases consist of three subfamilies: LonA, LonB and LonC and are distinguished by the number of individual domains in the protein [46,47]. All Lon proteases in these three subfamilies comprise of two conserved functional domains: The AAA+ domain that unfolds peptides *via* the hydrolysis of ATP and a proteolytic domain that cleaves the unfolded peptides with a catalytic dyad consisting of a Lysine and a Serine. Even though all three protease families have the same catalytic dyad, the neighboring amino acid sequences in the vicinity of active site vary significantly. Furthermore, LonA proteases have an additional third domain, the amino terminal domain that is proposed to recognize and bind to the peptide substrates. The N-terminal domain is remarkably variable in length where it differs from 300–330 amino acids in bacterial cells to 420–570 amino acids in eukaryotic cells. However even though LonB and LonC lack the functional N-terminal domain they can contain a number of subdomains and

inserts found in different Lon proteases. LonB proteases harbour an additional sequence called Membrane Anchoring (MA) insert that is found within the AAA+ module and enables the entry into the cell membrane. While LonC has a similar catalytic region as LonB they have an extended mainly α-helical insert called Lon Insertion Domain (LID) or Helical Harpin Extension (HHE) that bears no resemblance to the MA [48,49]. The majority of Lon proteases are situated in the mitochondrial matrix, including the Lon protease PIM1 in yeast [50]. PIM1 is essential for mitochondrial function and is constitutively expressed, with increased expression after exposure to heat stress, indicating a potential role in the heat shock response [51].

Human Lon

Proteases have been viewed originally as simply protein destruction machines that degrade dysfunctional or partially/fully unfolded proteins and peptides. Later studies have given new insights about the multifunctional role that proteases hold in not only degrading proteins [52–55]. They are also governing the dynamics, mitophagy and apoptosis of the cell, especially within mitochondria [56,57] where two evolutionary conserved ATP-dependent serine proteases are found, Lon and ClpXP. [58,59] These two proteases ensure a functional proteome in the matrix by maintaining and preserving the good health in the mitochondrion [1,35,60]. Lon, the protease that I have been working on is a ring-shaped self-compartmentalized protein and functions as a molecular motor driven by the energy released of ATP after hydrolysis, turning it to mechanical work. This mechanical work unfolds and subsequently cleaves the substrates of interest that are potentially harmful or dysfunctional to the cell [61].

The bacterial Lon protease was initially discovered in the early 1980s and was originally annotated as protease La. [62] A decade later, the eukaryotic homolog was reported with a size of approximately 100 kDa in the monomeric state [45]. The human Lon complex consists of a hexameric ensemble of six monomeric structures that are transported from the cell partially unfolded through the membrane of the mitochondrion with the assistance of mitochondrial targeting sequence (MTS)[63]. The MTS tag is cleaved of after the passage through the membranes, and the monomeric structures start folding and maturing followed by the formation of a hexameric complex in the mitochondrion. The monomeric structure is comprised of three unique domains. The amino-terminal domain (proposed to function in substrate recognition and binding), the AAA+ domain (ATP-binding and substrate unfoldase domain) harrboring the highly conserved Walker A and Walker B motif needed for ATP consumption [64], and finally

the protease domain (proteolytic active domain). The catalytic center of the protease domain houses the catalytic dyad comprised of S855 and K898 where the serine operates as the activating nucleophile. The dyad can cleave minor peptides without the assistance of ATP, but for bigger proteins it is necessary for the polypeptide to be unfolded prior cleavage, thus ATP hydrolysis is needed.

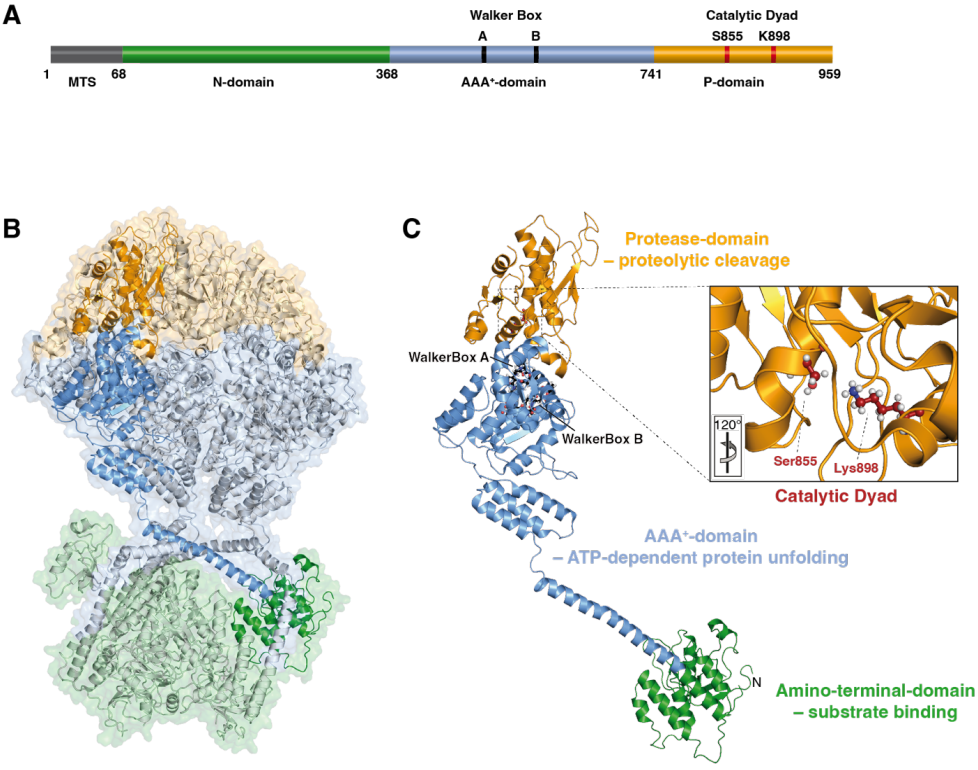

*Figure 2: **A)** Scheme of the used indicating the domain structure of the mature form of the human Lon protease. In addition, the residues belonging to the catalytic dyad as well as the Walker motif for ATP hydrolysis are indicated. **B)** Cryo-EM structure of the hexameric full-length human Lon (PDB-ID: 7NGL). The different domains (N-domain: green, AAA+-domain, blue, protease-domain: orange) are indicated. As a guide, one protomer of the hexameric assembly is not depicted transparent. **C)** Focus on one Lon protomer with a zoom-in on the catalytic dyad.*

Recognition and interaction with substrates

As mentioned previously the biggest difference between the Lon families is the N-terminal domain (NTD) that is only found in the Lon A family. It has been proposed that LonA proteases can interact both with and without the N-terminal domain being involved. This was shown by Tzeng et. al. for the mitochondrial Lon of *Meiothermus taiwanensis* that cleaved β-casein both with and without the NTD [65]. Even though this might be the case for *Meiothermus taiwanensis*, it has been showed that an truncated version of human Lon where the NTD is missing lack the

ability to cleave β-casein [66]. This points to the fact that different homologs of the LonA proteases might utilize the NTD differently and therefore it is of big interest to be able and understand the underlying mechanisms of the different types of Lon proteases and their interactions with their substrates. The NTD has been proven to be very difficult to work with in the full-length Lon due to its flexibility. Thus, gaining structural and dynamical properties of the isolated NTD is of big importance in solution to unravel detailed properties of the recognition and interaction patterns.

The regulation of mitochondrial gene expression

It is known from earlier studies that Lon is not only a protease as initially believed, but it also plays a complex role involving also DNA repair as well as the in regulation of the gene expression in the mitochondrion. Lon accomplishes these regulatory tasks with two different mechanisms, the degradation of mitochondrial RNase P Proteins 3 (MRPP3) and the degradation of Transcriptions Factor A, Mitochondria (TFAM) [67,68]. MRPP3 is the nuclease subunit of RNAseP (mitochondrial) that has a crucial role in the early RNA processing in the mitochondrion. TFAM is a protein that binds to mitochondrial DNA (mtDNA) and is crucial for maintaining the genome. The roles of TFAM involves regulating the packaging of mtDNA which determines its abundance within the mitochondria [56,57]. By degrading TFAM Lon controls the amount of TFAM present in the cell and indirectly regulate gene expression and mtDNA compaction.[70] When there is a high abundance of TFAM in the mitochondria, the gene expression decreases due to compaction of DNA hindering gene expression. When there is no DNA to bind or in cases where TFAM is dysfunctional, e.g phosphorylated and is incapable of binding DNA Lon degrades TFAM [72], resulting to the increase of gene expression. In paper three I show that Lon cleavage of TFAM is hindered in the presence of DNA while in absence of DNA 90% of TFAM is cleaved. This fits well with previous studies where they conduct cleavage assays with Lon and phosphorylated TFAM, that hinders TFAM from binding DNA and cleavage is reported.[71] .

Inhibition of Lon

As mentioned before, Lon has a catalytic dyad consisting of a Serine and a Lysine. To be able and study the active state of Lon in a steady state without any addition of substrate or ATP, that is needed for the activation of Lon, Bortezomib can be used. Bortezomib is an known protease inhibitor that is clinically used as cytostatic drug against several types of cancers such as myeloma and lymphoma. [73] In previous studies done by Su *et al.* [74] the authors could show that with bortezomib covalently attached to the catalytic serine of *Meithermus taiwanensis* Lon rearranges the conformation to the active state, this has also been recently confirmed by Shin *et al.* who could observe a similar rearrangement of the *Yersinia pestis* Lon toward the protease active conformation [75]. Bortezomib can be utilized for the analysis of structural and dynamical properties of the full-length Lon but also for the proteolytic domain since the interaction occurs with the catalytic dyad. This is more thoroughly outlined in conclusion chapter as well as in paper one where several analyses of the interaction with bortezomib have been conducted.

Chapter II: Methodology

This chapter comprises of all the methods used in my thesis that made this study possible. A more detailed explanation of the experimental details is provided in the methods sections of the accompanying manuscripts.

DNA cloning

The full-length Lon (68–959) plasmid in a pET28b(+) vector bearing an amino-terminal His_6-tag and a SUMO-solubility-tag, as well as the carboxy-terminal domain plasmid LonP (741–959) in a pET28b(+) vector with an N-terminal His6-tag and full-length TFAM (in a pET28b(+) vector harboring an N-terminal His_6-tag and a SUMO-solubility tag were purchased from GenScript codon optimized for *E. coli*. All other constructs and mutations used in this thesis derived from the different plasmids were constructed and produced by myself. The modifications mentioned involve changing individual amino acid residues, removing full or partial domains, or adding tags, such as addition of SUMO tag. To clone these new plasmids, a restriction-free cloning protocol was utilized, which allows for larger fragments of the plasmid to be removed or inserted without the use of classical restriction enzymes [76]. When inserting a new fragment, for example the SUMO tag, into a plasmid a "megaprimer" is created using standard PCR spanning the desired region of the template plasmid where the DNA alteration is intended. The megaprimer is then used as the primer pair in a standard PCR reaction, and the final product is subsequently treated with DpnI, a restriction enzyme that cleaves methylated DNA. This results in the digestion of the template plasmid used in the PCR reaction but not the newly synthesized plasmid [77]. All the primers used for the mutations were constructed by using the SerialCloner (http://serialbasics.free.fr/Serial_Cloner.html) software.

Protein expression and purification

All constructs used were chemically transformed from plasmid stocks harboring the different constructs and subsequently grown in 1l medium at 37°C in *E. coli* BL21 (λDE3) cells supplemented with 30µg/ml kanamycin due to the kanamycin resistance the cells have encoded. This means that only cells with the resistance will grow, thus minimizing the contamination risk, nor have other bacteria grow [50,78]. Upon reaching an OD_{600} of 0.6–0.8 the cells were induced with 0.4 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) [79] and proteins were expressed overnight (12–16 h) at 25°C. The cells were harvested by centrifugation at 4.500 x g for 20 minutes at 4 °C, resuspended in 70 ml lysis buffer (see manuscripts for detailed

information) and subsequently lysed *via* three passes through a high-pressure homogenizer (Emulsiflex, Avestin). Cell debris was removed by centrifugation at 20.000 x g for 1 hour at 4°C. Constructs were purified using a Ni^{2+}-Histrap (GE Healthcare) column equilibrated with lysis buffer and eluted with 500 mM imidazole. The fractions containing protein were pooled and dialyzed overnight against Buffer A (wash buffer) and subsequently loaded onto a 5 mL HeparinHP column (GE Healthcare) that has a high affinity to DNA binding proteins resulting to a separation of proteins and DNA. Elution from the heparin column was conducted with a linear gradient up to 1 M NaCl. Eluted fractions were pooled and concentrated using Vivaspin centrifugal concentrators (10k MWCO FF; Sartorius) before being applied to a gel filtration column (Superdex 75, 200 or Superose-6 Increase column (GE Healthcare)) depending on the size of the purified construct. Lastly the sample was equilibrated with NMR buffer (see manuscripts for detailed information) and the pure proteins were concentrated to a range of 0.3–1.0 mM and stored in -80°C until further usage. In the purification of the constructs harboring the SUMO tag, an additional step with cleavage of SUMO-tag while dialyzing over night was added after elution from the Ni^{2+}-HisTrap column. The dialyzed protein was loaded a second time on a Ni^{2+}-HisTrap column where cleaved construct was separated from the SUMO-solubility-tag.

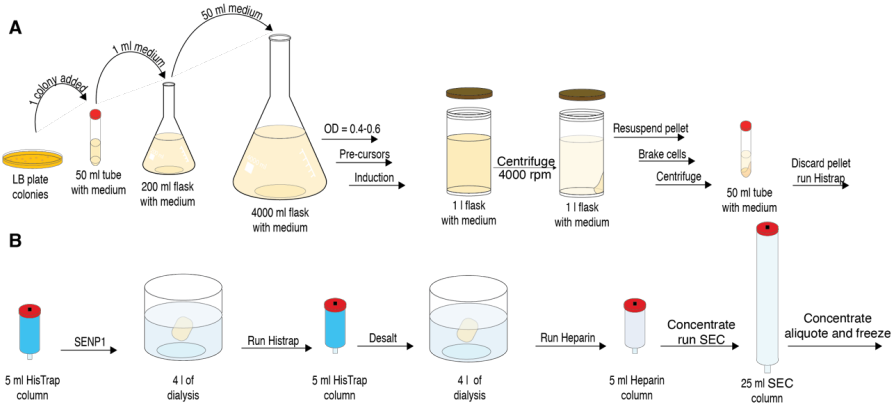

Figure 3: ***A)*** *A schematic figure showing the processes included in expression and initial purification steps separating proteins and smaller peptides from cell debris and other impurities.* ***B)*** *A schematic figure of the purification steps separating the protein of interest from unwanted proteins and smaller peptides and other impurities.*

Isotope labelling

NMR-active isotopes have to be used since all isotopes cannot be detected using NMR. The most common isotopes of hydrogen, ^{1}H and ^{2}H, are detectable and can be used in NMR. However, for the rest of the isotopes used in NMR such as nitrogen and carbon, the most naturally occurred isotopes ^{14}N and ^{12}C are not detectable with NMR. To solve this problem, the specific isotopes ^{15}N and ^{13}C have to be introduced during the growth of the cells expressing the protein of interest (explained in the section "Protein expression and purification" above). This can be achieved by supplementing the growth medium with ^{15}N-NH_4Cl and ^{13}C-glucose for ^{15}N and ^{13}C labelling, respectively. [80] Deuterium (^{2}H) can also be used instead of hydrogen (^{1}H) for labelling the protein. This is used if the protein of interest is above 25 kDa, which causes "slow tumbling" and will result in low signal-to-noise ratio and poor-resolution NMR spectra. The deuteration of the protein can help suppress dipolar interactions between heteronuclei and attached protons as well as ^{1}H-^{1}H interactions, resulting in decreased relaxation rates and improved spectral quality [81]. If the labelling of the backbone is not sufficient and a more detailed labelling scheme is needed, there are specific types of labelling that can be implemented on specific amino acids. Six of the amino acids contain a methyl-group, these are alanine, isoleucine, leucine, methionine, threonine and valine. Labeling of the methyl groups is achieved by supplementing the growth medium with isotopically labelled precursors of the methyl bearing residues taking advantage of the knowledge of amino acid synthesis pathways in *E. coli* [82–85]. By labelling specific amino acids you gain information that is isolated to only the methyl bearing amino acids of a protein. The amino acids that contain more than one methyl-group on their functional group, such as leucine and valine it gets a bit trickier since you won't know which of the methyl you are observing due to both of them giving the same signal. This problem can also be solved by labelling just one of the methyl's of leucine and valine by using stereo specific isotopes that are either *pro-R* or *pro-S* based [86,87,88].

Bio-layer Interferometry

Biolayer interferometry (BLI) is a label-free technology used to measure the interactions between biomolecules in real-time [89]. BLI relies on the principle of optical interferometry to monitor changes in the refractive index of a biomolecular layer that forms on the surface of a biosensor. The biosensor consists of a glass fiber optic tip coated with a layer of biocompatible material such as streptavidin [90]. The biomolecule of interest, e.g. a protein, is immobilized on the biocompatible layer. When the biosensor is placed in a sample containing its interacting ligand, the interaction between the biomolecules causes a change in the refractive index of the biolayer. This change is measured as a shift in the interference pattern (a change in wavelength) of the reflected light which is detected by the sensor. The magnitude of the shift in the interference pattern is governed by the number of biomolecules as well as the size of the biomolecules binding on the sensor surface. The resulting data can be used to extract kinetic parameters of the interaction, such as association and dissociation rate constants and binding affinity [91]. BLI is a highly sensitive and versatile technique that can be used to study a variety of biomolecular interactions, including protein-protein [92], protein-DNA [93,94], protein-ligand, and antibody-antigen interactions. It is also a fast and easy-to-use method that requires minimal sample preparation, making it a popular tool in many research and industry fields [89].

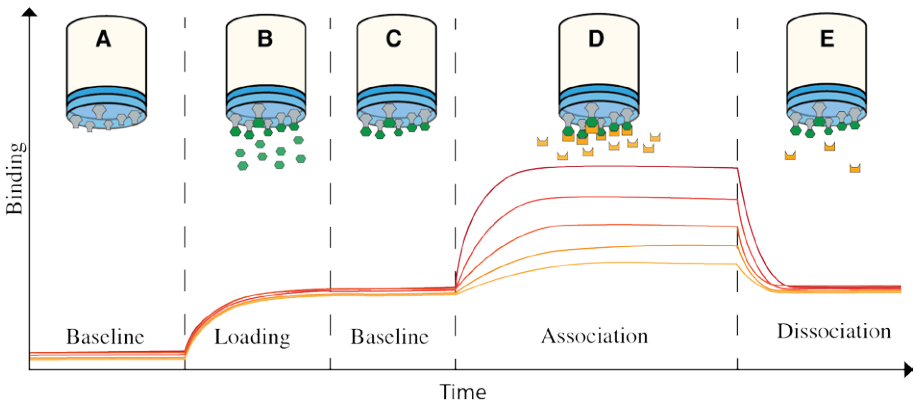

Figure 4: Schematic figure showing the different steps of al BLI run. For each step (A-E) on the top the sensor tip is shown depicting the interaction outplay. ***A)*** *The soaked tips in the buffer of usage, are submerged into wells of a 96-plate where a baseline is detected.* ***B) Next*** *the tips are submerged into wells containing the biotinylated protein. An increase is shown effected by the bounding of biotinylated protein to the surface of the tips.* ***C)*** *The tips are moved to wells with the same buffer and. a new baseline is set.* ***D)*** *Next step is association, meaning the tips are submerged into wells containing ligands with different concentrations that interact with the analyte thus, increasing the intensity of the signal.* ***E)*** *Lastly, the tips are moved into wells containing the same buffer as in the baseline steps. The ligands will dissociate and all signals, no matter the concentration of the ligands should align to the same intensity as in step* ***C****.*

Size-exclusion chromatography with multi-angle light scattering

Size-exclusion chromatography with multi-angle light scattering (SEC-MALS) is a widely used technique for the characterization of proteins and other macromolecules in solution. SEC-MALS combines two powerful analytical methods, size-exclusion chromatography (SEC) and multi-angle light scattering (MALS), to provide high-resolution information on the size, shape, and conformation of biomolecules [95]. SEC separates molecules based on their hydrodynamic size where smaller molecules elute later than larger molecules. As the sample flows through the SEC column the protein molecules are separated into different fractions based on their size. These fractions are then analyzed using the MALS detector, which measures the scattering of light by the protein in solution. The scattered light is detected at multiple angles, and the data is used to determine the molecular weight, size, and shape of the protein. SEC-MALS can be used to study protein aggregation, oligomerization, and stability, as well as to determine the purity and homogeneity of protein samples [96].

Fluorescence resonance energy transfer (FRET) assays

Fluorescence based technologies have revolutionized the field of protein-protein interaction (PPI) detection. One such technique is FLUOstar Optima, a microplate reader that is widely used for the detection of PPIs. FLUOstar Optima utilizes a variety of fluorescence-based assays to measure interactions between proteins in a high-throughput manner. These assays include Förster resonance energy transfer (FRET), time-resolved FRET (TR-FRET), and fluorescence polarization (FP). [97] Fluorescence polarization (FP), is a technique used to measure the movement and orientation of fluorescent molecules. It is based on the principle that a fluorophore that is excited with polarized light will emit light with the same polarization if it is not rotating rapidly. However, if the fluorophore is rotating rapidly the emitted light will be depolarized and give a lower intensity value. FRET the other hand is the interaction between two fluorophores in close proximity to each other with a distance less then 10 nanometers. This is accomplished when the fluorinated probes on a peptide, in this case casein is cleaved by a protease. That will release the fluorophores from the peptide making it possible to interact in a cleavage assay [98]. Cleavage assays are a type of biochemical assay that can be used to measure the activity of enzymes that break down proteins by cleaving peptide bonds [99] as shown in this thesis with Lon cleaving FITC (Fluorescein isothiocyanate-labeled)-casein.

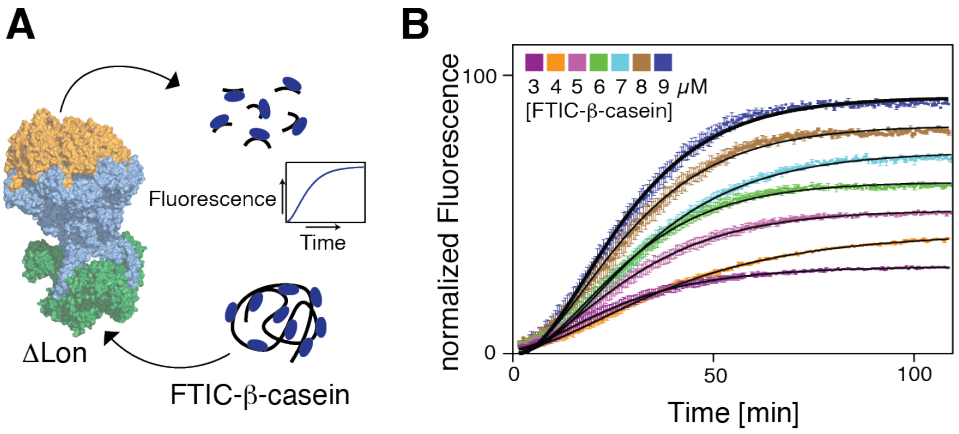

Figure 5: **A**) Scheme of a fluorescence cleavage assay. The fragments will discharge a higher source of fluorescence intensity due to the FRET-interactions of the FITC-tags compared to in folded casein. **B**: The graph shows the normalized RFU (relative fluorescence unit) on the y-axis and time (minutes) on the x-axis. Each color represents the concentration of FITC-β-casein cleaved by Lon over time. The RFU increases for each concentration due to more FITC-probes interacting with each other.

Nuclear Magnetic Resonance

History of NMR

The roots of nuclear magnetic resonance (NMR) spectroscopy go back to the early 20th century when scientists discovered that certain atoms and molecules could be excited by a magnetic field and emit radio waves [100]. In the 1940s, Isidor Rabi developed the technique of atomic beam magnetic resonance which allowed for the precise measurement of the magnetic moments of atomic nuclei. In 1946, Felix Bloch [101] and Edward Purcell [102] independently discovered NMR in liquids and solids respectively. Their discoveries revolutionized the study of atomic and molecular structure and led to numerous applications in chemistry, physics, biology, and medicine [100]. In the following decades, NMR spectroscopy was developed into a powerful analytical tool capable of providing detailed information about the chemical structure and dynamics of molecules. The introduction of Fourier transform techniques in the 1960s greatly improved the speed and accuracy of NMR measurements, and led to the development of multidimensional NMR experiments capable of providing detailed information about molecular structure [103]. NMR spectroscopy has since become a widely used technique in fields such as chemistry, biochemistry, and materials science. It has played a critical role in the development of new drugs, materials, and technologies. Today, NMR spectroscopy continues to evolve with advances in hardware and software allowing for more sensitive and precise

measurements, as well as new applications in fields such as metabolomics and structural biology. [104, 105,106]

The basics of NMR

The NMR technique relies on the magnetic properties of certain atomic nuclei to provide chemical information. The subatomic particles of an atom, such as protons and neutrons can be envisioned as rotating on their own axes. As described above, discussing isotope labeling some atoms e.g. ^{12}C the rotations are paired due to the same number of particles thus resulting in a nucleus with no overall spin, I=0. However, other atoms like ^{1}H, ^{13}C and ^{15}N the nucleus has an overall spin and thus are NMR active. This overall spin denoted as I tells us that the spin of a nucleus has 2I + 1 possible orientations, meaning that a nucleus with a I=1/2 such as ^{13}C or ^{1}H has two possible quantum states, m=+1/2 (high field) and m=-1/2 (low field) where m stand for the magnetic quantum number. These two orientations have the same energy while in absence of an external magnetic field (B_o). When an external magnetic field is applied the ratio of the energy states are determined by the thermodynamics described by the Boltzmann distribution and are equal until a certain level. The higher the applied external magnetic field is the bigger will be the difference between the two energy states become and the distributions of spins are favored towards the lower field state m=-1/2. The external magnetic field causes spins to resonate at a frequency denoted as Larmor frequency. The Larmor frequency that atoms experience depends on the properties of the gyromagnetic ratio that is unique for each nucleus. This results into different energy levels where these differences are directly connected to the gyromagnetic ratio of each atom and its surrounding local magnetic field that is governed by the shielding of electrons. This in turn will result to differences in resonance frequencies in each spin that are identified as chemical shifts. The specific chemical shifts for each atom are then detected as unique signals on a spectrum for further analysis.

Backbone assignment procedures using NMR

When you look at a [^{15}N,^{1}H]-TROSY-HSQC some general properties about the protein and its amino acids can be retrieved without knowing which peak belongs to which amino acid. You can determine if the protein is folded or partially/fully unfolded by the distribution of the peaks on the spectrum. The stability of a protein can also be concluded by observation of the peaks and how they disappear thus aggregating, precipitating or forming larger complexes with time, or by introducing different changes, such as temperature, buffer condition or addition of other interacting partners. The size, as in molecular weight, can also be retrieved by running special experiments that deduce the size of the protein quite accurately, more about the special experiments can be found below. But to be able to know which peak corresponds to which amino acid of the protein, the peaks have to be assigned. The sequential protein assignment technique was established in the Wüthrich lab in the 1980s where they used normal 2D ^{1}H COSY experiments and NOESY experiments [107–110]. COSY and NOESY experiments are very helpful in assigning aromatic residues and also to get the structural restraints. Although these methods are good, it can get very crowded in a spectrum where peaks might be difficult to separate since all the H-H interactions will be visible [111]. With time more pulse programs were developed and different ways of assigning more complex proteins were introduced such as triple resonance assigning methods. For these methods you have to label the sample of interest with at least ^{15}N and ^{13}C isotopes (see section about isotope labelling)[112–115]. For my backbone assignment experiments I used the [^{15}N,^{1}H]-TROSY-HSQC [116] experiment as a guide while several 3D experiments were used to elucidate the specific chemical shifts of the backbone atoms, 3D HNCO, 3D HNCA and 3D HNCACB as well as 3D CBCACONH experiments.[117] In an HNCO experiment, the magnetization is transferred from ^{1}H proton to ^{15}N amide and further onto the ^{13}C carbonyl of the preceding residue. It is of great use to obtain CO chemical shifts, but also to combine it with HN(CA)CO which detects the chemical shifts of both the preceding and the current carbonyls. HNCA reports on the chemical shifts of Cα of the current amino acid and Cα-1 of the preceding, whilst HNCACB reports on the chemical shifts of both Cα and Cα-1 as well as Cβ and Cβ-1 (Figure 6). If the signals for Cα-1 and Cβ-1 of the preceding amino acid are weak due to the longer distance the magnetization has to travel the experiment CBCACONH can be used. It focuses on these two signals that increases the accuracy and also confirms the assignments from HNCA and HNCACB. These interactions between amino acids can be identified through cross peaks which can be used for assigning missing peaks with the help of structural information from cryo-EM or X-ray crystallography.

Once the assignment have been conducted[118,119] secondary information can be obtained by calculation of the secondary chemical shifts using Cα and Cβ values. The POTENCI algorithm is used to retrieve the secondary chemical shifts relative to random coil values. [120]. To further refine the data and calculate more accurate shifts, a weighting function with weights 1-2-1 for residues *(i-1)-i-(i+1)* are applied. [121,122]

All the backbone-assignment data were processed using the mddNMR2.6 [123] and NMRPipe [124] software packages. Resonance assignments and spectral analyses were done in CARA [125].

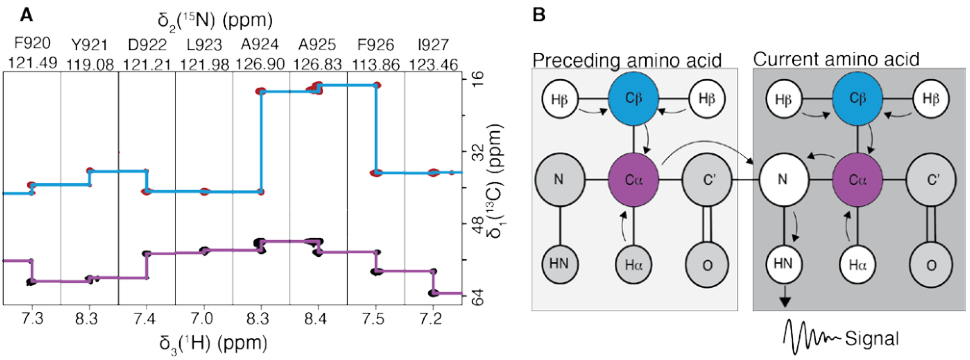

Figure 6 **A)** Representative backbone-assignment strip of a 3D TROSY-HNCACB of [*U*-^{2}H,^{15}N,^{13}C]-hLonP measured in 20 mM KPi, pH 6.8 at 37°C taken from Cara (software). Showing an example of how amino acids are connected to each other via the backbone by ^{13}C Cα and ^{13}C Cα-1(shown with purple line) and ^{13}C Cβ and ^{13}C Cβ-1 (shown with blue line). **B**) *A schematic figure showing the magnetization transfer of an HNCACB experiment. The magnetization starts at* ^{1}Hα and ^{1}Hβ of both the preceding and current amino acid through ^{13}Cβ and ^{13}Cα to ^{15}N of the current amino acid and finally reaching $^{1}N^{H}$ *for detection.*

As good as the backbone assignments experiments are there are some slight difficulties to take into account. Many of the amino acids have quite similar chemical shifts on their backbone Cα and Cβ, leading to complications in distinguishing them apart. Another problem is that the proline does not give a signal due to it lacking a backbone H^{N}-N signal that breaks the chain of magnetization transfer in the backbone. If the amino acid sequence contains a lot of prolines, it will also lead to shorter segments possible to assign which also adds to the insecurity. To come around these problems, there are other experiments that can be used as complements. In order to obtain further information, first an experiment called 3D HBHA(CO)NH is run as well as 3D (H)C(CCO)NH and 3D H(CCCO)NH that gives the Hα, Hβ, Hγ, Cα, Cβ and Cγ from the preceding amino acid. The assignments are then transferred to a 2D [^{13}C, ^{1}H]-HSQC spectrum and from there some of the carbons can be assigned. Many of the peaks for the

carbons in greater distance (for example Cγ) will not be visible or of such low intensity making it hard to assign. As a compliment to these, the experiments 3D HC(C)H-TOCSY and 3D (H)CCH-TOCSY are conducted [126]. These experiments are more sensitive to the carbons in greater distance and will make the assignments of the side chains possible. For larger complexes where the assignments are complicated due to loss of signal or to only observe specific amino acids, methyl-based NMR techniques can be implemented [127]. For these runs the sample has to be isotopically labelled with only the methyl bearing amino acids (alanine, methionine, leucine, isoleucine, valine and threonine) of interest. All the assignment techniques mentioned above have one thing in common. They all rely on correlations between the bonding to each other where the magnetization transfer travels through the direct connections of the amino acids. If some assignments still are missing or if the interactions through space in the three-dimensional space is of interest, Nuclear Overhauser effect (NOE) can be implemented. With this technique you retrieve the correlation between two or several amino acids in close vicinity to each other (no further then 5-6 Å).

Interaction studies using NMR titrations

NMR spectroscopy has various applications, including the extraction of atomic-level information on molecular interactions [128,129]. NMR can effectively capture protein-ligand interactions with binding affinities typically in the micro- to millimolar range [130]. Several NMR-based techniques such as 1D-^{1}H line broadening and chemical shift perturbations, are utilized to study protein-ligand interactions [131]. Chemical shift perturbations (CSP) can occur due to different factors, such as the proximity of aromatic rings or non-covalent interactions with ligands [132]. The perturbed chemical shifts of the protein can be evaluated by titrating the ligand towards the protein, using labelled ^{15}N or ^{13}C protein for [^{15}N,^{1}H]-TROSY-HSQC or 2D [^{13}C, ^{1}H]-HSQC titrations, respectively. This can involve ligands such as proteins [133], nucleic acids [134], or other molecules [135]. The changes in chemical shift during titration can be monitored through a series of 2D [^{1}H, ^{15}N]-HSQC spectra. Upon binding, specific cross peaks in the spectra are shifted from their original position depending on the exchange rate of labelled protein and substrate. With slow exchange, you see two distinct peaks corresponding the free protein and proteins-substrate complex, while with fast exchange you will only so the peak corresponding to the protein-substrate complex. Intermediate exchange is defined by one broadened peak that contains both the free protein peak as well as the protein-substrate complex peak. By utilizing sequence-specific resonance assignment (explained above) the displaced

cross peaks can be identified as residues in the protein located near the ligand binding site exhibiting significant ^{1}H-^{15}N CSP. Additionally, CSP can also occur from changes in backbone conformation[136] or allosteric effects due to ligand binding.[137] In this thesis the interactions of the Lon constructs with BTZ (known inhibitor), TFAM (known ligand) and several divalent ions were conducted via titrations studies using NMR. The CSP and the intensity difference data have been processed and analyzed using PINT software and are explained more in detail in the manuscripts.

Relaxation studies of proteins using NMR.

Structures of proteins are often depicted as static pictures as derived from cryo-EM studies or crystalized from X-ray studies. But contrary to this indicated rigidity proteins are much more mobile and dynamic [138,139]. The mobility of proteins, such as enzymes and proteases define the activity of the protein. These dynamic properties (time dependent changes) of a protein are usually rapid and are occurring on a range of timescales spanning from bond vibrations in the picosecond range to protein folding events in the range of seconds. By using NMR spectroscopy, these time depended dynamical changes can be readily detected with atomic resolution over these broad range of timescales [140].

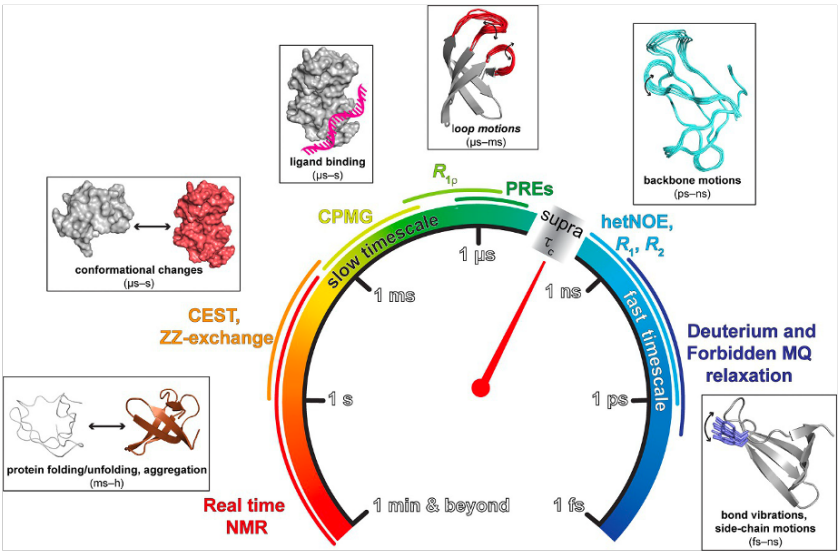

Figure 7 An overview of protein dynamics, including the timescales at which these motions occur, and highlights the available nuclear magnetic resonance (NMR) methods that can provide atomic-resolution information for a comprehensive understanding of biomolecular dynamics. Figure adapted from Kawale and Burmann [141]

The relaxation rates (R_1 and R_2) of proton-bound ^{13}C or ^{15}N nuclei in proteins are sensitive to protein motions occurring on different time scales. The information gained from an R_1 (also known as the longitudinal spin-lattice relaxation) experiment describes the recovery of the longitudinal magnetization reaching thermal equilibrium of the nuclear spins and is sensitive to motions on the pico- to nanosecond time scale, while R_2 (also called transverse spin-spin relaxation) describes the loss off phase coherence of nuclei's due to interactions with the local environment. This causes a decay of the transverse magnetization with time where the rate of decay is determined by R_2 relaxation time that is sensitive to motions on the micro- to millisecond time scale [142]. The experiment TROSY for rotational correlation times (TRACT) is used for determining $T_{2\alpha}(^{15}N)$ and $T_{2\beta}(^{15}N)$ that provide information about the molecular size of the molecules by giving an estimate of the NMR spin relaxation rates determining the value of τ_c.[143] Additionally, heteronuclear NOEs (hetNOEs) [144] sensitive to motions on the pico- to nanoseconds scale, provide information on the reorientation of the bond vector on the fast time scale. These parameters can be quantified using spectral density functions, which allow for the determination of key parameters such as the rigidity of the bond vector (expressed as the generalized order parameter S^2, the time scales of intra-molecular motions (expressed as the correlation time τ_e), the overall level of molecular tumbling (expressed as the rotational correlation time τ_c), and contributions to chemical exchange (R_{ex}).

Electrostatic Interactions

In order to gain insights into how proteins function, it is crucial to comprehend the behavior of individual side chains at key functional sites on proteins at the atomic level. Charged side chains and their electrostatic interactions play a fundamental role in molecular recognition, association, and catalysis by proteins, making them essential components to study in understanding protein mechanisms [145]. The functional activities of proteins rely on electrostatic interactions and hydrogen bonds which can be affected by the mobility of charged side chains. By conducting 2D [^{13}C,^{1}H] constant-time (CT)-HSQC experiment with 3-(Carbamoyl)-2,2,5,5-tetramethyl-1-pyrrolidineoxy (3-Carbamoyl-PROXYL; Merck) that is neutral or by addition of negatively charged 3-(Carboxy)-2,2,5,5-tetramethyl-1-pyrolidinyloxy (3- Carboxy-PROXYL; Merck) the direct effect of electrostatic interaction can be observed through changes in peak intensities and shifts [146].

Chapter III: Main results and discussion

In this section I will give a brief introduction to my thesis. I will discuss the objectives of the work chronologically and discuss the main findings that are explained in detail on the second part of this thesis consisting of the accompanying manuscripts.

The main objective when I started my PhD was to elucidate the functional details of Lon protease at the atomic level with different structural biology techniques with focus on NMR. When using NMR there is one bottleneck that complicates some experiments, that is if you use larger complexes (>25 kDa). In general, the bigger a protein complex is the larger its molecular tumbling will be resulting in broadening of peaks and loss of signal. To circumvent this issue, I used a divide-and-conquer-approach, where stable subdomains of a larger complex are constructed and investigated initially in isolation. In my case the hLonP$^{741–959}$ construct was purchased from GenScript and used for my initial experiments in this thesis (**Paper I**).

Investigating the proteolytic domain (hLonP) of the human Lon protease (Paper I)

As mentioned before, Lon in the mature hexameric complex consists of six monomeric units composed of three unique domains, the amino-terminal domain, the AAA+ domain and the proteolytic domain. I my first paper the goal was to probe the structural and dynamical properties of the LonP$^{741-959}$ domain.

When the purity and stability of the protein was confirmed, my initial step using NMR was to optimize sample conditions. [^{15}N,^{1}H]-TROSY-HSQC spectra were used as a sample quality control measure. After optimization of the conditions, I could obtain high-quality spectra showing well-dispersed resonances indicating the existence of a well-folded protein enabling subsequent 3D dimensional resonance-assignment experiments (**Figure 8A**).

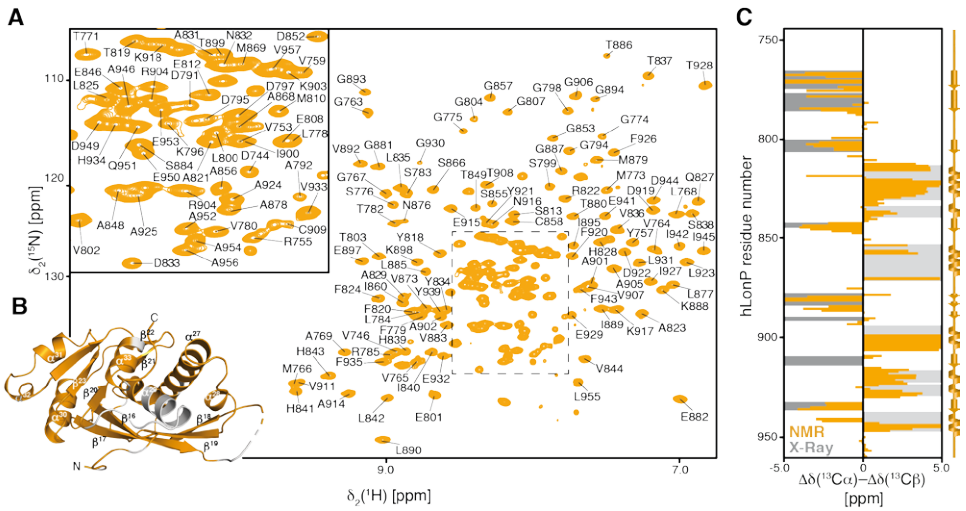

Figure 8 ***A:*** *A 2D [^{15}N,^{1}H]-TROSY-HSQC NMR spectrum of [U ^{2}H, ^{15}N,^{13}C]-hLonP$^{741-959}$. The sequence-specific resonance assignments were obtained from 3D TROSY-based backbone assignment experiments and are indicated with the specific amino acids followed by their position of the full amino acid sequence of Lon.* ***B:*** *A crystal structure of isolated hLonP (PDB: 2X36) shown in grey with annotated fully assigned stretches in selenium. The secondary structures of the protein are annotated indicating their secondary structure and numbering.* ***C:*** *Secondary backbone ^{13}C chemical shifts of hLonP plotted against the residue number in selenium. Positive values indicate α-helical structures whereas negative values indicate β-sheet structure. For comparison the secondary structure elements of isolated hLonP crystal structure are indicated in grey.*

The first conclusion we could draw on the information we got from the NMR assignments data comparing it to the available X-ray data conducted by J.García-Nafría et al [147], was that the secondary structure elements were generally well-maintained in solution (**Figure 8C**). This indicates that our solution structure of monomeric hLonP forms the same secondary structure as the crystalized hLonP, confirming the assumption that our protein behaves similar as seen in earlier studies. Next, I carried on with various backbone relaxation experiments to gain an understanding of the dynamical properties of hLonP. A series of R_1, R_2 and hetNOE relaxation experiments were conducted at two different magnetic fields and used in the analysis phase to employ the Lipari-Szabo Modell-Free approach[148] used to extract the S^2 (generalized order parameter reporting on the "rigidity" of the protein) as well as R_{ex} (chemical exchange contribution indicating possibly conformational exchange) by using the Tensor [149] fastMF programs[150] . We observed for the relaxation experiments mainly flat profiles that indicates high stability of the hLonP fold in general. The only regions that indicate some dynamical movements were the vicinity of the catalytic site (the close to the catalytic dyad serine 855 and lysine 898), and the strand β16 (**Figure 8B**). To get a more thorough understanding of the structure properties we in addition conducted methyl-methyl NOEs experiments on the

assigned MALVIT-hLonP. The NOEs revealed an extended network connections pointing once again to the stability of the protein. To investigate the dynamics of the methyl groups further we employed CPMG relaxation dispersion experiments that interestingly showed almost no exchange induced broadening that tells us the sidechains are not involved in the chemical exchange process that goes in line with protein stability.

Our next step was to investigate the potential effect pH could have on hLonP activity since it was reported earlier that the pH optimum is around 7.8 [151]. hLonP alone is in my hands also not proteolytically active in agreement with previous reports. Experimentally, I assessed the activity of full-length by applying cleavage assays both *via* SDS-PAGE as well as by the usage of fluorescence assays. **(Figure 9)**

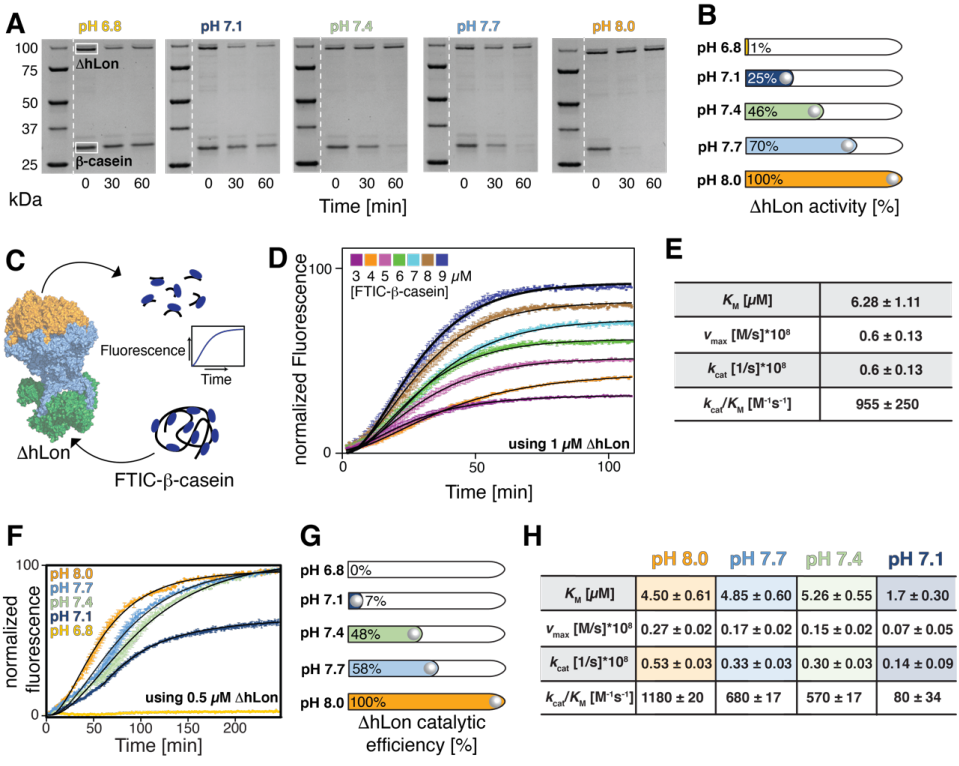

Figure 9 ***A****: SDS-PAGE gel cleavage assays conducted in several pH buffers.* ***B****) Comparison of the proteolytic efficiency of Lon towards b-casein at the different pH-values tested.* ***C****) Schematics of the fluorescence cleavage assay using the self-quenching fluorescein-5-isothiocyanate-(FTIC)-casein.* ***D****) Fluorescence assay with a range of (FTIC)-β-casein concentrations to gain robust fitting.* ***E****) Extracted enzymatic constants and catalytic rates from the data shown in panel D.* ***F****) Fluorescence assay at constant substrate concentration of 3 μM using 0.5 μM hLon using different pH-conditions.* ***G****) Analysis of the catalytic efficiency (k_{cat}/K_M) at the different pH-conditions used.* ***H****) pH-dependence of the extracted enzymatic constants and catalytic rates from the data shown in panel F.*

I first established SDS-PAGE gel analysis in five different pH ranging from 6.8-8.0. **(Figure 9A).** The proteolytic activity was measured by analyzing the intensity of the gel bands and determining the amount of casein Lon was possible to cleave. At pH 6.8 there was no cleavage activity noted while there was an increase in activity for each increased pH run reaching full cleavage at pH 8.0. **(Figure 9B)** To further asses the activity and gain enzymatic parameters we ran fluorescence cleavage assays using a constant concentration of Lon and different concentrations of [FITC]-β-casein we obtained a catalytic efficiency (k_{cat}/K_M) of ~960 ± 250 $M^{-1}s^{-1}$ in the presence of 1 μM ΔhLon, which fits well with previous reports ranging from 1,000 – 3,000 $M^{-1}s^{-1}$ [152,2] **(Figure 9D-E)**. I also noticed a remarkable decrease in proteolytic activity by the change of pH where a decrease of 0.3 in pH reduced the v_{max} (the maximum velocity) by almost 50% **(Figure 9H)**. The effect we saw from the pH changes made us think if divalent ions could have an effect on the activity as well. Even though human Lon is not a metalloprotease it does need Mg^2 for its unfolding reactions v*ia* the AAA+-domain. And with the importance of ion-homeostasis in cells, especially in mitochondria we carried out several experiments with different divalent ions, to see if they would have an impact. We ran a series of NMR titrations with $CaCl_2$, $ZnSO_4$ and $CuSO_4$ titrated to [*U*-^{15}N]-labeled hLonP as well as in-gel and fluorescence cleavage assays as outlined for the pH-dependent experiments using ΔhLon. Ca^{2+} had a delayed effect on the cleavage decreasing the v_{max} approximately 40% whilst Cu^{2+} and Zn^{2+} completely deactivated proteolytic activity (see Figure 5 in MS1). These findings are of high importance especially for a protease such as Lon that has a high involvement in many different fields and is of great importance for the well-being of cells.

Structure and dynamics of the amino-terminal domain (NTD) of human Lon (Paper II)

The amino terminal domain of Lon found in the Lon A family (see introduction) is of high importance for the recognition and interaction of potential aggregates as well as substrates. From previous studies conducted with X-ray and Cryo-EM it could be shown that this domain can sample multiple orientation impairing structural refinement of the NTD due to its flexible properties. Thus, I started to conduct exploit the applicability of advanced high-resolution NMR-spectroscopy to the human Lon NTD to gain additional information of its structural as well as the dynamical properties. The initial step was to obtain an isolated hLon by implementing the divide-and-conquer approach. This was accomplished and a fairly-stable constructs denoted hLonN$^{68-368}$ was designed. The purification and the initial molecular weight were determined by usage of SDS-PAGE and SEC-MALS (see methodology), where a defined range of oligomeric states was identified (monomer, dimer and trimer) **(Figure 10).**

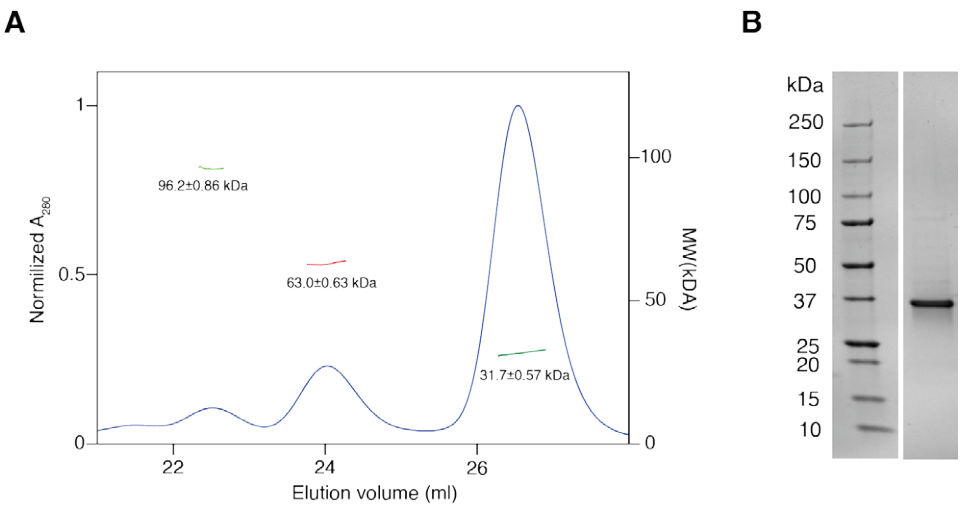

Figure 10 ***A:*** *SEC-MALS elution profile of hLonN recorded on a 400 µM protein sample in NMR-buffer at room temperature.* ***B:*** *SDS-PAGE of purified hLonN indicating a single band at ~33 kDa.*

To further investigate the structural and dynamical properties of hLonN we initiated a series of NMR experiments, as outlined before for the hLonP domain. The initial ^{15}N HSQC spectrum

looked promising with well dispersed peaks with a subset of resonances showing an intensity increase in the middle region. This observation indicated that hLonN possesses flexible regions, indicating the presence of local unfolded parts such as intrinsically disordered regions besides the well-folded parts of the protein evident by the well-dispersed spectrum. A set of backbone assignment experiments facilitating the sequence-specific resonance assignment. Due to the multiple oligomeric states hLonN exhibits in combination with the highly dynamic regions thus far only approximately 25% of the resonances could be assigned **(Figure 11A)**. As shown in **Figure 11A** the vast majority of assignments are located between 7.5–8.5 ppm, the typical ^{1}H region reporting on amide moieties outside of stable secondary structure elements. When comparing the assigned regions to the AlphaFold2 structural model most of the obtained assignments belong to the loop regions further evidencing the assumption that hLonN is highly flexible in solution as well as in previous studies conducted. **(Figure 11B)**

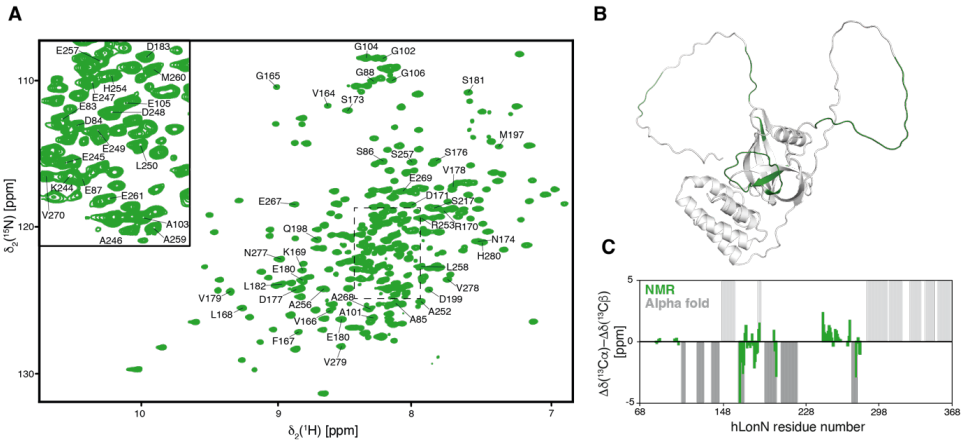

Figure 11 A: 2D [^{15}N, ^{1}H]-NMR spectrum of the [U-^{2}H,^{15}N, ^{13}C]-hLonN (NTD). The sequence specific resonance assignment based on triple resonance experiments. B: The assignments plotted on the Alpha Fold structure. C: Secondary structure elements derived from the combined $^{13}C\alpha$ and $^{13}C\beta$ chemical shifts (green), plotted against the calculated chemical shifts of Alpha fold (grey).

To validate the assumption that the assigned peaks are indeed in intrinsically disordered regions exhibiting random coil structure, we analyzed the secondary chemical shifts of the assigned peaks as explained in methodology section. We noted that most of the assigned peaks except a small strand consisting of β-sheet formation were of random coil formation in agreement with the structural model **(Figure 11C).** To get a deeper understanding of the inherent dynamic properties we ran specifically labeled methionine, alanine, leucine, valine and isoleucine

(MALVI) methyl spectra. We chose to label those amino acids due to the good distribution throughout hLonN. The [^{13}C,^{1}H]-NMR spectra were of very good quality and when overlayed with a specifically labeled Leucine and Valine (LV)*-hLonN sample yielded to full identification of the alanine, isoleucine, leucine and valine as well as the methionine by measuring a constant time [^{13}C,^{1}H]-HSQC inverting the signal of the methionine methyl-groups. To further analyze the methyl groups, we conducted CPMG relaxation dispersion experiments that senses motions of the micro- to milliseconds timescale giving information of conformational exchange processes between residues. In summary the initial analysis does not point to global conformational exchange that would mean the protein is instable but rather to some methyl groups involvement in activities in the regulatory process modulating hLonN engagement with substrate. In conclusion these initial experiments clearly show that structural and dynamical studies of isolated hLonN are feasible by usage of NMR spectroscopy.

Dynamics and allosteric regulation of full-length human Lon as seen by NMR spectroscopy (Paper III)

In this paper we explored the possibility if we could characterize the full-length Lon employing solution NMR-spectroscopy even though it has a hexameric size of 600 kDa. In most cases when conducting NMR experiments on big complexes the signal is expected to be low and centered as a "blob" in the middle region of the spectrum in the random coil region 7.5–8.5 ppm of the ^{1}H-dimension as it has been reported for comparable multimeric complexes previously.[153] In contrast we observed a remarkably good spectrum taking into account the size of the ΔhLon hexamer. Since we had obtained the sub-spectra of hLonP (proteolytic domain) (Paper I) and hLonN (Paper II) already earlier, I could directly overlay the spectra to observe eventual matching resonance peak patterns. Initially, we expected to get a match with hLonN due to it being very flexible as previously seen from earlier studies [75] and also reported in paper II by our investigation. To our surprise this was not the case and we got a fairly good match with hLonP spectrum. **(Figure 12A)**. This would suggest that the proteolytic domain of ΔhLon in absence of ATP or other substrates is flexible. This hypothesis has to a certain extent been suggested in a previous study for *Yersinia pestis* Lon where the authors hypothesized that an inactive state of Lon induces a formation of a helical staircase for the full-length protein breaking stabilizing contact between adjacent proteolytic domains.[154]

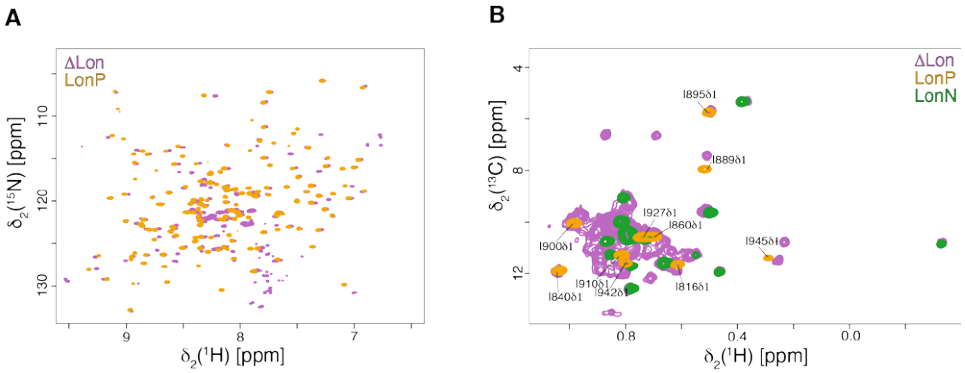

Figure 12 ***A:*** *Amide spectra of ΔhLon. 2D [^{15}N,^{1}H]-NMR spectrum of [U-^{2}H,^{15}N]- ΔhLon (purple) overlayed with a 2D [^{15}N,^{1}H]-NMR spectrum of the [U-^{2}H,^{15}N]-hLonP (orange). Protein concentrations were ~150 μM and the samples were measured in NMR buffer at 310 K on an 18.8 T Bruker AvanceIII magnet with a ^{1}H-frequency of 800 MHz.* ***B:*** *2D [^{13}C,^{1}H]-NMR spectrum of [U-^{2}H,^{15}N, Ile-δ_1-^{13}CH$_3$]-ΔhLon (purple) overlayed with 2D [^{13}C,^{1}H]-NMR spectra focusing on the isoleucine methyl region of [U-^{2}H, Ile-δ_1-^{13}CH$_3$, Leu-δ,Val-γ-^{13}CH$_3$-^{12}CD$_3$, Ala-β-^{13}CH$_3$, Thr-γ_2-^{13}CH$_3$, Met-ε-^{13}CH$_3$]-hLonP (orange) and [U-^{2}H, Ile-δ_1-^{13}CH$_3$, Leu,Val-^{13}CH$_3$-^{12}CD$_3$, Ala–β–^{13}CH$_3$, Met-ε-^{13}CH$_3$]-hLonN (green). Protein concentrations were ~250 μM and the samples were measured in NMR buffer at 310 K on a 21.1 T Bruker AvanceIII magnet with a ^{1}H-frequency of 900 MHz.*

The observations from the ^{15}N HSQC data made us question if we would be able and gain high quality information from methyl labeled spectra as-well. We therefore labeled in a next step the isoleucine methyl groups of ΔhLon as they are evenly distributed across the ΔhLon sequence. After we established the specific labeling and got our initial spectrum, we observed 47 peaks (in purple) out of 48 expected isoleucines peaks that ΔhLon contains. Further, we overlayed the isoleucine methyl spectrum with the hLonP and hLonN methyl spectra **(Figure 12B)** yielding a high number of overlapping peaks. This opens the possibility of transferring directly assignments from the respective subdomains to larger protein complexes and further enforces feasibility of the divide-and-conquer approach for this system.

Next, I explored if we can obtain analyzable initial data by running several interaction assays with Lon and its known natural substrate TFAM. We conducted cleavage assays in the same manner as outlined in Paper I but with TFAM as the substrate instead on β-casein. As previously shown, we could see that ΔhLon cleaves TFAM when no DNA is present, but also that Bortezomib, the known protease inhibitor blocks the cleavage by covalently binding to the serine of the catalytic dyad. Further we wanted to gain enzymatic data of the binding to TFAM. Since we were only interested in the binding and not the proteolytic activity, we constructed an inactive mutant by substituting serine 855 to an alanine. We ran the experiments using Bio-Layer Interferometry (see methodology) and gained an initial K_D value of 360 nM when ATP was added and 76 nM with the addition of ADP. This indicates that the interaction is rather tight and could be implemented in the future to run ΔhLon with TFAM bound in complex using solution NMR experiments to further elucidate the recognition and interaction parameters of Lon and TFAM.

Structure and dynamics of the mitochondrial DNA-compaction factor Abf2 from *S. cerevisiae* (Paper IV)

The micron-sized mitochondria organelles are of high importance for the eukaryotic cells in production of energy via the oxidative phosphorylation (OXPHOS) system. The mitochondrial genome (mtDNA) encodes several of the subunits used in the OXPHOS system and together with subunits govern the OXPHOS system. In the budding yeast *Saccharomyces cerevisiae* approximately 50 copies of mainly linear mtDNA molecules/cell are found which are in need of packaging into spherical shapes called nucleoids due to the extensive length in uncoiled state. Thus, the mitochondria are depended on a DNA condensing mechanism that confines the size of mtDNA. ARS-binding factor 2 (Abf2) is a protein encoded by the nucleus that governs the packaging of mtDNA in the mitochondria via the two DNA binding domains, high mobility group-box 1 (HMG1) and high mobility group-box 2 (HMG2). Recent studies by X-ray crystallography revealed that full-length Abf2 and its homolog Transcription Factor A, Mitochondria (TFAM) found in human cells bind to DNA via these HMG domains. However, it is still unclear how these mitochondrial nucleoprotein complexes are formed and regulated in the molecular level. In this paper a detailed biophysical characterization of Abf2 was conducted with NMR spectroscopy and other biophysical techniques such as Bio-layer Interferometry. The research revealed new knowledge of the Abf2-DNA interactions, as well as the interactions of isolated HMG domains with DNA by implementing the divide and conquer approach. Interestingly, it was showed that the isolated HMG boxes differ a lot in stability where HMG1 meta-stability first initially binds to DNA followed by the HMG2 enhancing the affinity in full length Abf2 to DNA five-fold in compared to HMG1 alone. It was also showed that isolated HMG2 shows weak if no binding to DNA alone, that further more goes in line with the theory that the initial recognition and binding to DNA is governed by HMG1 followed by assistance of HMG2. Thus, the present study will possibly be an important foundation for future studies into the contribution of Abf2 into the formation of mitochondrial nucleoids in yeast.

NMR approaches to reveal the intricate details of the dynamic underlying protein quality control (Paper V)

In this book chapter we focus on the contributions Nuclear Magnetic Resonance (NMR) spectroscopy has had in elucidating structural and functional details of chaperone:client complexes as well as the multimeric proteases and their respective involvement in the protein quality control (PQC) system. The PQC system is mother nature's way of insuring that the homeostasis of the cell is governed and taken care of in all kingdoms of life. The path of a protein going from newly translated unfolded structure to functionally folded protein is filled with potential errors and road bumps. Even though many proteins are fully capable of folding themselves the mechanism governing these actions can misfire leading to incomplete folding and help is needed. Molecular chaperones are specialized in not just helping misfolded proteins to refold but also transporting them to the designated area where the protein in question resides. If the refolding is not possible or by any other reasons the protein is too damaged it has to be discarded. Proteases are well known for their unfolding properties as well as their ability to cleave peptide bonds. These two molecular machine classes are crucial in the PQC system and has been a prevalent theme in research for decays. Many different techniques have been implemented in the search of understanding the underlying mechanisms to chaperones and proteases such as X-ray crystallography as well as Electron Microscopy. The two techniques combined has generated a great amount of data increasing the general understanding of how these complexes are structured. But to fully comprehend how the underlying mechanisms of chaperones and proteases function the dynamical properties are needed. In the last 10–15 years NMR spectroscopy has emerged as an extremely powerful technique not only to gain structural information of proteins but also assessing dynamical as well and interacting properties. The chapter reviews the current state of research with a particular focus on the bacterial chaperone system in the biogenesis of integral outer membrane protein on the one hand as well as mitochondrial serine proteases on the other hand.

Chapter IV: Future perspectives of Lon studies

Lon proteases have been studied since the first protein was discovered in the early 80s, accumulating to almost 45 years of research. Even though this immense time of research has been dedicated to Lon proteases there is still much more that has not been unraveled. To understand how complex machineries such as Lon proteases, especially the human Lon, work demands deep analysis of the underlying mechanisms governing Lons role within the PQC system as discussed in the introduction as well as in paper V.

In **paper I** we could see that hLonP (proteolytic domain) structural as well as the dynamical properties play a crucial role of Lon pH-dependent activation. The indication that histidine 839 and histidine 841 participates in the inhibitory binding point to a potential involvement due to their pH-sensitive properties. These observations are of high interest for future investigation taking to account that a second active site has been purposed for hLon.

In **paper II** we could show that the divide and conquer approach is applicable while using NMR spectroscopy to gain initial insight in the dynamics and structure of hLonN (amino terminal domain). Due to the oligomerization properties of hLonN as well as hLonN harboring highly dynamic loops as shown in paper II complicates deeper investigations. A future aspect would be to experiment with the buffer conditions to gain stability but also introducing mutations to gain a monomeric state that would facilitate in revealing the underlying contributions of hLonN in recognition and interaction with clients.

In **paper III** we conducted several NMR experiments proving that even though hexameric human Lon has a size of 600 kDa it was feasible to gain information. This taking into account that research of large complexes with NMR has historically been a struggle due to the molecular tumbling and loss off signal. Proving that we could gain initial data of full-length Lon using NMR spectroscopy opens many doors in future research, such as specifically label aromatic groups or the usage of fluor labelling experiments.

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References

1. Goldberg, A. L., Moerschell, R. P., Hachung, C. & Maurizi, M. R. [25] ATP-dependent protease La (Lon) from Escherichia coli. in *Methods in Enzymology* vol. 244 350–375 (1994).
2. Lee, I. & Suzuki, C. K. Functional mechanics of the ATP-dependent Lon protease- lessons from endogenous protein and synthetic peptide substrates. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* **1784**, 727–735 (2008).
3. Ambro, L., Pevala, V., Bauer, J. & Kutejová, E. The influence of ATP-dependent proteases on a variety of nucleoid-associated processes. *Journal of Structural Biology* **179**, 181–192 (2012).
4. Callender, R. & Dyer, R. B. The Dynamical Nature of Enzymatic Catalysis. *Accounts of Chemical Research* **48**, 407–413 (2015).
5. Moseley, P. Stress proteins and the immune response. *Immunopharmacology* **48**, 299–302 (2000).
6. Aravind, L. Conserved domains in DNA repair proteins and evolution of repair systems. *Nucleic Acids Research* **27**, 1223–1242 (1999).
7. Lentz, B. R., Malinin, V., Haque, M. E. & Evans, K. Protein machines and lipid assemblies: current views of cell membrane fusion. *Current Opinion in Structural Biology* **10**, 607–615 (2000).
8. Alberts, B. The Cell as a Collection of Protein Machines: Preparing the Next Generation of Molecular Biologists. *Cell* **92**, 291–294 (1998).

9. Martinus, R. D. *et al.* Selective Induction of Mitochondrial Chaperones in Response to Loss of the Mitochondrial Genome. *European Journal of Biochemistry* **240**, 98–103 (1996).
10. Chen, B., Retzlaff, M., Roos, T. & Frydman, J. Cellular Strategies of Protein Quality Control. *Cold Spring Harbor Perspectives in Biology* **3**, a004374–a004374 (2011).
11. Tatsuta, T. Protein Quality Control in Mitochondria. *Journal of Biochemistry* **146**, 455–461 (2009).
12. Czarnecka, A. M., Campanella, C., Zummo, G. & Cappello, F. Mitochondrial chaperones in cancer: From molecular biology to clinical diagnostics. *Cancer Biology & Therapy* **5**, 714–720 (2006).
13. Liyanagamage, D. S. N. K. & Martinus, R. D. Role of Mitochondrial Stress Protein HSP60 in Diabetes-Induced Neuroinflammation. *Mediators of Inflammation* **2020**, 1–8 (2020).
14. Stuart, R. A., Cyr, D. M. & Neupert, W. Hsp70 in mitochondrial biogenesis: From chaperoning nascent polypeptide chains to facilitation of protein degradation. *Experientia* **50**, 1002–1011 (1994).
15. Huttlin, E. L. *et al.* Architecture of the human interactome defines protein communities and disease networks. *Nature* **545**, 505–509 (2017).
16. Wagner, I., Arlt, H., van Dyck, L., Langer, T. & Neupert, W. Molecular chaperones cooperate with PIM1 protease in the degradation of misfolded proteins in mitochondria. *The EMBO Journal* **13**, 5135–5145 (1994).
17. Szczepanowska, K. & Trifunovic, A. Mitochondrial matrix proteases: quality control and beyond. *The FEBS Journal* **289**, 7128–7146 (2022).
18. Warshel, A., Naray-Szabo, G., Sussman, F. & Hwang, J. K. How do serine proteases really work? *Biochemistry* **28**, 3629–3637 (1989).

19. Di Cera, E. Serine proteases. *IUBMB Life* **61**, 510–515 (2009).

20. Hedstrom, L. Serine Protease Mechanism and Specificity. *Chemical Reviews* **102**, 4501–4524 (2002).

21. Reiss, K. & Saftig, P. The "A Disintegrin And Metalloprotease" (ADAM) family of sheddases: Physiological and cellular functions. *Seminars in Cell & Developmental Biology* **20**, 126–137 (2009).

22. Sieber, S. A., Niessen, S., Hoover, H. S. & Cravatt, B. F. Proteomic profiling of metalloprotease activities with cocktails of active-site probes. *Nature Chemical Biology* **2**, 274–281 (2006).

23. Barrett, A. J. & Rawlings, N. D. Families and Clans of Serine Peptidases. *Archives of Biochemistry and Biophysics* **318**, 247–250 (1995).

24. Rawlings, N. D. & Barrett, A. J. Evolutionary families of peptidases. *Biochemical Journal* **290**, 205–218 (1993).

25. Bota, D. A. & Davies, K. J. A. Lon protease preferentially degrades oxidized mitochondrial aconitase by an ATP-stimulated mechanism. *Nature Cell Biology* **4**, 674–680 (2002).

26. Consolato, F., Maltecca, F., Tulli, S., Sambri, I. & Casari, G. *m* -AAA and *i* -AAA complexes work coordinately regulating OMA1, the stress-activated supervisor of mitochondrial dynamics. *Journal of Cell Science* jcs.213546 (2018).

27. Meissner, C., Lorenz, H., Weihofen, A., Selkoe, D. J. & Lemberg, M. K. The mitochondrial intramembrane protease PARL cleaves human Pink1 to regulate Pink1 trafficking: Rhomboid protease PARL cleaves human Pink1. *Journal of Neurochemistry* **117**, 856–867 (2011).

28. Alavi, M. V. Targeted OMA1 therapies for cancer. *International Journal of Cancer* **145**, 2330–2341 (2019).

29. Quirós, P. M., Mottis, A. & Auwerx, J. Mitonuclear communication in homeostasis and stress. *Nature Reviews Molecular Cell Biology* **17**, 213–226 (2016).

30. Hrdinka, M. & Yabal, M. Inhibitor of apoptosis proteins in human health anddisease. *Genes & Immunity* **20**, 641–650 (2019).

31. Kraut, J. Serine Proteases: Structure and Mechanism of Catalysis. *Annual Review of Biochemistry* **46**, 331–358 (1977).

32. Ekici, Ö. D., Paetzel, M. & Dalbey, R. E. Unconventional serine proteases: Variations on the catalytic Ser/His/Asp triad configuration. *Protein Science* **17**, 2023–2037 (2008).

33. Confalonieri, F. & Duguet, M. A 200-amino acid ATPase module in search of a basic function. *BioEssays: News and Reviews in Molecular, Cellular and Developmental Biology* **17**, 639–650 (1995).

34. Neuwald, A. F., Aravind, L., Spouge, J. L. & Koonin, E. V. AAA+: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. *Genome Research* **9**, 27–43 (1999).

35. Sauer, R. T. & Baker, T. A. AAA+ Proteases: ATP-Fueled Machines of Protein Destruction. *Annual Review of Biochemistry* **80**, 587–612 (2011).

36. Ogura, T. & Wilkinson, A. J. AAA+ superfamily ATPases: common structure--diverse function. *Genes to Cells: Devoted to Molecular & Cellular Mechanisms* **6**, 575–597 (2001).

37. Voos, W. & Röttgers, K. Molecular chaperones as essential mediators of mitochondrial biogenesis. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **1592**, 51–62 (2002).

38. Park, H.-M. *et al.* The serine protease HtrA2/Omi cleaves Parkin and irreversibly inactivates its E3 ubiquitin ligase activity. *Biochemical and Biophysical Research Communications* **387**, 537–542 (2009).

39. Belostotsky, R. *et al.* Mutations in the Mitochondrial Seryl-tRNA Synthetase Cause Hyperuricemia, Pulmonary Hypertension, Renal Failure in Infancy and Alkalosis, HUPRA Syndrome. *The American Journal of Human Genetics* **88**, 193–200 (2011).

40. Rai, M., Curley, M., Coleman, Z. & Demontis, F. Contribution of proteases to the hallmarks of aging and to age-related neurodegeneration. *Aging Cell* **21**, (2022).

41. Hua, Y. & Nair, S. Proteases in cardiometabolic diseases: Pathophysiology, molecular mechanisms and clinical applications. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* **1852**, 195–208 (2015).

42. Park, K. C., Dharmasivam, M. & Richardson, D. R. The Role of Extracellular Proteases in Tumor Progression and the Development of Innovative Metal Ion Chelators That Inhibit Their Activity. *International Journal of Molecular Sciences* **21**, 6805 (2020).

43. Inui, T. *et al.* A novel mutation in the proteolytic domain of LONP1 causes atypical CODAS syndrome. *J Hum Genet* **62**, 653–655 (2017).

44. Besse, A., Brezavar, D., Hanson, J., Larson, A. & Bonnen, P. E. LONP1 de novo dominant mutation causes mitochondrial encephalopathy with loss of LONP1 chaperone activity and excessive LONP1 proteolytic activity. *Mitochondrion* **51**, 68–78 (2020).

45. Wang, N., Gottesman, S., Willingham, M. C., Gottesman, M. M. & Maurizi, M. R. A human mitochondrial ATP-dependent protease that is highly homologous to bacterial Lon protease. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 11247–11251 (1993).

46. Perona, J. J. & Craik, C. S. Structural basis of substrate specificity in the serine proteases. *Protein Science* **4**, 337–360 (2008).

47. Rotanova, T. V. *et al.* New insights into structural and functional relationships between LonA proteases and ClpB chaperones. *FEBS Open Bio* **9**, 1536–1551 (2019).

48. Li, J.-K. *et al.* The N-terminal substrate-recognition domain of a LonC protease exhibits structural and functional similarity to cytosolic chaperones. *Acta Crystallogr D Biol Crystallogr* **69**, 1789–1797 (2013).

49. Liao, J.-H. *et al.* Structures of an ATP-independent Lon-like protease and its complexes with covalent inhibitors. *Acta Crystallographica Section D Biological Crystallography* **69**, 1395–1402 (2013).

50. Studier, F. W. & Moffatt, B. A. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *Journal of Molecular Biology* **189**, 113–130 (1986).

51. Van Dyck, L., Pearce, D. A. & Sherman, F. PIM1 encodes a mitochondrial ATP-dependent protease that is required for mitochondrial function in the yeast Saccharomyces cerevisiae. *Journal of Biological Chemistry* **269**, 238–242 (1994).

52. López-Otín, C. & Bond, J. S. Proteases: Multifunctional Enzymes in Life and Disease. *Journal of Biological Chemistry* **283**, 30433–30437 (2008).

53. López-Otín, C. & Hunter, T. The regulatory crosstalk between kinases and proteases in cancer. *Nat Rev Cancer* **10**, 278–292 (2010).

54. Turk, B., Turk, D. & Turk, V. Protease signalling: the cutting edge: Protease signalling. *The EMBO Journal* **31**, 1630–1643 (2012).

55. Quirós, P. M., Langer, T. & López-Otín, C. New roles for mitochondrial proteases in health, ageing and disease. *Nature Reviews Molecular Cell Biology* **16**, 345–359 (2015).

56. Koppen, M. & Langer, T. Protein Degradation within Mitochondria: Versatile Activities of AAA Proteases and Other Peptidases. *Critical Reviews in Biochemistry and Molecular Biology* **42**, 221–242 (2007).

57. Anand, R., Langer, T. & Baker, M. J. Proteolytic control of mitochondrial function and morphogenesis. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **1833**, 195–204 (2013).

58. Rugarli, E. I. & Langer, T. Mitochondrial quality control: a matter of life and death for neurons: Mitochondrial quality control and neurodegeneration. *The EMBO Journal* **31**, 1336–1349 (2012).

59. Goard, C. A. & Schimmer, A. D. Mitochondrial matrix proteases as novel therapeutic targets in malignancy. *Oncogene* **33**, 2690–2699 (2014).

60. Van Melderen, L. & Aertsen, A. Regulation and quality control by Lon-dependent proteolysis. *Research in Microbiology* **160**, 645–651 (2009).

61. Erzberger, J. P. & Berger, J. M. EVOLUTIONARY RELATIONSHIPS AND STRUCTURAL MECHANISMS OF AAA+ PROTEINS. *Annual Review of Biophysics and Biomolecular Structure* **35**, 93–114 (2006).

62. Chung, C. H. & Goldberg, A. L. The product of the lon (capR) gene in Escherichia coli is the ATP-dependent protease, protease La. *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4931–4935 (1981).

63. Omura, T. Mitochondria-Targeting Sequence, a Multi-Role Sorting Sequence Recognized at All Steps of Protein Import into Mitochondria. *Journal of Biochemistry* **123**, 1010–1016 (1998).

64. Walker, J. E., Saraste, M. & Gay, N. J. E. coli F1-ATPase interacts with a membrane protein component of a proton channel. *Nature* **298**, 867–869 (1982).

65. Tzeng, S.-R. *et al.* Molecular insights into substrate recognition and discrimination by the N-terminal domain of Lon AAA+ protease. *eLife* **10**, e64056 (2021).

66. Kereïche, S. *et al.* The N-terminal domain plays a crucial role in the structure of a full-length human mitochondrial Lon protease. *Scientific Reports* **6**, 33631 (2016).

67. Li, F., Liu, X., Zhou, W., Yang, X. & Shen, Y. Auto-inhibitory Mechanism of the Human Mitochondrial RNase P Protein Complex. *Scientific Reports* **5**, 9878 (2015).

68. Münch, C. & Harper, J. W. Mitochondrial unfolded protein response controls matrix pre-RNA processing and translation. *Nature* **534**, 710–713 (2016).

69. Kang, I., Chu, C. T. & Kaufman, B. A. The mitochondrial transcription factor TFAM in neurodegeneration: emerging evidence and mechanisms. *FEBS Letters* **592**, 793–811 (2018).

70. Matsushima, Y., Goto, Y. & Kaguni, L. S. Mitochondrial Lon protease regulates mitochondrial DNA copy number and transcription by selective degradation of mitochondrial transcription factor A (TFAM). *Proceedings of the National Academy of Sciences of the United States of America* **107**, 18410–18415 (2010).

71. Lu, B. *et al.* Phosphorylation of Human TFAM in Mitochondria Impairs DNA Binding and Promotes Degradation by the AAA+ Lon Protease. *Molecular Cell* **49**, 121–132 (2013).

72. Kunkel, G. H., Chaturvedi, P. & Tyagi, S. C. Mitochondrial pathways to cardiac recovery: TFAM. *Heart Failure Reviews* **21**, 499–517 (2016).

73. Chen, D., Frezza, M., Schmitt, S., Kanwar, J. & P. Dou, Q. Bortezomib as the First Proteasome Inhibitor Anticancer Drug: Current Status and Future Perspectives. *Current Cancer Drug Targets* **11**, 239–253 (2011).

74. Su, S.-C. *et al.* Structural Basis for the Magnesium-Dependent Activation and Hexamerization of the Lon AAA+ Protease. *Structure* **24**, 676–686 (2016).

75. Shin, M. *et al.* Structures of the human LONP1 protease reveal regulatory steps involved in protease activation. *Nature Communications* **12**, 3239 (2021).

76. Geiser, M., Cébe, R., Drewello, D. & Schmitz, R. Integration of PCR Fragments at Any Specific Site within Cloning Vectors without the Use of Restriction Enzymes and DNA Ligase. *BioTechniques* **31**, 88–92 (2001).

77. Panavas, T., Sanders, C. & Butt, T. R. SUMO Fusion Technology for Enhanced Protein Production in Prokaryotic and Eukaryotic Expression Systems. in *SUMO Protocols* (ed. Ulrich, H. D.) vol. 497 303–317 (2009).

78. Rosano, G. L. & Ceccarelli, E. A. Recombinant protein expression in Escherichia coli: advances and challenges. *Frontiers in Microbiology* **5**, (2014).

79. Hansen, L. H., Knudsen, S. & Sørensen, S. J. The Effect of the lacY Gene on the Induction of IPTG Inducible Promoters, Studied in Escherichia coli and Pseudomonas fluorescens. *Current Microbiology* **36**, 341–347 (1998).

80. Sambrook, J., Fritsch, E. & Maniatis, T. *Molecular cloning: A laboratory manual: Vol. 2*. (1989).

81. Sattler, M. & Fesik, S. W. Use of deuterium labeling in NMR: overcoming a sizeable problem. *Structure* **4**, 1245–1249 (1996).

82. Goto, N. K., Gardner, K. H., Mueller, G. A., Willis, R. C. & Kay, L. E. [No title found]. *Journal of Biomolecular NMR* **13**, 369–374 (1999).

83. Mas, G., Crublet, E., Hamelin, O., Gans, P. & Boisbouvier, J. Specific labeling and assignment strategies of valine methyl groups for NMR studies of high molecular weight proteins. *Journal of Biomolecular NMR* **57**, 251–262 (2013).

84. Callon, M., Burmann, B. M. & Hiller, S. Structural Mapping of a Chaperone-Substrate Interaction Surface. *Angewandte Chemie International Edition* (2014) doi:10.

85. Tzeng, S.-R., Pai, M.-T. & Kalodimos, C. G. NMR Studies of Large Protein Systems. in *Protein NMR Techniques* (eds. Shekhtman, A. & Burz, D. S.) vol. 831 133–140 (2012).

86. Gans, P. *et al.* Stereospecific isotopic labeling of methyl groups for NMR spectroscopic studies of high-molecular-weight proteins. *Angewandte Chemie - International Edition* **49**, 1958–1962 (2010).

87. Rosenzweig, R. & Kay, L. E. Bringing dynamic molecular machines into focus by methyl-TROSY NMR. *Annual Review of Biochemistry* **83**, 291–315 (2014).

88. Tugarinov, V. & Kay, L. E. An isotope labeling strategy for methyl TROSY spectroscopy. *Journal of Biomolecular NMR* **28**, 165–72 (2004).

89. Concepcion, J. *et al.* Label-Free Detection of Biomolecular Interactions Using BioLayer Interferometry for Kinetic Characterization. *Combinatorial Chemistry & High Throughput Screening* **12**, 791–800 (2009).

90. Rich, R. Advances in surface plasmon resonance biosensor analysis. *Current Opinion in Biotechnology* **11**, 54–61 (2000).

91. Leonard, P., Hearty, S., Ma, H. & O'Kennedy, R. Measuring Protein–Protein Interactions Using Biacore. in *Protein Chromatography* (eds. Walls, D. & Loughran, S. T.) vol. 1485 339–354 (2017).

92. Shah, N. B. & Duncan, T. M. Bio-layer Interferometry for Measuring Kinetics of Protein-protein Interactions and Allosteric Ligand Effects. *Journal of Visualized Experiments* 51383 (2014).

93. Okeke, D. C., Lidman, J., Matečko-Burmann, I. & Burmann, B. M. *Thumb-domain dynamics modulate the functional repertoire of DNA-Polymerase IV (DinB)*. http://biorxiv.org/lookup/doi/10.1101/2022.10.16.512411 (2022).

94. Kawale, A. A. & Burmann, B. M. UvrD helicase–RNA polymerase interactions are governed by UvrD's carboxy-terminal Tudor domain. *Communications Biology* **3**, 607 (2020).

95. Wyatt, P. J. Light scattering and the absolute characterization of macromolecules. *Analytica Chimica Acta* **272**, 1–40 (1993).

96. Sahin, E. & Roberts, C. J. Size-Exclusion Chromatography with Multi-angle Light Scattering for Elucidating Protein Aggregation Mechanisms. in *Therapeutic Proteins* (eds. Voynov, V. & Caravella, J. A.) vol. 899 403–423 (2012).

97. Rao, V. S., Srinivas, K., Sujini, G. N. & Kumar, G. N. S. Protein-Protein Interaction Detection: Methods and Analysis. *International Journal of Proteomics* **2014**, 1–12 (2014).

98. Jares-Erijman, E. A. & Jovin, T. M. FRET imaging. *Nature Biotechnology* **21**, 1387–1395 (2003).

99. Bayot, A. *et al.* Towards the control of intracellular protein turnover: Mitochondrial Lon protease inhibitors versus proteasome inhibitors. *Biochimie* **90**, 260–269 (2008).

100. van Rossum, B.-J., Förster, H. & de Groot, H. J. M. High-Field and High-Speed CP-MAS13C NMR Heteronuclear Dipolar-Correlation Spectroscopy of Solids with Frequency-Switched Lee–Goldburg Homonuclear Decoupling. *Journal of Magnetic Resonance* **124**, 516–519 (1997).

101. Bloch, F. Nuclear Induction. *Physical Review* **70**, 460–474 (1946).

102. Purcell, E. M., Torrey, H. C. & Pound, R. V. Resonance Absorption by Nuclear Magnetic Moments in a Solid. *Physical Review* **69**, 37–38 (1946).

103. Levitt, M. H. *Spin dynamics: basics of nuclear magnetic resonance*. (2008).

104. Deppe, M. H., Teh, K., Parra-Robles, J., Lee, K. J. & Wild, J. M. Slice profile effects in 2D slice-selective MRI of hyperpolarized nuclei. *Journal of Magnetic Resonance* **202**, 180–189 (2010).

105. Kay, L. E. NMR studies of protein structure and dynamics. *Journal of Magnetic Resonance* **173**, 193–207 (2005).

106. Tugarinov, V. & Kay, L. E. An Isotope Labeling Strategy for Methyl TROSY Spectroscopy. *Journal of Biomolecular NMR* **28**, 165–172 (2004).

107. Wüthrich, K., Wider, G., Wagner, G. & Braun, W. Sequential resonance assignments as a basis for determination of spatial protein structures by high resolution proton nuclear magnetic resonance. *Journal of Molecular Biology* **155**, 311–319 (1982).

108. Billeter, M., Braun, W. & Wüthrich, K. Sequential resonance assignments in protein 1H nuclear magnetic resonance spectra. *Journal of Molecular Biology* **155**, 321–346 (1982).

109. Wüthrich, K. *NMR of proteins and nucleic acids*. (Wiley, 1986).

110. Campbell, I. D. The evolution of protein NMR. *Biomedical Spectroscopy and Imaging* **2**, 245–264 (2013).

111. *Protein NMR spectroscopy: principles and practice*. (Elsevier Academic Press, 2007).

112. Marion, D. *et al.* Overcoming the overlap problem in the assignment of proton NMR spectra of larger proteins by use of three-dimensional heteronuclear proton-nitrogen-15 Hartmann-Hahn-multiple quantum coherence and nuclear Overhauser-multiple quantum coherence spectroscopy: application to interleukin 1.beta. *Biochemistry* **28**, 6150–6156 (1989).

113. Messerle, B. A., Wider, G., Otting, G., Weber, C. & Wüthrich, K. Solvent suppression using a spin lock in 2D and 3D NMR spectroscopy with H2O solutions. *Journal of Magnetic Resonance (1969)* **85**, 608–613 (1989).

114. Ikura, M., Kay, L. E. & Bax, A. A novel approach for sequential assignment of proton, carbon-13, and nitrogen-15 spectra of larger proteins: heteronuclear triple-resonance three-dimensional NMR spectroscopy. Application to calmodulin. *Biochemistry* **29**, 4659–4667 (1990).

115. Kay, L. E., Ikura, M., Tschudin, R. & Bax, A. Three-dimensional triple-resonance NMR spectroscopy of isotopically enriched proteins. *Journal of Magnetic Resonance* **89**, 496–514 (1990).

116. Pervushin, K., Riek, R., Wider, G. & Wuthrich, K. Attenuated T2 relaxation by mutual cancellation of dipole-dipole coupling and chemical shift anisotropy indicates an avenue to NMR structures of very large biological macromolecules in solution. *Proceedings of the National Academy of Sciences* **94**, 12366–12371 (1997).

117. Salzmann, M., Pervushin, K., Wider, G., Senn, H. & Wuthrich, K. TROSY in triple-resonance experiments: New perspectives for sequential NMR assignment of large proteins. *Proceedings of the National Academy of Sciences* **95**, 13585–13590 (1998).

118. Gorman, S. D., Sahu, D., O'Rourke, K. F. & Boehr, D. D. Assigning methyl resonances for protein solution-state NMR studies. *Methods* **148**, 88–99 (2018).

119. Tugarinov, V., Choy, W.-Y., Orekhov, V. Yu. & Kay, L. E. Solution NMR-derived global fold of a monomeric 82-kDa enzyme. *Proceedings of the National Academy of Sciences* **102**, 622–627 (2005).

120. Nielsen, J. T. & Mulder, F. A. A. POTENCI: prediction of temperature, neighbor and pH-corrected chemical shifts for intrinsically disordered proteins. *Journal of Biomolecular NMR* **70**, 141–165 (2018).

121. Burmann, B. M., Wang, C. & Hiller, S. Conformation and dynamics of the periplasmic membrane-protein–chaperone complexes OmpX–Skp and tOmpA–Skp. *Nature Structural & Molecular Biology* **20**, 1265–1272 (2013).

122. Morgado, L., Burmann, B. M., Sharpe, T., Mazur, A. & Hiller, S. The dynamic dimer structure of the chaperone Trigger Factor. *Nature Communications* **8**, 1992 (2017).

123. Jaravine, V., Ibraghimov, I. & Yu Orekhov, V. Removal of a time barrier for high-resolution multidimensional NMR spectroscopy. *Nature Methods* **3**, 605–607 (2006).

124. Delaglio, F. *et al.* NMRPipe: A multidimensional spectral processing system based on UNIX pipes. *Journal of Biomolecular NMR* **6**, (1995).

125. Keller, R. L. J. Optimizing the process of nuclear magnetic resonance spectrum analysis and computer aided resonance assignment. 147 S. (ETH Zurich, 2005).

126. Sattler, M. Heteronuclear multidimensional NMR experiments for the structure determination of proteins in solution employing pulsed field gradients. *Progress in Nuclear Magnetic Resonance Spectroscopy* **34**, 93–158 (1999).

127. Kerfah, R., Plevin, M. J., Sounier, R., Gans, P. & Boisbouvier, J. Methyl-specific isotopic labeling: a molecular tool box for solution NMR studies of large proteins. *Current Opinion in Structural Biology* **32**, 113–122 (2015).

128. Takeuchi, K. & Wagner, G. NMR studies of protein interactions. *Current Opinion in Structural Biology* **16**, 109–117 (2006).

129. Barile, E. & Pellecchia, M. NMR-Based Approaches for the Identification and Optimization of Inhibitors of Protein–Protein Interactions. *Chemical Reviews* **114**, 4749–4763 (2014).

130. Almeida, T. B., Panova, S. & Walser, R. NMR Reporter Assays for the Quantification of Weak-Affinity Receptor–Ligand Interactions. *SLAS Discovery* **26**, 1020–1028 (2021).

131. Williamson, M. P. Using chemical shift perturbation to characterise ligand binding. *Progress in Nuclear Magnetic Resonance Spectroscopy* **73**, 1–16 (2013).

132. Shapiro, M. J. & Wareing, J. R. High resolution NMR for screening ligand/protein binding. *Current Opinion in Drug Discovery & Development* **2**, 396–400 (1999).

133. Gao, G., Williams, J. G. & Campbell, S. L. Protein–Protein Interaction Analysis by Nuclear Magnetic Resonance Spectroscopy. in *Protein-Protein Interactions* vol. 261 079–092 (2004).

134. Varani, G., Chen, Y. & Leeper, T. C. NMR Studies of Protein–Nucleic Acid Interactions. in *Protein NMR Techniques* vol. 278 289–312 (2004).

135. Harner, M. J., Frank, A. O. & Fesik, S. W. Fragment-based drug discovery using NMR spectroscopy. *Journal of Biomolecular NMR* **56**, 65–75 (2013).

136. Frank, A. T., Law, S. M., Ahlstrom, L. S. & Brooks, C. L. Predicting Protein Backbone Chemical Shifts From Cα Coordinates: Extracting High Resolution Experimental Observables from Low Resolution Models. *Journal of Chemical Theory and Computation* **11**, 325–331 (2015).

137. Grutsch, S., Brüschweiler, S. & Tollinger, M. NMR Methods to Study Dynamic Allostery. *PLOS Computational Biology* **12**, e1004620 (2016).

138. Kern, D. & Zuiderweg, E. R. The role of dynamics in allosteric regulation. *Current Opinion in Structural Biology* **13**, 748–757 (2003).

139. Gunasekaran, K., Ma, B. & Nussinov, R. Is allostery an intrinsic property of all dynamic proteins? *Proteins: Structure, Function, and Bioinformatics* **57**, 433–443 (2004).

140. Kawale, A. A. & Burmann, B. M. Characterization of backbone dynamics using solution NMR spectroscopy to discern the functional plasticity of structurally analogous proteins. *STAR Protocols* **2**, 100919 (2021).

141. Kawale, A. A. & Burmann, B. M. Advanced NMR spectroscopy methods to study protein structure and dynamics. in *Advanced Spectroscopic Methods to Study Biomolecular Structure and Dynamics* 125–152 (Elsevier, 2023).

142. Szyperski, T., Wider, G., Bushweller, JohnH. & W□thrich, K. 3D 13C-15N-heteronuclear two-spin coherence spectroscopy for polypeptide backbone assignments in 13C-15N-double-labeled proteins. *Journal of Biomolecular NMR* **3**, (1993).

143. Lee, D., Hilty, C., Wider, G. & Wüthrich, K. Effective rotational correlation times of proteins from NMR relaxation interference. *Journal of Magnetic Resonance* **178**, 72–76 (2006).

144. Zhu, G., Xia, Y., Nicholson, L. K. & Sze, K. H. Protein Dynamics Measurements by TROSY-Based NMR Experiments. *Journal of Magnetic Resonance* **143**, 423–426 (2000).

145. Nguyen, D., Chen, C., Pettitt, B. M. & Iwahara, J. NMR Methods for Characterizing the Basic Side Chains of Proteins: Electrostatic Interactions, Hydrogen Bonds, and Conformational Dynamics. in *Methods in Enzymology* vol. 615 285–332 (2019).

146. Yu, B., Pletka, C. C. & Iwahara, J. Protein Electrostatics Investigated through Paramagnetic NMR for Nonpolar Groups. *The Journal of Physical Chemistry B* **126**, 2196–2202 (2022).

147. García-Nafría, J. *et al.* Structure of the catalytic domain of the human mitochondrial Lon protease: proposed relation of oligomer formation and activity. *Protein Science: A Publication of the Protein Society* **19**, 987–999 (2010).

148. Lipari, G. & Szabo, A. Model-free approach to the interpretation of nuclear magnetic resonance relaxation in macromolecules. 1. Theory and range of validity. *Journal of the American Chemical Society* **104**, 4546–4559 (1982).

149. Dosset, P., Hus, J.-C., Blackledge, M. & Marion, D. Efficient analysis of macromolecular rotational diffusion from heteronuclear relaxation data. *Journal of Biomolecular NMR* **16**, 23–28 (2000).

150. Cole, R. FAST-Modelfree: A program for rapid automated analysis of solution NMR spin-relaxation data. *Journal of Biomolecular NMR* **26**, 203–213 (2003).

151. Bota, D. A. & Davies, K. J. A. Protein degradation in mitochondria: implications for oxidative stress, aging and disease: *Mitochondrion* **1**, 33–49 (2001).

152. Venkatesh, S., Lee, J., Singh, K., Lee, I. & Suzuki, C. K. Multitasking in the mitochondrion by the ATP-dependent Lon protease. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **1823**, 56–66 (2012).

153. Šulskis, D., Thoma, J. & Burmann, B. M. Structural basis of DegP protease temperature-dependent activation. *Science Advances* **7**, eabj1816 (2021).

154. Shin, M. *et al.* Structural basis for distinct operational modes and protease activation in AAA+ protease Lon. *Science Advances* **6**, eaba8404 (2020).